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Endothelial cell-derived chemotactic activity for mouse peritoneal macrophages and the effects of modified forms of low density lipoprotein

(atherosclerosis/foam cells/lipid peroxides/acetyl-low density lipoprotein)

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ABSTRACT **Cultured rabbit and bovine aortic endothelial cells generated chemotactic activity for mouse resident peritoneal macrophages, demonstrable in the conditioned medium. This chemotactic activity was heat stable and was not extracted into chloroform/methanol. It was inhibited by addition of endothelial cell-modified low density lipoprotein (EC-modified LDL), a form of LDL shown previously to contain peroxidized lipids, increased lysophosphatidylcholine, and partially degraded apoprotein B. The chemotactic activity was also inhibited by LDL previously oxidized in the absence of cells with 5 μM Cu^{2+}. Inhibitory activity was present in the lipid extract of EC-modified LDL but not in that of native LDL, presumably representing peroxidized lipid components. EC-modified LDL also inhibited the chemotactic activity of zymosan-activated serum. Because EC-modified LDL is taken up in part by way of the acetyl-LDL receptor, the effects of acetyl-LDL were tested. Rather than inhibiting chemotaxis, acetyl LDL showed intrinsic positive chemotactic activity as did also fucoidin and polyinosinic acid, both of which also interact with the acetyl-LDL receptor. These studies suggest mechanisms by which macrophages may be recruited into the subendothelial space by endothelial cell-derived chemotactic factors or by natural polyanions structurally related to fucoidin or polyinosinic acid and then become "trapped" there because of the inhibitory effects of peroxidized lipid components in modified forms of LDL.**

A growing body of evidence indicates that many or most of the lipid-laden foam cells in the atherosclerotic lesion are derived from monocyte/macrophages (1–3). One of the earliest events in experimental atherosclerosis is the adherence of monocytes to the arterial endothelium and penetration into the subendothelial space (3). The mechanisms by which macrophages take up lipoproteins and store cholesterol have been intensively studied over the past several years. These studies show that the macrophage has only a limited of number of receptors for the specific uptake of native low density lipoprotein (LDL) but that it avidly takes up certain chemically modified forms of LDL via an alternative specific, saturable receptor—the acetyl-LDL receptor (4). We have shown previously that LDL incubated overnight with cultured endothelial cells in F-10 medium (endothelial cell-modified LDL; EC-modified LDL) is biologically modified to a form that is also more rapidly degraded by macrophages, in part by the same acetyl-LDL receptor (5–7). Macrophages also express a specific receptor for a modified form of very low density lipoprotein (VLDL) that accumulates in cholesterol-fed animals, the so-called β-VLDL receptor (8). Finally, it has been shown that the macrophage secretes lipoprotein lipase, which facilitates uptake and storage of lipids derived from very low density, triglyceride-rich lipoproteins (9, 10).

It remains unclear what factors lead to the recruitment and retention of the monocyte/macrophage in the artery wall. Many chemotactic factors for monocytes have been characterized, including thrombin (11), plasminogen activator (12), fibronectin (13), platelet-derived growth factor (14), the fifth component of complement (15), kallikrein (12), and fragments of elastin and collagen (16, 17). Chemotactic activities, not yet fully identified, have been observed in smooth muscle cell-conditioned medium (18) and in fibroblast-conditioned medium (19). Recently, Gerrity *et al.* reported that extracts from areas of the swine aorta known to be more susceptible to experimental atherosclerosis contain chemotactic activity for monocytes isolated from hyperlipidemic swine (but not for monocytes from normal swine) (20). Whether the chemotactic factors in the artery originated from plasma or were endogenously produced was not determined.

The present studies stemmed from our speculation that lipoproteins themselves, or products generated as a result of their interaction with cells of the artery wall, might act as chemotactic factors for the monocyte/macrophage. Preliminary studies showed that native LDL was without chemotactic activity and that incubation of LDL with EC failed to generate chemotactic activity. However, the conditioned medium from EC incubated *in the absence* of LDL did contain potent chemotactic activity. We report some of the properties of this EC-derived chemotactic activity and show that its activity (and that of zymosan-activated serum) is inhibited by EC-modified LDL. We show further that several substances that interact with the acetyl-LDL receptor (acetyl LDL, fucoidin, and polyinosinic acid) themselves act as mouse macrophage chemotactic factors.

MATERIALS AND METHODS

Ham's F-10 medium and Dulbecco's modified Eagle's medium (DMEM) were obtained from GIBCO; fetal bovine serum was from HyClone (Logan, UT); female Swiss Webster mice were from Simonsen Laboratories (Gilroy, CA); butylated hydroxytoluene (BHT) was from Baker. Bovine serum albumin, zymosan A, fucoidin, polyinosinic acid, and platelet-activating factor (PAF) were from Sigma.

Cells and Conditioned Media. The rabbit aortic EC were from a line established and characterized by Buonassisi and Venter (21). These cells were grown in Ham's F-10 medium/15% fetal bovine serum containing epidermal

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Abbreviations: LDL, low density lipoprotein; VLDL, very low density lipoprotein; EC, endothelial cell(s); l-PtdCho, lysophosphatidylcholine; PAF, platelet-activating factor; BHT, butylated hydroxytoluene; CI, chemotactic index.

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growth factor at 10 ng/ml in 60-mm plastic culture dishes and were used for experiments at confluence. Bovine aortic EC were from a strain (11-BAEC) that was a gift of Michael A. Gimbrone (Department of Pathology, Harvard University). These cells were cultured in DMEM/10% fetal bovine serum in 60-mm plastic culture dishes and were used for experiments at confluence. EC were washed three times with serum-free medium and then incubated with 2.0 ml of Ham's F-10 medium at 37°C. At the end of the incubation, the medium was aspirated and any detached cells were removed by low-speed centrifugation.

Resident peritoneal macrophages were isolated from female Swiss Webster mice by lavage with Hanks' balanced salt solution (HBSS). After centrifugation, the cells were resuspended in HBSS containing 0.2% bovine serum albumin at a concentration of 2.0×10^6 cells per ml and used in the chemotaxis assays.

Lipoproteins. LDL (ρ = 1.019–1.063) was isolated by ultracentrifugation from pooled normal human plasma collected in EDTA (1 mg/ml). Protein was determined by the Lowry method (22) with bovine serum albumin as a standard. EC-modified LDL was prepared by first washing the cells three times with serum-free Ham's F-10 medium and then incubating at 37°C with 2 ml of the same medium containing native LDL (100 μg of protein per ml). At the end of the incubation, the medium was harvested and any detached cells were removed by low-speed centrifugation. Reisolated EC-modified LDL was prepared by flotation at ρ = 1.2 for 24 hr. In some experiments the medium containing EC-modified LDL was incubated with defatted bovine serum albumin (10 mg/ml) for 24 hr at 37°C to remove lysophosphatidylcholine (l-PtdCho) and free fatty acids. The LDL was then reisolated as before by centrifuging at ρ = 1.21 g/ml. Acetyl-LDL was prepared by reaction with acetic anhydride as described by Basu *et al.* (23). Copper-oxidized LDL was prepared by incubating 100 μg of LDL per ml with 10 μM Cu^{2+} in a total volume of 2 ml in F-10 medium for 24 hr at 37°C (24). The LDL was then dialyzed against phosphate-buffered saline containing EDTA. Lipid extractions were carried out by using $CHCl_3$/MeOH according to the method of Bligh and Dyer (25). The lipids were dissolved in 20 μl of EtOH and then added to 0.8 ml of F-10 medium for chemotaxis assay. The upper phase was dialyzed against F-10 medium for 24 hr before assay.

Chemotaxis Assay. Chemotaxis was assayed in a 48-well modified Boyden micro chemotaxis chamber (Neuro Probe, Inc., Cabin John, MD) as described by Falk *et al.* (26). The bottom wells contained 25 μl of the solution to be assayed; previously isolated mouse peritoneal macrophages were added to the upper wells in a volume of 50 μl (1.0×10^5 cells per well). Assays were run in duplicate or triplicate. After a 5-hr incubation at 37°C in humidified air containing 7% CO_2, the filter sheet was removed and the nonmigrated cells were scraped from its top side. The filter was then stained with Diff-Quick (Harleco, Gibbstown, NJ) and mounted on a glass slide. The migrated cells were counted with a 40× objective and a 10× ocular containing a 10-mm^2 counting grid. 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. Five to seven grid areas were counted per sample and averaged. Average variation among the counted fields was 10% ± 4% for the EC-conditioned F-10 medium and 19% ± 6% for the unincubated control medium. Results shown are in every case representative of two or more replicate protocols.

RESULTS

Chemotactic Activity in EC-Conditioned Medium. After incubation with monolayers of rabbit aortic EC, the conditioned incubation medium was consistently found to contain chemotactic activity for mouse peritoneal macrophages. In 25 experiments the CI averaged 8.2 ± 5.5 (mean ± SD); it exceeded 5 seventeen times and was never less than 3. The absolute number of cells migrating ranged from 43 to 118 per field for the EC-conditioned F-10 medium and 15 to 33 for the control F-10 medium. To assess chemotactic and chemokinetic activity, studies were done with EC-conditioned F-10 medium only in the lower chamber, only in the upper chamber, or in both; the CIs were 13.2, 2.0, and 6.4, respectively. These results indicate both chemokinetic and chemotactic activities.

The concentration of chemotactic activity in the medium increased with time of incubation; the CI rose from 1.1 at 1 hr to 5.6 at 8 hr and to 13.5 at 24 hr (results not shown). Most studies were carried out by using rabbit aortic EC cultured in Ham's F-10 medium for 24 hr. However, a similar release of chemotactic activity was found by using DMEM. Similar results were also obtained by using a line of bovine aortic EC (CI = 3.0 and 6.1 in two studies).

The chemotactic activity was stable to boiling for 10 and even 30 min, was not dialyzable, and was not destroyed by adjusting to pH 2.5 or to pH 12 for 10 min followed by neutralization and testing (Table 1). No activity was extracted into the $CHCl_3$ phase after acidic $CHCl_3$/MeOH extraction; the activity was apparently almost fully recovered in the upper phase. However, in these preliminary studies systematic testing of dilutions was not done and the results should be considered only semiquantitative. Diluting EC-conditioned F-10 medium 1:1 only reduced the CI by about 10%; diluting 1:2 reduced the CI by about 60%.

Since PAF has been shown to have chemoattractant activity for neutrophils and is secreted by EC (27, 28), we tested authentic PAF but found no activity for mouse peritoneal macrophages under the conditions of our assay. Further studies on the nature of the chemotactic factor(s) are necessary.

Effects of Lipoproteins. Native LDL added to control F-10 medium showed no chemotactic activity (Table 2, exp. 1) nor was native LDL inhibitory when added to EC-conditioned F-10 medium (Table 2, exp. 2). However, when native LDL was included in the F-10 medium and was present throughout the conditioning incubation, the EC-conditioned medium (now containing EC-modified LDL) failed to show chemotactic activity (Table 2, exp. 3). In fact, the number of cells migrating was sharply reduced (CI = 0.5), suggesting an

Table 1. Properties of chemotactic activity in EC-conditioned medium

Exp.	Sample of rabbit EC-conditioned F-10 medium	CI
1	Untreated sample	13.4
	Boiled 10 min	9.8
	Acid-treated (pH 2.5, 10 min)	11.6
	Alkali-treated (pH 11.0, 10 min)	11.0
2	Untreated sample	23.3
	Boiled 30 min	21.8
3	Untreated sample	6.1
	Lipid phase from $CHCl_3$/MeOH extraction	1.1
	Aqueous phase from $CHCl_3$/MeOH extraction	5.9

EC were incubated with Ham's F-10 medium for 24 hr and chemotactic activity was measured. Acid and alkali treatment was carried out with 1 M HCl and 1 M NaOH, respectively; after 10 min the pH was adjusted to 7.4 and chemotactic activity was measured. See text for lipid extraction procedure.

Table 2. Effects of LDL and modified forms of LDL on the chemotactic activity of EC-conditioned F-10 medium

Exp.	Sample	CI
1	Native LDL, 100 μg/ml, added to control F-10 medium	1.0
2	EC-conditioned F-10 medium	13.8
	+ Native LDL, 50 μg/ml (added *after* incubation)	15.6
3	EC-conditioned F-10 medium	6.6
	Prepared in presence of 100 μg of LDL per ml	0.5
	Prepared in presence of 100 μg of LDL per ml + 20 μM BHT	7.4
4	EC-conditioned F-10 medium	9.3
	+ EC-modified LDL, 50 μg/ml	0.4
5	EC-conditioned DMEM	5.0
	Prepared in presence of 100 μg of LDL per ml	5.0
6	EC-conditioned F-10 medium	9.3
	+ Cu^{2+}-oxidized LDL, 50 μg/ml	0.4
7	EC-conditioned F-10 medium	5.8
	Zymosan-activated rat serum	10.8
	+ EC-modified LDL, 50 μg/ml	3.0

See text for assay conditions. BHT was added in 20 μl of EtOH to yield a final concentration of 20 μM. LDL, EC-modified LDL, and Cu^{2+}-oxidized LDL were added to EC-conditioned F-10 medium in equal volumes to give a final LDL concentration of 50 μg/ml. Results are from representative experiments and are compared to the appropriate conditioned medium control in that experiment.

inhibitory effect. This was confirmed (Table 2, exp. 4) by mixing EC-conditioned medium prepared in the usual way (i.e., in the absence of LDL) with medium that had contained LDL throughout the EC incubation. The chemotactic activity in the former was inhibited by admixture with the latter. Inhibition of chemotactic response to EC-conditioned medium was a function of EC-modified LDL concentration—about 50% at 25 μg/ml and >80% at 100 μg/ml. The migration of unstimulated macrophages in the Boyden chamber was also inhibited by reisolated EC-modified LDL (CI = 0.48 ± 0.5; n = 5).

We have shown previously that EC modification of LDL entails peroxidative changes and can be prevented by tocopherol or BHT (24). As shown in Table 2, exp. 3, the inhibitory effect seen when LDL was present in the medium throughout the conditioning incubation was abolished if the medium also contained BHT during the incubation. We have also shown previously that EC modification of LDL does not occur if one uses DMEM in place of F-10 medium, probably because of the lower concentrations of divalent cations in the former (24). When DMEM was substituted for F-10 medium, there was still appreciable chemotactic activity in the conditioned DMEM (Table 2, exp. 5) but now the presence of LDL during the incubation no longer masked the chemotactic activity. The results suggested strongly that the LDL must undergo the oxidative changes associated with EC modification in order to become inhibitory. Though EC-modified LDL is toxic to some cells, the macrophages incubated 5 hr with 50 μg of EC-modified LDL per ml remained viable (trypan blue exclusion).

Incubation of LDL in the absence of cells but in presence of high concentrations of Cu^{2+} yields an oxidatively damaged LDL (24). This LDL shares a number of properties with EC-modified LDL, including cell toxicity (29, 30) and enhanced degradation by macrophages (24). As shown in Table 2, exp. 6, Cu^{2+}-oxidized LDL (prepared in the absence of cells) potently inhibited the chemotactic activity in EC-conditioned medium. As already mentioned, native, unincubated LDL was not inhibitory under similar conditions (Table 2, exp. 2).

To test whether the inhibitory effect of EC-modified LDL was specific to the chemotactic activity generated by EC, we tested its ability to inhibit the chemotactic activity of zymosan-activated rat serum. As shown in Table 2, exp. 7, EC-modified LDL was again strongly inhibitory.

Role of the Acetyl-LDL Receptor. Since both EC-modified LDL and Cu^{2+}-oxidized LDL are preferentially degraded by macrophages and, at least partially, by way of the same receptor (the acetyl-LDL receptor), we considered the possibility that the inhibition of macrophage chemotaxis might simply require interaction with that receptor. However, as shown in Table 3, acetyl-LDL failed to inhibit macrophage migration in response to the EC-conditioned medium. In fact, acetyl-LDL added to control F-10 medium actually stimulated macrophage migration (CI = 5.5).

Brown *et al.* have shown that fucoidin, polyinosinic acid, and a number of other negatively charged macromolecules compete with acetyl-LDL, presumably by interaction with the same specific receptor (31). As shown in Table 3, both fucoidin and polyinosinic acid shared with acetyl-LDL the ability to stimulate macrophage motility. EC-modified LDL partially inhibited the chemotactic effects of fucoidin. The data shown in Table 4 indicate that the predominant effects were truly chemotactic, although polyinosinic acid may have some chemokinetic activity as well.

Basis for the Inhibitory Effects of EC-Modified LDL. EC modification of LDL to a form recognized by the macrophage acetyl-LDL receptor is associated with extensive changes in its composition (24, 32). These include extensive hydrolysis of LDL phosphatidylcholine to l-PtdCho, degradation of apoprotein B, and a marked increase in lipid peroxides (thiobarbituric acid-reactive material) (31). The l-PtdCho and free fatty acids generated during EC modification can largely be removed by incubating the EC-modified LDL with albumin and then recovering the EC-modified LDL by ultracentrifugation. As shown in Table 5, EC-modified LDL incubated previously for 24 hr in the presence of albumin lost its ability to inhibit the chemotactic activity of EC-conditioned F-10 medium (Table 5, sample 3). EC-modified LDL incubated in the absence of albumin (but otherwise under identical conditions and reisolated in the same way) did not lose its inhibitory effect (Table 5, sample 2). Unstimulated migration was affected similarly (Table 5, samples 5 and 4). These studies suggested that one or more of the oxidized lipid components of the EC-modified LDL were responsible for its inhibitory effect. This was demonstrated explicitly by extracting the lipids from EC-modified LDL and showing that the lipid extract contained the inhibitory material(s) (Table 6)

Table 3. Chemotactic activity of acetyl-LDL, fucoidin, and polyinosinic acid

Exp.	Sample	CI
1	Control F-10 medium	(1.0)
	EC-conditioned F-10 medium	8.7
	+ Acetyl-LDL, 100 μg/ml	7.6
	Control F-10 medium	
	+ Acetyl-LDL, 100 μg/ml	5.5
	+ Fucoidin, 50 μg/ml	6.7
	+ Fucoidin + EC-modified LDL	2.0
2	Control F-10 medium	(1.0)
	+ Polyinosinic acid, 50 μg/ml	16.8
	EC-conditioned F-10 medium	17.4

Zymosan-activated serum was prepared by incubating zymosan (1 mg/ml) in rat serum for 1 hr at 37°C. A 17,600 × g supernatant was used for the assay of chemotactic activity.

Table 4. Chemotactic activity of acetyl-LDL, fucoidin, and polyinosinic acid

Addition to lower chamber	Addition to upper chamber	CI
Acetyl-LDL	None	4.0
Acetyl-LDL	Acetyl-LDL	0.96
None	Acetyl-LDL	0.92
Fucoidin	None	2.2
Fucoidin	Fucoidin	0.9
None	Fucoidin	0.9
Polyinosinic acid	None	5.1
Polyinosinic acid	Polyinosinic acid	1.6
None	Polyinosinic acid	1.7

Acetyl-LDL was added to control (unincubated) F-10 medium at 100 μg/ml; fucoidin and polyinosinic acid were at 50 μg/ml.

whereas lipid extracts prepared from native LDL were not inhibitory.

DISCUSSION

These studies demonstrate the production by cultured rabbit aortic EC of a chemotactic factor (or factors). Such a factor could play a role in the recruitment of monocytes into the artery wall and thus in the development of atherosclerotic lesions, particularly if production is favored, directly or indirectly, by hyperlipidemia. It will be important now to determine in what ways production of this material is regulated physiologically and pathophysiologically. While these studies were in progress, Berliner and coworkers reported preliminary observations of a chemotactic activity for normal circulating human monocytes (rather than resident mouse peritoneal macrophages) released by cultured bovine and human umbilical vein EC (33). It differs in at least one important respect from the activity described here in that it is heat labile rather than heat stable.

The present studies were initiated with the thought that LDL itself might be chemotactic or that it might stimulate the generation of chemotactic activity from EC. Instead, what was found was that native LDL has no intrinsic chemotactic activity nor does it affect the chemotactic activity in EC-conditioned medium. In fact, the presence of LDL during the incubation of EC *prevented* expression of chemotactic activity. This was shown to be due to the EC-modified LDL, a product generated as a result of metal-dependent peroxidation of LDL accompanied by hydrolysis of LDL phospholipids and degradation of apoprotein B (32). The present studies show that this inhibitory effect of EC-modified LDL resides in the lipid fraction and can be abolished by incubating it overnight with serum albumin to remove l-PtdCho and free fatty acids. EC-modified LDL prevented the migration of macrophages not only in response to the factor(s) produced by EC but also in response to zymosan-activated rat serum, suggesting that the effect was not specifically on the interaction between chemotactic factor and receptor but rather on subsequent events, events that may be more generally involved in macrophage motility. This is also suggested by the decrease in migration of unstimulated cells induced by EC-modified LDL. The inhibition of cell motility may well be accompanied by additional effects on macrophage functions.

Table 5. Treatment of EC-modified LDL with bovine serum albumin abolishes its inhibitory effect on chemotactic activity

Sample	CI
EC-conditioned F-10 medium	5.0
+ Reisolated EC-modified LDL (albumin *not* present)	0.5
+ Albumin-treated, reisolated EC-modified LDL	4.8
Control F-10 medium	
+ Reisolated EC-modified LDL (albumin *not* present)	0.1
+ Albumin-treated, reisolated EC-modified LDL	0.9

Albumin treatment of EC-modified LDL was carried out in order to remove free fatty acids and lysophosphatides. Reisolated EC-modified LDL was adjusted to a concentration of 100 μg/ml in F-10 medium for the chemotactic assay. Results from a duplicate experiment were in close agreement.

Table 6. Inhibition of chemotaxis by lipids extracted from EC-modified LDL

Sample	CI
EC-conditioned F-10 medium	4.0
+ EC-modified LDL, 50 μg/ml	0.3
Lipid extract of native LDL	0.5
EC-conditioned F-10 medium + lipid extract of native LDL	4.0
Lipid extract of EC-modified LDL	0.2
EC-conditioned F-10 medium + lipid extract of EC-modified LDL	0.5

The possibility that the inhibitory effect of EC-modified LDL might depend on its interaction with the acetyl-LDL receptor led to the testing of other macromolecules recognized by that receptor (23). Three such compounds—acetyl-LDL, fucoidin, and polyinosinic acid—not only failed to inhibit but were actually found to be chemotactic in their own right. Furthermore, treatment of EC-modified LDL with albumin to remove l-PtdCho and free fatty acids did not prevent its interaction with the acetyl-LDL receptor yet it prevented the inhibitory action on macrophage movement, presumably by removing peroxidized free fatty acids or other oxidized components. It is quite possible that if *all* of the inhibitory components could be removed from EC-modified (oxidized) LDL, it might then be chemotactic in its own right, like the other molecules that bind to the acetyl-LDL receptor.

The inhibitory effect of EC-modified LDL on macrophage motility could have significance with respect to atherogenesis. One might visualize a normal steady state in which monocytes penetrate into the subendothelial space at some rate but leave it at a comparable rate. EC-modified LDL generated in the subendothelial space might then reduce the rate at which monocytes can leave and thus exert a "trapping" effect. This phenomenon might be more marked when plasma LDL levels are elevated and the generation of EC-modified LDL therefore enhanced. Other inhibitors of macrophage motility have been recognized and have been postulated to play a role in the general inflammatory process at other sites by an analogous trapping phenomenon (34).

How the present observations relate to macrophage recruitment *in vivo* remains to be determined. Thus far only the resident mouse peritoneal macrophage has been used in our studies. This cell differs, of course, in a number of ways from the circulating monocyte. For example, the circulating monocyte expresses fewer acetyl-LDL receptors (4) and secretes less lipoprotein lipase (10). On the other hand, monocytes, once in the subendothelial space, may well acquire properties like those of other resident macrophages, in which case chemotactic substances recognized by the acetyl-LDL receptor might act to prevent their exiting from the artery wall. In summary, the chemotactic activity of the factor(s) released by EC may contribute to the recruitment and/or retention of macrophages in developing atherosclerotic lesions. Modified forms of LDL (such as acetyl-LDL) and natural polyanionic macromolecules analogous to fucoidin may also play a role. Once oxidized, however, modified forms of LDL, including EC-modified LDL, actually *inhibit* macrophage motility and thus may exert a

"trapping effect" favoring macrophage accumulation in developing lesions.

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