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CORRELATION OF C3b-RECEPTOR ACTIVITY AND DIPHENYLHEXATRIENE POLARIZATION IN A MURINE MACROPHAGE CELL LINE

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SUMMARY: Treatment of a macrophage cell line with lymphokine (LK)-rich lymphocyte culture supernatants activates the cells for C3b receptor-mediated phagocytosis and leads to simultaneous depolarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) incorporated into the cells. Maximal effects were observed after 14 hours of incubation with LK. DPH polarization in liposomes prepared from lipids of untreated and LK-treated cells were the same. Furthermore, LK-treatment had no significant effects on phospholipid fatty acid composition, cholesterol content, or phospholipid composition. These observations indicate LK induces alterations in the organization and dynamics of the plasma membrane that lead to facilitation of phagocytosis.

INTRODUCTION. Macrophages bind C3b-coated particles via the C3b receptor but must be stimulated or activated in order to express C3b-receptor-mediated phagocytosis (1). Such activation of unstimulated murine macrophages can be brought about by exposing the cells to lymphokine (LK)-rich culture supernatants (2,3). The underlying mechanism for this activation is not understood. A possible role for lipids in endocytosis and nonspecific phagocytosis has been reported (4,5). Increased lateral diffusion of a lipid probe in membranes of elicitated macrophages have been described (6), and it has also been suggested that receptor mobility is a prerequisite for C3b-coated particle ingestion (3) implying that a change in dynamics of plasma membrane constituents underlies lymphokine activation. Here, we have studied the correlation between induction of C3b-receptor activity by LK and fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene in the macrophage cell line RAW 264.7. We have found that maximal phagocytic activity is exhibited at minimal polarization values of the probe. Since microviscosity of liposomes prepared from lipids isolated from LK-treated cells and those of untreated cells were the same and since cellular

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lipid composition had not significantly changed, it is concluded that LK-induced activation produces changes in the organization and dynamics of the plasma membrane.

MATERIALS AND METHODS

The macrophage cell line RAW 264.7 (7) was grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics at 37°C in a humidified 5% CO_2 environment with a generation time of about 20 hours. The LK preparation was derived from spleen cells of Balb/c mice with a chronic BCG infection following *in vitro* stimulation with 50 µg/ml of PPD according to Ruco and Meltzer (8). Control preparations were obtained from normal spleen cells incubated with PPD. The LK preparations activate macrophages for C3b-receptor-mediated ingestion at dilutions up to 1:1500 with 1:1500 being the optimum. This LK preparation can activate mouse macrophage for C3b-receptor-mediated ingestion and to inhibit the intracellular replication of *T. gondii* (9). The cells were grown in petri dishes until the end of log phase and then at various times prior to harvest the LK preparation was added at a dilution of 1:500. Each plate yielded about $2x10^7$ cells. Control plates consisted of cultures that had received no treatment (0 time) or received control supernates. The trypan blue test showed that cells were greater than 90% viable. C3b-receptor-mediated ingestion was assayed as previously described (2,10). The ingestion index was calculated as the product of the number of cells which ingest C3b-coated erythrocytes times the number of ingested particles per cell.

To label with DPH, cells were harvested, washed with cold phosphate buffered saline (PBS), and resuspended in the same solution at 10^6 cells/ml. Cells were labeled with DPH and polarization determined as described before (11,12) except that 1 ml of the cell suspension was added to 0.6 µg of DPH and no glass beads were used. Liposomes were prepared also as described before (11) after adding 1 ml of PBS to a dried film of lipids isolated from 10^6 cells and 0.6 µg of DPH.

Procedures for lipid isolation, phospholipid fatty acid analysis, and lipid phosphorus determination have been described (13,14). Cholesterol was measured according to Mahoney and Scott (15), using a 3% SP-2300 on supelcoport column. To determine incorporation of ^{32}P into the phospholipids, at the time of addition of LK (14 hours prior to harvest) 0.33 $mC^{32}P$ were added to all cultures. Cells were harvested and washed twice with PBS. Lipids were extracted and fractionated according to Rouser et al. (16), using standard phospholipids for identification. Gel spots containing phospholipids were scraped and their radioactivities counted as described previously (17).

RESULTS AND DISCUSSION

The time-dependent induction of phagocytic activity and decrease in DPH polarization in the cells by LK-rich lymphocyte culture supernatants is shown in Figure 1. There was no significant phagocytosis during the first 4 hours of treatment. Then, a significant increase in activity was observed reaching a maximal level after 14 hours of incubation with LK such that the index of ingestion increased from 10 at 0 time to 450 at 14 hours. Concomitantly, there was a gradual decrease in DPH polarization in the same cell cultures,

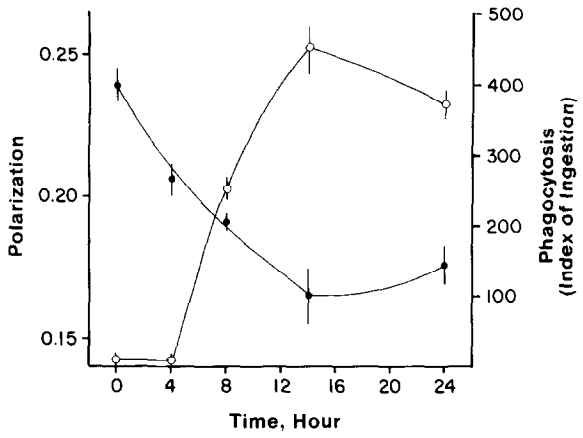

Figure 1. Effect of incubation of RAW 264.7 cells with lymphokine-rich lymphocyte culture supernatants on phagocytosis by the C3b receptor (O) and polarization of DPH (●) incorporated into the cells.

from 0.24 ± 0.01 at 0 time to 0.16 ± 0.01 after 14 hours of incubation, which is statistically significant at $P < 0.001$. To establish that the observed effects on phagocytic activity and DPH depolarization were due to presence of LK in the lymphocyte culture supernatant, control experiments were carried out in which control (LK-deficient) supernatant was added to cultures of the cells. These cells did not acquire phagocytic activity and failed to show any decrease in DPH polarization demonstrating a relationship between changes in the plasma membrane which lead to the depolarization of DPH and increases in phagocytic activity. Such changes could be brought about by changes in cellular lipid composition (18) or reorganization of constituents of the plasma membrane. Experiments, therefore, were carried out to distinguish between these possibilities.

First, DPH polarization was compared in liposomes prepared from lipids isolated from untreated and LK-treated cells. As shown in Table I, DPH polarization was the same in the two types of liposomes indicating identical microviscosity of the lipids probably due to the same lipid composition. The lipid compositions of these cells were determined (Table II). Treatment with LK had no effects on the fatty acid composition of phospholipids which showed 15-18% C16:0, 8% C16:1, 17% 18:0 and 45% 18:1. Likewise, the cholesterol

TABLE I: Effect of lymphokine treatment on polarization of DPH incorporated into cells and liposomes prepared from lipid extracts.

	Polarization		
Treatment[a]	Cells	Liposomes	
+	0.20 ± 0.05	0.20 ± 0.02	
-	0.31 ± 0.02	0.19 ± 0.01	$P < 0.01$
	$P < 0.01$		

a. 14 hours prior to harvest lymphokines were added to the culture (+); (-), no additions.

content of the cells remained unchanged at approximately 0.8 - 0.9 $\mu g/10^6$ cells. Treatment with lymphokines resulted in a reduction of phospholipid content by 35%, from 19.8 ± 2.6 µg lipid-phosphorus per 10^6 cells in untreated cultures to 12.6 ± 2.4. Such a decrease in phospholipid content of elicitated peritoneal cells have already been reported (19). The significance of this change is not understood but it cannot explain the increased depolarization of DPH in membranes exposed to LK since only a substantial increase in phospholipid content could explain a decrease in DPH polarization (18). Finally, we found that LK treatment did not result in altered phospholipid composition since it had no effect on the extent of incorporation of ^{32}P into various classes of phospholipids (Table II).

These studies show that activation of the macrophage cell line with LK did not result in any significant compositional changes in lipids that could produce a bilayer with lower microviscosity. Recently, it has been reported that mouse peritoneal macrophages activated with LK-rich leukocyte culture supernatants for tumor cytotoxicity have altered lipid composition (20). The differences between those studies and our findings may be related to our use of a macrophage cell line and/or to their measurement of tumor cytotoxicity as a parameter of activation. Cytotoxicity of LK-treated macrophages requires additional signals which may facilitate their observed lipid compositional changes (21,22).

TABLE II: Effect of incubation with lymphokines on lipid content and composition of RAW 246.7 cells.

Measurement	Control	LK Treated
Cholesterol ($\mu g/10^6$ cells)	0.88 ± 0.18	0.84 ± 0.12
Phospholipid ($\mu g/10^6$ cells)	19.8 ± 2.6	12.6 ± 2.4
^{32}P Incorporation (% Total)		
Cardiolipin	16 ± 2	16 ± 2
Lecithin	60 ± 2	54 ± 2
Lysolecithin	1	1
Phosphatidylethanolamine	10 ± 2	10 ± 2
Phosphatidylserine	7 ± 3	12 ± 2
Sphingomyelin	6 ± 1	6 ± 1
Phospholipid Fatty Acid Composition (% Total)[a]		
C16:0	15	18
C16:1	7	8
C18:0	17	17
C18:1	45	44

a. 14:0, 18:2, 18:3, 20:3 and 22:0 each constituted 1 to 3%.

It is clear that the observed "fluidizing" effect of LK cannot be explained in terms of alterations in overall cellular lipid composition, and, therefore, must be explained in terms of reorganization of the plasma membrane. Such a reorganization could be brought about by any of a number of likely rearrangement. More fluid components of the lipids could be inserted into the plasma membrane. Alternatively, LK could induce a clustering of proteins leaving large areas of the bilayer deplete of proteins and with lower fluidity, or it could cause vertical displacement of proteins (18) minimizing interaction between the probe in the inner core of the bilayer and intrinsic proteins (23, 24) thus registering a greater depolarization of DPH. These possibilities are being investigated.

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