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Lysophosphatidylcholine: A chemotactic factor for human monocytes and its potential role in atherogenesis

(low density lipoprotein/oxidation/atherosclerosis/platelet-activating factor/inflammation)

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ABSTRACT **Native low density lipoprotein (LDL) does not affect monocyte/macrophage motility. On the other hand, oxidatively modified LDL inhibits the motility of resident peritoneal macrophages yet acts as a chemotactic factor for circulating human monocytes. We now show that lysophosphatidylcholine (lyso-PtdCho), which is generated by a phospholipase A_2 activity during LDL oxidation, is a potent chemotactic factor for monocytes. It is not chemotactic for neutrophils or for resident macrophages. Platelet-activating factor, after treatment with phospholipase A_2, becomes chemotactic for monocytes, whereas the intact factor is not. Synthetic 1-palmitoyl-lyso-PtdCho showed chemotactic activity comparable to that of the lyso-PtdCho fraction derived from oxidized LDL. The results suggest that lyso-PtdCho in oxidized LDL may favor recruitment of monocytes into the arterial wall during the early stages of atherogenesis. Generation of lyso-PtdCho, either from LDL itself or from membrane phospholipids of damaged cells, could play a more general role in inflammatory processes throughout the body.**

One of the characteristics of the earliest atherosclerotic lesion is the intimal accumulation of lipid-laden foam cells derived predominantly from monocyte/macrophages (1–5). Exactly how these cells are recruited and retained in the artery wall remains unclear, but it seems likely that adhesion molecules and chemotactic factors are involved. Although many different chemotactic factors for monocytes have been described (6–9), their relative importance in relation to atherogenesis remains to be determined. The possibility that a high plasma level of low density lipoprotein (LDL) might play a role in monocyte recruitment seemed attractive in view of the fact that a high LDL level is the proximate consequence of LDL receptor deficiency in homozygous familial hypercholesterolemia and, therefore, must somehow account for the genesis of the fatty streak lesions (10). However, native untreated LDL has no chemotactic activity for monocytes (9). On the other hand, LDL previously incubated with cultured endothelial cells (11–13), smooth muscle cells (11, 14–17), or macrophages (18) undergoes a complex oxidative modification that converts it to a form readily taken up by macrophages, partly via the acetyl LDL or "scavenger" receptor described by Basu *et al.* (19). Such oxidatively modified LDL inhibited the motility of resident peritoneal macrophages (20). Surprisingly, it had quite the opposite effect on human circulating monocytes—i.e., it was chemotactic (9). The activity resided in the lipid component of the oxidized LDL. We have now isolated and identified lysophosphatidylcholine (lyso-PtdCho) as a major chemotactic lipid component of oxidized LDL. Since as much as 40% of the PtdCho of LDL can be converted to lyso-PtdCho during oxidative modification (21), oxidation of LDL in the subendothelial space could play a significant role in recruitment of monocyte/macrophages during early stages of atherogenesis. Since lyso-PtdCho is generated in connection with cytotoxicity more generally, it could play a general role in recruitment of monocytes to sites of tissue injury and repair.

MATERIALS AND METHODS

Materials. Ham's F-10 medium was from Whittaker M.A. Bioproducts (Walkersville, MD); fetal bovine serum was from HyClone (Logan, UT); Ficoll/Hypaque, bovine serum albumin, Hanks' balanced salt solution, 1-caproyl lyso-PtdCho, 1-palmitoyl lyso-PtdCho, 1-myristoyl lyso-PtdCho, 1-linoleoyl 2-palmitoyl PtdCho, 1-*O*-alkyl 2-acetyl-*sn*-glycero-3-phosphorylcholine (platelet-activating factor, PAF), L-α-lysophosphatidylserine, L-α-lysophosphatidylethanolamine, 1-monopalmitate, and dipalmitoyl PtdCho were from Sigma; and female Swiss–Webster mice (27–30 g) were from Simonsen Laboratories (Gilroy, CA).

Cells. The rabbit aortic endothelial cells were from a line established and characterized by Buonassisi and Venter (22). These cells were grown in Ham's F-10 medium/15% fetal bovine serum containing epidermal growth factor at 10 μg/ml in 60-mm plastic culture dishes and were used at confluence.

Human monocytes were isolated from normal human blood by the method of Kumagai *et al.* (23) as described (9). The cells were resuspended in Hanks' balanced salt solution/0.2% bovine serum albumin at 2×10^6 cells per ml. The resultant cell suspension contained >90% monocytes, as determined by nonspecific esterase staining, which were >95% viable, as determined by trypan blue exclusion.

Human neutrophils were isolated by the method of Ferrante and Thong (24) using Ficoll/Hypaque (density = 1.114 g/ml). The neutrophils were collected, washed with and suspended in Hanks' balanced salt solution containing 0.2% bovine serum albumin, and used for the chemotaxis assay. Purity was evaluated by light microscopy, and viability was assessed by trypan blue exclusion (both always >95%).

Nonelicited resident peritoneal macrophages were harvested from female Swiss–Webster mice by lavage with phosphate-buffered saline according to the method of Cohn and Benson (25). For the chemotaxis assay, the macrophages were resuspended in Hanks' balanced salt solution containing 0.2% bovine serum albumin at 2×10^6 cells per ml. Viability was assessed by trypan blue exclusion (always >95%).

Lipoproteins. LDL (density = 1.019–1.063 g/ml) was isolated by ultracentrifugation from pooled normal human plasma collected in EDTA (1 mg/ml) (26). Protein was determined by the Lowry method (27) using bovine serum

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Abbreviations: LDL, low density lipoprotein; PtdCho, phosphatidylcholine; lyso-PtdCho, lysophosphatidylcholine; PAF, platelet-activating factor; CI, chemotactic index.

albumin as a standard. Endothelial cell-modified LDL was prepared by incubating LDL (100 μg/ml) in F-10 medium with rabbit aortic endothelial cells as described (28). Copper-oxidized LDL was prepared by incubating LDL (100 μg/ml) with 5 μM Cu^{2+} in F-10 medium at 37°C for 24 hr (29).

Lipid Extractions. Lipid extractions were carried out according to the method of Bligh and Dyer (30). The chloroform phase was evaporated under N_2 and the lipids were resuspended in appropriate solvents. For the chemotaxis assays, the lipids were dissolved in 10 μl of ethanol and the appropriate volumes were added to Hanks' balanced salt solution to match the original concentrations represented in intact LDL.

Silicic Acid Column Chromatography. Lipids from 2 mg of oxidized LDL were applied in 1.0 ml of hexane to a 1 × 13 cm column of silica gel (60–200 mesh; J. T. Baker Chemical, Phillipsburg, NJ) equilibrated with hexane. The column was eluted sequentially with 8 ml of hexane/ether, 3:1 (vol/vol); 8 ml of hexane/ether, 1:1 (vol/vol); 8 ml of hexane/ether, 1:3 (vol/vol); 8 ml of ether; 8 ml of $CHCl_3$; 8 ml of $CHCl_3/CH_3OH$, 1:1 (vol/vol); 8 ml of $CHCl_3/CH_3OH/H_2O$, 5:4:1 (vol/vol); and 16 ml of CH_3OH. Eluting solvents were collected as single fractions and dried under N_2 before resuspending in appropriate solvents. For the chemotaxis assay, the lipids were dissolved in 10 μl of ethanol, and the appropriate volumes of Hanks' balanced salt solution were added to match the original concentration of the lipid represented in intact LDL.

TLC. Lipids were fractionated on 20 × 20 cm silica gel G plates (Analtech, Newark, DE) that had been activated at 110°C for 1 hr. Plates were developed in $CHCl_3/CH_3OH/H_2O$, 65:35:6 (vol/vol). Lipid bands were detected by lightly staining with I_2 vapor. The plates were then divided into appropriate zones, which were scraped off and eluted three times with 5 ml of $CHCl_3/CH_3OH$, 1:1 (vol/vol). Samples were then dried under N_2 before being taken up in other solvents. Inorganic phosphorus was measured according to the method of Bartlett (31) as modified by Marinetti (32).

Phospholipase A_2 Treatment of LDL. Native LDL (400 μg) was incubated in 1.0 ml of 100 mM Tris buffer (pH 7.4) containing 2 mM $CaCl_2$ and 4.2 μg of cobra venom phospholipase A_2 (*Naja naja naja;* a generous gift from E. A. Dennis, University of California, San Diego) for 2 hr at 37°C. Control LDL was incubated without enzyme. The products were extracted by the method of Bligh and Dyer (30), the chloroform layer was dried, and the lipids were used for TLC, silica gel chromatography, and chemotaxis assays. PAF and PtdCho were treated with phospholipase A_2 under similar conditions.

Chemotaxis Assay. Chemotaxis was measured in a 48-well modified Boyden microchemotaxis chamber (NeuroProbe, Cabin John, MD) as described earlier (20). Assays were run in triplicate, using a 2-hr incubation for the monocytes and neutrophils and a 5-hr incubation for the macrophages. Chemotactic activity is expressed as chemotactic index (CI), defined as the number of cells migrating in response to the test substance divided by the number migrating when unincubated control medium was present in both chambers. Seven or eight grid areas were counted per sample, and averaged results shown are in every case representative of two or more replicate protocols.

RESULTS

Identification of Lyso-PtdCho as a Chemoattractant from Oxidized LDL. As shown in Table 1, the unfractionated lipids extracted from oxidized LDL (but not from native LDL) were strongly chemotactic for human monocytes, confirming our previous report (9). After fractionation, the activity was found only in the relatively polar fractions, fractions 5 and 6 (Table 1). No additional lipid and no additional chemotactic activity eluted with a solvent of still greater polarity (100% CH_3OH). Similar results in all respects were obtained with either endothelial cell-modified LDL or Cu^{2+}-oxidized LDL; the general term oxidized LDL is therefore used hereafter to refer collectively to either endothelial cell-modified LDL or Cu^{2+}-oxidized LDL.

Table 1. Fractionation of oxidized LDL by silica gel column chromatography

Sample	Lipid class	CI
Lipid extract of native LDL		1.1 ± 0.1
Lipid extract of oxidized LDL		10.5 ± 0.3
Silica column eluates		
(Column fraction, eluting solvent)		
1. Hexane/ether (3:1)	Cholesteryl esters, triglycerides	1.0 ± 0.1
2. Hexane/ether (1:1)	Cholesterol	1.1 ± 0.1
3. Hexane/ether (1:3)	Cholesterol	1.0 ± 0.1
4. $CHCl_3$	None	1.0 ± 0.1
5. $CHCl_3/CH_3OH$ (1:1)	Free fatty acids (FFA), oxidized FFA	10.4 ± 1.0
6. $CHCl_3/CH_3OH/H_2O$ (5:4:1)	Phospholipids	11.3 ± 1.1
7. CH_3OH	None	1.0 ± 0.2

Identification of lipid class is based on TLC compared to authentic standards. The CI for each sample is expressed as mean ± SD (seven fields counted); the results are representative of those from five separate identical experiments in which LDL was oxidized.

Further fractionation of column fraction 6 by TLC showed that all of the attractant activity was in the band running at about R_f 0.09, almost exactly with the lyso-PtdCho standard (Table 2). No other areas of the lane showed any chemotactic activity. Synthetic lyso-PtdCho was then evaluated for activity. As shown in Fig. 1, synthetic 1-palmitoyl lyso-PtdCho stimulated chemotaxis of human monocytes in a dose-dependent manner with maximal activity at 25 μM. As with a number of other chemotactic factors, the response fell off at higher concentrations (33, 34). Whether this reflects "saturation" of the response systems (33) or cytotoxicity is not known. A modified checkerboard analysis of the response demonstrated that it was primarily chemotactic (Table 3). Lyso-PtdCho generated from phospholipase A_2 treatment of dipalmitoyl PtdCho exhibited essentially the same dose–response relationship as the synthetic 1-palmitoyl-*sn*-glycero-3-phosphorylcholine (Fig. 1). Synthetic PtdCho standards (1-linoleoyl 2-palmitoyl PtdCho and dipalmitoyl PtdCho) (Fig. 1) or PtdCho isolated and purified from previously oxidized LDL by TLC all lacked chemoattractant activity. Unfractionated lipids from oxidatively modified LDL also stimulated chemotaxis in a dose-dependent manner (data not shown). The dose–response relationship was essentially as would have

Table 2. TLC fractionation of column fraction 6 (see Table 1)

Sample	Major phospholipids	CI
Unfractionated silica gel column fraction 6	All	18.8 ± 2.3
TLC fractions		
1. R_f 0.0 to 0.06	None	1.0 ± 0.3
2. R_f 0.06 to 0.12	Lyso-PtdCho	17.5 ± 3.3
3. R_f 0.12 to 0.28	Sphingomyelin	1.0 ± 0.3
4. R_f 0.28 to 0.50	PtdCho	1.0 ± 0.3
5. R_f 0.50 to 1.0	None	1.3 ± 0.3

Phospholipids were detected by lightly staining with I_2 vapor and identified by TLC compared to authentic standards. The CI for each sample is expressed as mean ± SD (seven fields counted); the results are representative of those from three separate experiments.

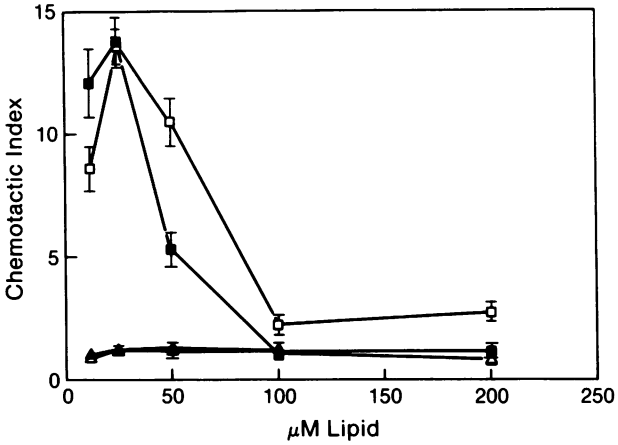

FIG. 1. Human monocyte chemotactic response to synthetic 1-palmitoyl lyso-PtdCho (■), phospholipase A_2-treated PtdCho (□), dipalmitoyl PtdCho (▲), and 1-palmitoyl, 2-linoleoyl PtdCho (△). The CI for each sample is expressed as mean ± SD (seven fields counted); the results are representative of those from three separate experiments.

been predicted on the basis of the lyso-PtdCho content (determined by phosphate analysis of TLC scrapings). These results establish that lyso-PtdCho is indeed the active factor in column fraction 6 and may be responsible for the major part of the activity of intact oxidized LDL. However, as shown in Table 1, there was also chemotactic activity in fraction 5. Since both fractions showed activity about the same as that of the unfractionated lipids, it would appear that they are not strictly additive (at least at the concentrations tested).

Phospholipase A_2-Treated LDL. As previously shown, oxidation of LDL is associated with degradation of lecithin by a phospholipase A_2-like activity (21). To test whether the chemotactic activity of oxidized LDL eluted in fraction 5 (Table 1) might represent fatty acids released by phospholipase A_2 activity, nonoxidized LDL was incubated with purified phospholipase A_2, as described in *Materials and Methods*. Analysis by TLC showed that there had been complete conversion of PtdCho to lyso-PtdCho (>94% as determined by TLC and phosphorus analysis). Fractionation of the extracted lipids by silica gel chromatography showed chemotactic activity only in fraction 6 (Table 4); there was none in fraction 5. The results suggest that the activity found in fraction 5 when oxidized LDL is fractionated (Table 1) probably represents a lipid fragment generated during oxidation. As a control, phospholipase A_2 treatment of the LDL was also carried out in the presence of 20 μM butylated hydroxytoluene; the same results were obtained as in the absence of antioxidants. These studies provide further evidence that the lyso-PtdCho generated from the PtdCho mixture as it occurs in circulating LDL is indeed a monocyte chemoattractant. Oxidation may be necessary only to facilitate hydrolysis of the fatty acids from the 2 position; oxidative modification of the rest of the molecule is not obligatory.

Table 3. Chemotactic and chemokinetic activities of lyso-PtdCho

Addition to lower chamber	Addition to upper chamber	CI
Control medium	Control medium	1.0 ± 0.1
Lyso-PtdCho	Control medium	7.5 ± 0.7
Lyso-PtdCho	Lyso-PtdCho	0.6 ± 0.1
Control medium	Lyso-PtdCho	0.5 ± 0.1

The CI for each sample is expressed as mean ± SD (seven fields counted); the results are representative of those from three separate experiments. Lyso-PtdCho was tested at 25 μM. The medium used for control was Hanks' balanced salt solution.

Table 4. Fractionation of phospholipase A_2-treated LDL by silica acid gel column chromatography

Sample	Lipid class	CI
Lipid extract of native LDL		1.1 ± 0.1
Lipid extract of phospholipase A_2-treated LDL		4.7 ± 0.5
Silica column eluates (Column fraction, eluting solvent)		
1. Hexane/ether (3:1)	Cholesteryl esters, triglycerides	0.9 ± 0.1
2. Hexane/ether (1:1)	Cholesterol	1.0 ± 0.2
3. Hexane/ether (1:3)	Cholesterol	1.0 ± 0.1
4. $CHCl_3$	None	1.2 ± 0.2
5. $CHCl_3/CH_3OH$ (1:1)	Free fatty acids (FFA), oxidized FFA	0.9 ± 0.1
6. $CHCl_3/CH_3OH/H_2O$ (5:4:1)	Phospholipids	4.7 ± 0.5

Identification of lipid class is based on TLC compared to authentic standards. The CI for each sample is expressed as mean ± SD (seven fields counted); the results are representative of those from three separate experiments.

In the absence of any acceptor, lyso-PtdCho generated during oxidative modification remains bound to the LDL as reported (29). The Boyden chamber assay was carried out with 0.2% albumin in the upper well along with the monocytes. Since lyso-PtdCho has a high affinity for albumin, we considered the possibility that this might affect the gradient sensed by the cells. The studies were therefore repeated with graded concentrations of albumin in the lower chamber (0.05%, 0.1%, and 0.2%) but this did not significantly alter the chemotactic responses (data not shown).

Structural Specificity. The effect of acyl chain length on the chemotactic activity of lyso-PtdCho was examined. Both 1-palmitoyl lyso-PtdCho (C-16) and 1-myristoyl lyso-PtdCho (C-14) had chemoattractant activity with peak activity at the same concentration (25 μM). However, the maximal activity for 1-myristoyl lyso-PtdCho was ≈30% lower than that for 1-palmitoyl lyso-PtdCho. On the other hand, 1-caproyl lyso-PtdCho (C-6) had no chemotactic activity at any of the concentrations tested (6–400 μM). It is important to note that 1-caproyl lyso-PtdCho, even at the highest concentration tested (400 μM), was still at a concentration far below its critical micellar concentration (CMC), while the other forms of lyso-PtdCho tested were nearer their respective CMCs.

Although very little lysophosphatidylserine and lysophosphatidylethanolamine are associated with the LDL particle, we tested whether they were chemotactic for the monocyte. However, neither of these lysophospholipids was active over the concentration range tested (12–50 μM) (data not shown).

Since PAF is a known chemotactic factor for eosinophils (35) and neutrophils (34, 36) and has structural similarities to lyso-PtdCho, we examined PAF for monocyte chemotactic activity. PAF lacked chemotactic activity for monocytes when tested over a wide concentration range (6–200 μM) (Fig. 2). However, when PAF was first treated with phospholipase A_2 to remove the 2-acetyl group (forming lyso-PAF), dose-dependent chemotactic activity was generated (Fig. 2).

It has been shown that lyso-PtdCho and cholesterol interact in solution to form lamellar structures, and this has been postulated to reduce the lytic activity of lyso-PtdCho (37). Therefore, we studied the effect of added cholesterol on the chemotactic activity of 25 μM lyso-PtdCho. Adding cholesterol with lyso-PtdCho at ratios of 0.25:1, 0.5:1, 0.75:1, and 1:1 (cholesterol/lyso-PtdCho) had no significant effect on the monocyte chemotactic response (CI = 9.8 ± 0.4, 9.9 ± 0.8, 10.3 ± 0.6, and 10.2 ± 0.6, respectively; mean ± SD;

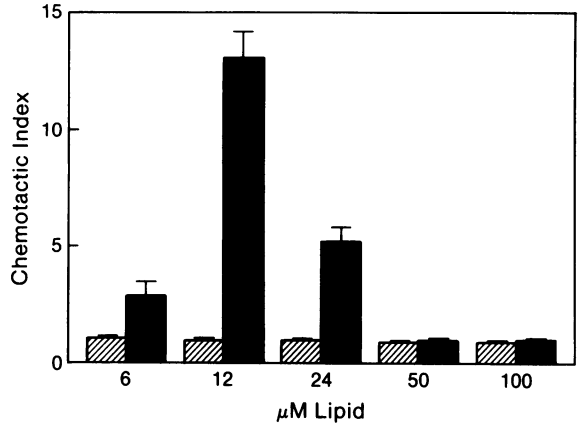

FIG. 2. PAF (▨) becomes chemotactic after treatment with phospholipase A_2 (■). The CI for each sample is expressed as mean ± SD (seven fields counted); the results are representative of those from three separate experiments.

n = 7) compared to the response with 25 μM lyso-PtdCho alone (CI = 10.1 ± 0.6; mean ± SD; n = 7). These results further demonstrate that the chemotactic response to lyso-PtdCho is not simply due to a nonspecific detergent effect, but they do not rule out subtle membrane effects that may be specifically involved.

DISCUSSION

In these studies, we have identified lyso-PtdCho as a chemotactic factor for human circulating monocytes. Previous studies by Hoffman *et al.* (38) have shown that lyso-PtdCho is a chemoattractant for mouse lymphoblastic cells (mouse thymic lymphoma 6C3HED cells and concanavalin A-stimulated mouse spleen lymphocytes) but the activity was seen only at much higher concentrations—concentrations 20 times those used in the present studies. In the present studies, we also tested the response of human neutrophils to similar concentrations of lyso-PtdCho and found no effect, in agreement with Hoffman *et al.* (38). Since lyso-PtdCho is generated during oxidation of LDL, it could play a role in atherogenesis. Since neutrophils are not normally associated with the atherosclerotic lesion, the observed monocyte specificity of lyso-PtdCho is consonant with such a role.

The specific mechanism(s) of action of lyso-PtdCho as a chemotactic factor remains to be established, but the present findings allow us to say at least something about the possibilities. The fact that phospholipase A_2-treated PAF, which has an ether-linked fatty acid in the 1 position, was fully active shows that further metabolism to remove the fatty acid from the 1 position is not a prerequisite. The possibility that the chemotactic effect is simply a function of the detergent effect of lyso-PtdCho is also unlikely. PAF also has detergent properties and they are equivalent to those of lyso-PtdCho (39); yet, intact PAF lacked chemotactic activity. Furthermore, complexing of cholesterol with lyso-PtdCho is recognized to reduce its detergent effects, but such complexes showed chemotactic activity equivalent to that of lyso-PtdCho alone.

Several new findings *in vivo* support the proposal that oxidation of LDL may play a role in fatty streak formation. LDL has recently been shown to be present in the normal rabbit aortas at a higher concentration in areas known to develop fatty streaks soon after an atherogenic diet is started (40). Thus, at these lesion-susceptible sites the stage might be set for the generation of a higher chemotactic gradient than at lesion-resistant areas. Moreover, since monocyte/macrophages themselves can oxidize LDL (18, 41), there might be an almost autocatalytic crescendo of activity at these susceptible sites that could account for the selective localization. Gerrity *et al.* (7) have reported that extracts of areas of the swine aorta susceptible to atherosclerosis contain more chemotactic activity than extracts of resistant areas, but the nature of the chemoattractant activity has not been determined. Also relevant is the finding that concentrations of lyso-PtdCho in the artery wall increase early in the development of the lesion (42). The total concentrations of lyso-PtdCho in the lesions of atherosclerosis (42) and in the plasma (43) are quite high, but there is uncertainty as to the exact concentration of unbound lyso-PtdCho. In any case, the relevant concentration gradients would be generated over a very minute distance—the distance from the immediate subendothelial space out to the luminal end of the intercellular channels through which the monocytes penetrate. Direct tests of the proposed role of lyso-PtdCho as a chemoattractant under *in vivo* conditions will be difficult to design. However, there is indirect evidence at least compatible with the hypothesis. Carew *et al.* (44) recently reported that treatment with an antioxidant compound (probucol) slowed the rate of progression of fatty streak lesions in receptor-deficient rabbits with extremely high plasma concentrations of LDL (WHHL rabbit). The effect was not due to a lowering of cholesterol levels since a control group with comparable cholesterol levels provided the reference control. Those studies also showed that the rate of degradation of circulating LDL in macrophage-rich lesions was decreased, whereas degradation in lesion-free areas (which contain no macrophages) was unaffected. The results support the proposition that LDL must first undergo oxidative modification before it can be taken up at a maximal rate by intimal monocyte/macrophages. Blocking oxidative modification may thus have also inhibited monocyte recruitment. It should be stressed that many factors may contribute to the recruitment of monocytes at the sites of atherogenesis. Endothelial cells themselves (8, 9, 20) and smooth muscle cells (45, 46) have been shown to secrete factors that are chemotactic for monocytes. As mentioned, many factors have been identified as chemotactic for monocytes. However, most of them are also chemotactic for the neutrophil, whereas lyso-PtdCho is not. Atherosclerotic lesions, particularly at the early stages, contain almost no neutrophils, only monocyte/macrophages and a few T lymphocytes (2–5). It is this specificity of lyso-PtdCho, in part, that makes the hypothesis that it plays a significant role in fatty streak formation an attractive one.

Finally, the importance of lyso-PtdCho as a chemotactic factor may be much more general, being relevant in many situations where there is an inflammatory response. LDL in inflammatory fluids has been shown to share a number of properties with oxidized LDL (47). Necrotic cells release phospholipase A_2 activity and the membrane phospholipids undergo oxidation and degradation that could generate significant concentrations of lyso-PtdCho at any site of inflammation (48, 49). This simple compound, generated ubiquitously in response to inflammatory stimuli, may thus play a general role in monocyte recruitment, including recruitment to sites of atherogenesis.

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