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PARTICIPATION OF SERUM PROTEINS IN THE INFLAMMATION-PRIMED ACTIVATION OF MACROPHAGES[1]

NOBUTO YAMAMOTO,[2-4] NORMAN P. WILLETT,[5] and DWIGHT D. LINDSAY[5]

[2]*Department of Biochemistry*
[3]*Fels Institute for Cancer Research and Molecular Biology*
[5]*Department of Microbiology and Immunology*
Temple University School of Medicine
Philadelphia, Pennsylvania 19140

Abstract—Inflamed lesions release degradation products of membrane lipids, lysophospholipids, and inflamed tumor tissues release alkylglycerols. Macrophages were activated by administration of lysophosphatidylcholine (lyso-Pc) or dodecylglycerol (DDG) to mice. In vitro treatment of mouse peritoneal cells (mixture of nonadherent and adherent cells) with lyso-Pc or DDG in fetal calf serum supplemented medium for 30 min, followed by 3-h cultivation of adherent cells (macrophages) alone, resulted in greatly enhanced Fc-receptor mediated phagocytic activity and superoxide generating capacity of macrophages. The tumor lipid metabolite, DDG, is far more potent (400-fold) than lyso-Pc in terms of doses required for the maximal levels of macrophage activation. The inflammation-primed macrophage activation required a serum factor, vitamin D binding protein, as a precursor for the macrophage activating factor. Treatment of mouse peritoneal cells with 1 μg lyso-Pc/ml or 50 ng DDG/ml in a serum-free 0.1% egg albumin supplemented medium for 30 min, followed by 3-h cultivation of the treated peritoneal cells in a medium supplemented with a very small amount (0.0005–0.05%) of ammonium sulfate [20–50% saturated $(NH_4)_2SO_4$] precipitable protein fraction of FCS, resulted in greatly enhanced superoxide generating capacity of macrophages. The ammonium sulfate precipitable fraction was found to contain vitamin D binding protein.

INTRODUCTION

Microbial infections cause inflammation, which attracts and activates phagocytes and induces immunity against the infectious agents, implying that biochemical

[1] Supported in part by NIH grant AI-32140 to N.Y.
[4] Present address: Laboratory of Cancer Immunology and Molecular Biology, Albert Einstein Cancer Center, Philadelphia, Pennsylvania 19141.

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signals should be radiating out from the inflamed lesions. Inflamed tissues produce lysophospholipids, degradation products of membranous phospholipids, as a consequence of cellular damage (1–3). Inflammation of cancerous tissues (e.g., melanoma and bladder cancer), induced by local administration of BCG (*Bacillus Calmette-Guerin*) or other bacterial cells, can result in regression of local as well as metastasized tumors, suggesting development of specific immunity against the cancerous cells (4–6). However, injection of these bacteria to normal (noncancerous) tissues in cancer-bearing hosts does not cause tumor regression. Inflamed cancerous tissues produce alkyl-lysophospholipids and alkylglycerols as well as lysophospholipids (3, 7), because cancerous cells contain alkylphospholipids and monoalkyldiacylglycerols (8, 9). Lysophospholipids and alkylglycerols are potent chemotactic and macrophage stimulating agents (1–3, 7). Administration of lysophospholipids or alkylglycerols to mice activates macrophages to phagocytize target antigens or cells via the Fc-receptor but not the C3b receptor (1–3, 7). In vitro treatment of mouse peritoneal cells (mixture of adherent and nonadherent cells) with lysophosphatidylcholine (lyso-Pc) or dodecylglycerol (DDG) for a few hours produces a markedly enhanced Fc-receptor mediated phagocytic activity of macrophages (1–3, 7, 10). Treatment of adherent cells (macrophages) alone with lyso-Pc or DDG results in no activation of macrophages, suggesting participation of nonadherent cells (B and T cells) in macrophage activation. Cocultivation of untreated macrophages with lyso-Pc- or DDG-treated nonadherent cells in medium RPMI 1640 containing 10% fetal calf serum (FCS medium) produces a greatly enhanced Fc-receptor mediated phagocytic activity of macrophages in 3 h (7, 10–12). Thus, this in vitro activation mechanism of macrophages with lipid metabolites reproduced the in vivo inflammation-primed activation of macrophages and provided us with a tool to identify the factors involved in the major inflammation-primed macrophage activation cascade.

When a small amount of FCS (1%) was added to 0.1% egg albumin supplemented medium RPMI 1640 (EA medium) for cultivation of lyso-Pc- or DDG-treated peritoneal cells, a greatly increased Fc-receptor mediated phagocytic activity of macrophages was observed as compared with serum protein-free EA medium (13, 14). Studies with serum fractionation using preparative starch block electrophoresis of FCS and adult human serum showed that α_2-globulin fraction is required for macrophage activation (12–14). A serum factor in α_2-globulin fraction can be modified by lyso-Pc- or DDG-treated B cells and untreated T cells to yield a macrophage activating factor (12, 13). Thus, a precursor of the macrophage activating factor exists in the α_2-globulin fraction (12–15). We identified vitamin D_3 binding protein (DBP; human DBP is known as group-specific component or Gc) as the precursor for macrophage activating factor (13, 15). DBP is a glycoprotein and can be converted to a

potent macrophage activating factor by stepwise interaction with β-galactosidase of lyso-Pc- or DDG-treated B cells and sialidase of untreated T cells (15).

In inflammatory diseases, the DBP concentration levels in patient sera increase (16). Thus, evaluation of the DBP concentration in patient sera may provide a means to identify disease stages of infection and inflammation. In contrast, DBP concentration levels in sera of liver cirrhosis and chronic hepatitis patients decrease as much as five-fold and 50-fold, respectively (16). Since DBP is produced in the liver, acute liver diseases and hepatotoxic therapeutic agents are likely to cause a decrease in DBP levels in patient sera. Quantitation of concentration levels of DBP in patient sera might prove useful for diagnosis, classification, and/or predictive clinical purposes as well as monitoring the effects of therapy. Therefore, a method to quantitate DBP concentrations in individual serum should also be established for diagnostic parameters of disease states.

Recently we found that human DBP is precipitable by 20–50% saturated ammonium sulfate. Ammonium sulfate precipitation of fetal calf serum strikingly increases the precursor activity for macrophage activating factor, suggesting that this fractionation eliminates serum inhibitors. Although 50% saturated ammonium sulfate is known to precipitate larger serum proteins such as γ-globulin but not smaller proteins such as albumin (mol wt 65,000), 20–50% ammonium sulfate precipitates even smaller protein DBP (mol wt 52,000). Thus, this fractionation procedure with ammonium sulfate is sufficient to approximate the serum content of DBP as the precursor activity of serum for the macrophage activating factor.

In this communication we report lipid metabolites of cancerous membranes as potent macrophage stimulating agents that activate macrophages for enhanced phagocytosis and superoxide generation. This inflammation-primed activation of macrophages requires an ammonium sulfate precipitable serum protein, vitamin D_3 binding protein.

MATERIALS AND METHODS

Animals. Female BALB/c mice, 7–12 weeks of age weighing about 20 g, were obtained from the Jackson Laboratories, Bar Harbor, Maine. The mice were housed in AALAC-accredited animal quarters with temperature and light control and were fed Purina Mouse Chow and water ad libitum.

Chemicals and Reagents. Lysophosphatidylcholine and dodecylglycerol were purchased from Sigma Chemical Co. (St. Louis, Missouri). Gamma-globulin-free fetal calf serum (FCS) was obtained from GIBCO Laboratories, Gaithersburg, Maryland.

Fractionation of Fetal Calf Serum by Ammonium Sulfate Precipitation. Various amounts (0.5, 1, or 2 ml) of saturated ammonium sulfate were added to 2 ml of γ-globulin-free fetal calf serum and incubated for 1 h at room temperature. Supernatant and precipitate were separated by

low-speed centrifugation for 10 min. The pellet was suspended in 1–1.5 ml of saline (0.85% NaCl) and dialyzed three times against a large volume (500 ml) of saline at 4°C. The dialyzed fraction was adjusted by adding saline to yield the same volume (2 ml) as the original volume of the serum used for ammonium sulfate precipitation. Thus, the concentration of the protein of interest per volume corresponded to that in the original serum used. These fractions were filtered through a Millipore filter (type HA 0.45 μm) for sterilization.

Isolation of Mouse Peritoneal Cells and Enumeration of Adherent Macrophages. The detailed procedures for isolation and cultivation of mouse peritoneal cells (mixture of adherent and non-adherent cells) and enumeration of adherent macrophages were described previously (7, 10). Briefly, resident peritoneal cells were collected by peritoneal lavage with PBS supplemented with heparin. The cells were then washed three times in cold PBS and resuspended in FCS medium or EA medium. The desired cell number (6–9 × 10^5/ml) of the peritoneal cells was determined and 0.5-ml aliquots of the cells were laid onto 12-mm cover glasses (Bellco, Vineland, New Jersey), which had been placed in the 16-mm-diameter wells of tissue culture plates (Costar, Cambridge, Massachusetts). The cells were incubated at 37°C in a humidified CO_2 incubator for 30 min to allow the adherence of macrophages to the cover glasses. About 35% of resident peritoneal cells are adherent cells. Macrophages were identified by morphological criteria and ability to ingest latex particles (0.81 μm in diameter) (17). About 96% of the adhering cells were macrophages by the above criteria.

Treatment of Mouse Peritoneal Cells with Lyso-Pc or DDG. Lyso-Pc (20 μg/ml) or DDG (50 ng/ml) was added to the peritoneal cells in FCS medium and mixed gently. After 30 min of incubation, the adherent cells were washed with PBS to remove residual lyso-Pc or DDG and nonadherent cells. The washed adherent macrophages alone were cultured for 3 h prior to phagocytosis assay.

Alternatively, peritoneal cells were treated with 1 μg lyso-Pc/ml or 50 ng DDG/ml in EA medium for 30 min. To remove residual lyso-Pc or DDG, the nonadherent and adherent cells were separately washed with PBS and admixed in FCS medium or EA medium supplemented with various amounts of ammonium sulfate precipitate. These treated peritoneal cells (mixture of treated non-adherent and adherent cells) were cultured for 3 h prior to phagocytosis assay.

Cocultivation of Untreated Adherent Cells with Lyso-Pc- or DDG-Treated Nonadherent Cells. Peritoneal nonadherent cells were treated with various concentrations of lyso-Pc or DDG in EA medium for 30 min and washed with PBS. The washed nonadherent cells were admixed with untreated adherent cells. Various amounts of FCS or 20–50% ammonium sulfate precipitate were added to the EA medium for cocultivation of untreated adherent cells with the lyso-Pc- or DDG-treated nonadherent cells. After 3 h of cultivation, phagocytic activity and superoxide generating capacity of macrophages were assayed.

Phagocytosis Assay. Fc-receptor mediated phagocytosis was measured using sheep erythrocytes (E) coated with immunoglobulin G (IgG) of anti-E (hemagglutinin), EIgG conjugate, as described previously (7, 11). Briefly, washed E (Dutchland, Denver, Pennsylvania) were coated with subagglutinating dilutions of purified rabbit anti-E IgG fraction (Cordis Laboratories, Miami, Florida). Each macrophage-attached cover glass was overlaid with 0.5 ml of a 0.5% suspension of the above EIgG conjugate in medium RPMI 1640 and incubated at 37°C in a humidified 5% CO_2 incubator for 1 h. Noninternalized erythrocytes were lysed by immersing the cover glass in a hypotonic solution (1:5 PBS) for 5–10 sec. The macrophages were fixed with methanol, air dried, and stained with Giemsa and phagocytosis was quantified microscopically. The data are expressed as the ingestion index described by Bianco et al. (18). Ingestion index = (% macrophages with phagocytized E) × (average number of E phagocytized per macrophage).

Assayed for Generation of Superoxide in Activated Macrophages. A modified assay method of Pick and Mizel (19) was used to determine levels of superoxide production. After washing peritoneal cells, the desired number of the cells (6 × 10^5–3 × 10^6/ml) were suspended in EA medium and placed in 16-mm culture wells and incubated at 37°C in a humidified 5% CO_2 incubator

for 30 min to allow macrophage adherence to plastic substrata. The nonadherent cells were isolated and treated with various concentrations of lyso-Pc or DDG in EA medium for 30 min. The treated nonadherent cells were washed and added to untreated adherent cells and cultured for 3 h in EA medium supplemented with 0.1–10% FCS or 0.0005–0.5% of 20–50% saturated ammonium sulfate precipitate. The adherent macrophages were washed twice with Krebs-Ringer phosphate buffer solution containing 1% glucose to remove nonadherent cells. Immediately after removal of the second wash, the macrophages (2×10^5–1×10^6 macrophages/ml) were overlaid with 20 μg cytochrome *c*/ml and incubated for 10 min. About 30 min after addition of phorbol-12-myristate acetate (1–5 μg/ml) (20), the superoxide generating activity of the macrophages was determined spectrophotometrically (21). The data are expressed as nanomoles superoxide produced per minute per 10^6 cells.

RESULTS

Macrophage Activation by In Vitro Treatment of Peritoneal Cells with DDG or Lyso-Pc in Medium Supplemented with or without FCS. In vitro treatment of peritoneal cells (mixture of nonadherent and adherent cells) with 50 ng DDG/ml or 20 μg lyso-Pc/ml in FCS medium for 30 min, followed by 3 h cultivation of the washed adherent cells (macrophages) alone in either FCS or EA medium, resulted in development of a greatly enhanced phagocytic activity of macrophages (Table 1, lines a and c). However, treatment of peritoneal cells with 50 ng DDG/ml in EA medium for 30 min, followed by 3 h cultivation of adherent cells alone regardless of the presence of FCS in the media, resulted in no activation of macrophages as shown in Table 1, line b. Thus, signal transmission between nonadherent and adherent cells must occur during the 30-min DDG-treatment period, if serum is present (3, 7). When peritoneal cells were treated with 50 ng DDG/ml in EA medium for 30 min, and washed with PBS and cultured for 3 h in FCS medium, a greatly enhanced phagocytic activity was observed, as can be seen in Table 1, line b. Thus, cocultivation of the treated nonadherent cells and adherent cells in the presence of serum allows generation and transmission of a signal "macrophage activating factor" from the nonadherent cells to macrophages (7, 11).

Treatment of peritoneal cells with 20 μg lyso-Pc/ml in EA medium for 30 min, followed by 3-h cultivation of adherent cells alone in FCS medium or 3-h cultivation of the washed peritoneal cells (mixed of treated nonadherent and adherent cells) regardless of the presence of FCS in the media, did not activate macrophages and showed rather reduced phagocytic activities as compared with untreated peritoneal cells as shown in Table 1, line d. This appears to be due to the cytotoxicity of the high lyso-Pc dose to peritoneal cells in EA medium. Therefore, peritoneal cells were treated with various lower concentrations of lyso-Pc in EA medium (data not shown). When peritoneal cells were treated with 1 μg lyso-Pc/ml in EA medium for 30 min and the washed peritoneal cells

Table 1. Effect of Serum Protein on In Vitro Macrophage Activation during Treatment of Mouse Peritoneal Cells with Lysophosphatidylcholine (Lyso-Pc) and Dodecylglycerol (DDG) in FCS and EA Medium or Posttreatment Cultivation.[a]

During treament of peritoneal cells	Ingestion index with medium of posttreatment cultivation,			
Dose of agent in FCS or EA medium	FCS medium		EA medium	
	Ad. alone	Ad. + Nonad.	Ad. alone	Ad. + Nonad.
0 μg (no agent)	60 ± 13	62 ± 11	59 ± 14	66 ± 15
a. 50 ng DDG/ml (FCS)	296 ± 44	311 ± 59	290 ± 42	272 ± 53
b. 50 ng DDG/ml (EA)	58 ± 12	318 ± 63	63 ± 15	78 ± 14
c. 20 μg lyso-Pc/ml (FCS)	316 ± 41	339 ± 56	276 ± 48	248 ± 46
d. 20 μg lyso-Pc/ml (EA)	18 ± 5	12 ± 6	11 ± 4	14 ± 7
e. 1 μg lyso-Pc/ml (EA)	65 ± 12	322 ± 53	77 ± 24	65 ± 21
f. 1 μg lyso-Pc/ml (FCS)	95 ± 19	98 ± 21	93 ± 19	87 ± 22

[a] The peritoneal cells were treated with lyso-Pc or DDG in FCS medium or EA medium for 30 min and washed with PBS to remove the agent and the nonadherent cells. The adherent macrophages alone were cultured for 3 h in either FCS medium or EA medium prior to phagocytic assays. Alternatively, the treated nonadherent cells and macrophages were washed separately and admixed for 3 h of cultivation in FCS medium or EA medium prior to phagocytic assays. Values represent mean ± *SEM* of triplicate assays. FCS, FCS medium; EA, EA medium; Ad., adherent cells; Nonad., nonadherent cells.

were cultured in FCS medium for 3 h, a greatly increased phagocytic activity of macrophages was observed (Table 1, line e). However, treatment of peritoneal cells with 1 μg lyso-Pc/ml in FCS medium, followed by 3-h cultivation of the treated peritoneal cells regardless of the presence of FCS in the media, did not develop significant phagocytic capacity of macrophages as shown in Table 1, line f. This can be explained by the fact that serum albumin binds most lysophospholipid molecules (22–24). Since the extent of macrophage activation after treatment of peritoneal cells with 20 μg lyso-Pc/ml in FCS medium is equivalent to that with 1 μg lyso-Pc/ml in EA medium, a 20-fold increased potency of lyso-Pc in EA medium over that of lyso-Pc in FCS medium suggests albumin–lyso-Pc binding equilibrium, leaving about 5% unbound lyso-Pc molecules that can interact with peritoneal cells for macrophage activation. Treatment of peritoneal cells with 50 ng DDG/ml in either FCS medium or EA medium, followed by 3-h cultivation of the treated peritoneal cells in EA medium or FCS medium, respectively, resulted in a greatly enhanced phagocytic activity (Table 1, lines a and b). Thus, DDG-treatment of peritoneal cells with or without the presence of serum resulted in the same level of macrophage activation as long as serum is present either during treatment or the post-treatment coculti-vation period. This equal effectiveness of DDG-treatment with or without the

presence of serum also suggests that serum components do not bind DDG and only 50 ng DDG/ml is sufficient for stimulation of nonadherent cells regardless of the presence or absence of serum during the treatment.

Dose Effects of Lyso-Pc and DDG on Activation of Macrophages for Superoxide Generation. Activated macrophages produce superoxide immediately after phagocytosis of microbes or eukaryotic cells, resulting in bactericidal and cytocidal effects. Thus, the superoxide generating capacity of macrophages was used as a parameter for the inflammation-primed activation of macrophages. Lyso-Pc and DDG are lipid membrane perturbing agents (25). Treatment of macrophages with membrane perturbing agents such as unsaturated fatty acids (26) or sodium dodecyl sulfate (27, 28) can induce superoxide. To avoid direct contact of macrophages with lyso-Pc and DDG, peritoneal nonadherent cells were treated with EA medium containing various concentrations of lyso-Pc or DDG for 30 min, washed with PBS, and added to untreated adherent cells in 1% FCS supplemented EA medium for 3-h cultivation. Superoxide generating capacity of macrophages was analyzed as shown in Figures 1 and 2. Treatment

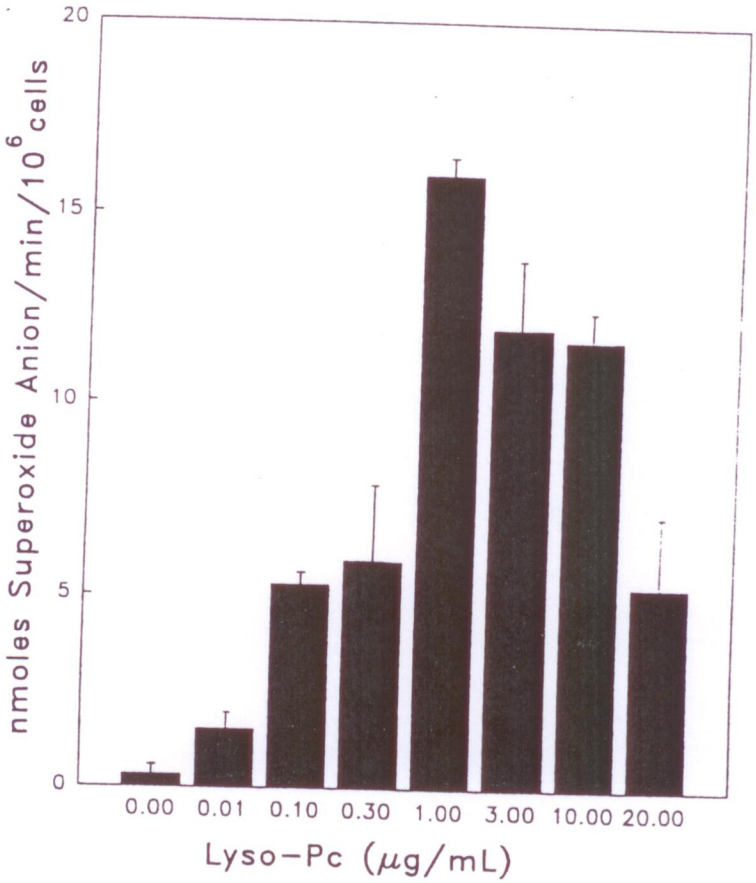

Fig. 1. Dose effect of lyso-Pc on in vitro activation of macrophages for generation of superoxide. Nonadherent cells were treated with various concentrations of lyso-Pc in EA medium for 30 min. The treated nonadherent cells were added to untreated macrophages and incubated for 3 h prior to superoxide generation assays. Values represent mean of triplicate assays.

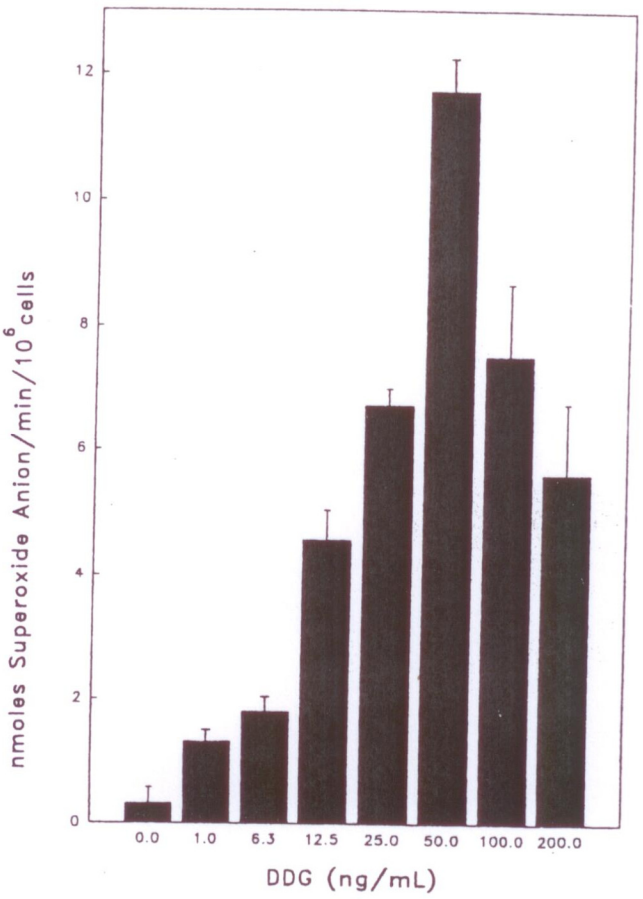

Fig. 2. Dose effect of DDG on in vitro activation of macrophages for generation of superoxide. Nonadherent cells were treated with various concentrations of DDG in EA medium for 30 min. The treated nonadherent cells were added to untreated macrophages and incubated for 3 h prior to superoxide generation assays. Values represent mean of triplicate assays.

of peritoneal nonadherent cells with 1 μg lyso-Pc/ml or 50 ng DDG/ml in EA medium resulted in a greatly enhanced superoxide generation of macrophages. The dose effect patterns and the most effective doses of these lipid metabolites for superoxide generation of the activated macrophages correspond well with those for phagocytic activity of the activated macrophages (2, 7). The dose effects of serum on lyso-Pc-primed activation of macrophages for superoxide generating capacity also correspond to that for phagocytic capacity, as shown in Figure 3. About 1% of FCS supported the maximal level of macrophage activation for phagocytic and superoxide generating capacities.

Effects of Ammonium Sulfate Precipitable Fraction of Fetal Calf Serum on Generation of Macrophage Activating Factor. A serum factor in the α_2-globulin fraction, vitamin D_3 binding protein (DBP), is a precursor for the macrophage activating factor (15). Human DBP was shown to be precipitable by treatment of whole serum with 50% saturated ammonium sulfate (13, 29). Although 50% saturated ammonium sulfate ordinarily precipitates larger serum

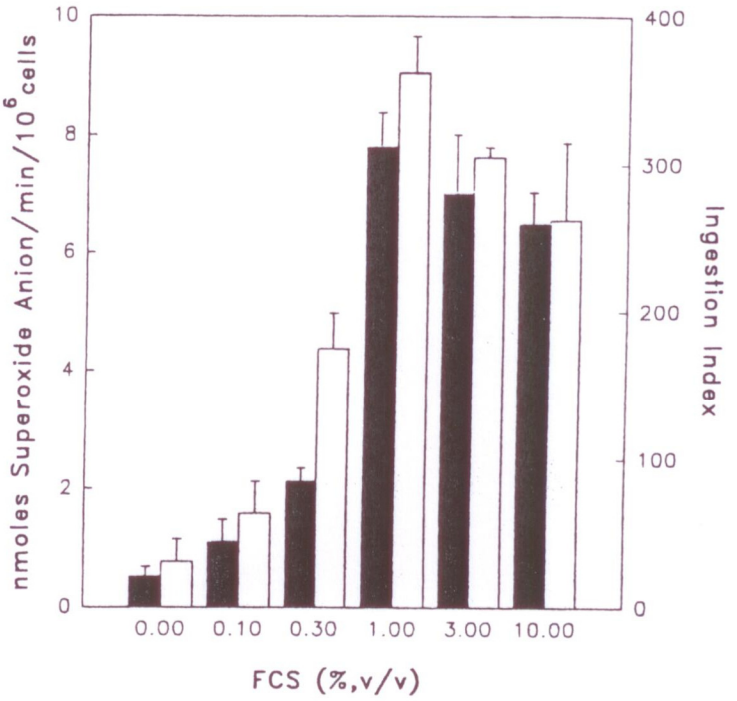

Fig. 3. Dose effect of fetal calf serum on in vitro activation of macrophages for phagocytic capacity and generation of superoxide. Nonadherent cells were treated with 1 μg lyso-Pc/ml in EA medium for 30 min. The treated nonadherent cells were added to untreated macrophages and incubated for 3 h in a medium supplemented with various concentrations of FCS prior to phagocytic (open bars) and superoxide (solid bars) generation assays. Values represent mean of triplicate assays.

proteins but not smaller proteins such as albumin, this procedure appeared to precipitate DBP, which is smaller than albumin. As shown in Table 2, a minute amount (as low as 0.0005%, v/v) of 50% ammonium sulfate precipitate of FCS in culture media efficiently supported lyso-Pc-primed macrophage activation as demonstrated by a greatly increased superoxide generating capacity. This level of macrophage activation can not be achieved by a medium supplemented with 1–10% FCS (Table 2). Thus, the ammonium sulfate precipitation greatly increases macrophage activating capacity of the serum factor. The DBP concentration of the 0.0005% ammonium sulfate precipitate is about 1.3 ng/ml. Since cocultivation of the treated nonadherent cells and untreated macrophages in EA medium supplemented with 1–2 ng purified DBP/ml produced a greatly enhanced macrophage activation (14), the macrophages are efficiently activated with the presence of ammonium sulfate precipitate of FCS as with purified DBP.

Differential Fractionation of Serum Factor by Various Concentrations of Ammonium Sulfate. As shown in Table 2, both 30% and 50% saturated ammonium sulfate precipitates equally supported lyso-Pc-primed macrophage activation. Moreover, as low as 20% saturated ammonium sulfate precipitate supported macrophage activation, although to a lesser extent. This near-threshold concentration, 20%, of ammonium sulfate for precipitation of DBP is most useful for

Table 2. Macrophage Activation by Cocultivation of Untreated Macrophages with Lyso-Pc-Treated Nonadherent Cells in Presence of Various Concentrations of $(NH_4)_2SO_4$ Precipitates.[a]

Saturated $(NH_4)_2SO_4$ used for fractionation of fetal calf serum (%)	Concentration (%) used for cultivation of a mixture of treated nonadherent cells and adherent cells	Superoxide nmol/min/10^6 cells	
		Lyso-Pc-pretreated nonadherent cells mixed with adherent cells	Lyso-Pc-treatment of mixture of nonadherent and adherent cells
20	0.05	16.02 ± 0.9	12.66 ± 0.25
30	0.05	30.23 ± 0.12	21.87 ± 0.04
50	0.05	32.45 ± 0.6	31.10 ± 0.3
50	0.005	10.11 ± 0.3	
50	0.0005		12.66 ± 0.25
FCS	10.0	6.48 ± 0.12	
FCS	1.0	7.80 ± 0.05	
FCS	0.1	1.10 ± 0.02	
FCS	0.05	0.73 ± 0.11	0.76 ± 0.08
None	saline	0.35 ± 0.09	0.47 ± 0.05

[a] Nonadherent cells were treated with 1 μg lyso-Pc/ml in EA medium for 30 min. After washing, the nonadherent cells were admixed with untreated adherent macrophages and cultured for 3 h in EA medium supplemented with 0.05, 0.005 or 0.0005% or 0.0005% of 20%, 30% or 50% $(NH_4)_2SO_4$ precipitates prior to superoxide generation assay. Values represent mean ± *SEM* of triplicate assays.

serum fractionation because of reduced amounts of coprecipitable serum proteins.

DISCUSSION

Injection of inflammation metabolites of membranous lipids, lysophospholipids, and alkylglycerols, into tissues establishes an aseptic inflammatory environment that attracts and activates macrophages. Both lysophospholipids and alkylglycerols act on B cells to increase membrane fluidity (2, 25). A fluidity increase in B cell membranes induces β-galactosidase, which removes galactose from serum DBP to yield a macrophage proactivating factor that can be further converted to the macrophage activating factor by the action of sialidase of untreated T cells (15). Thus, a membrane fluidity increase in B cells triggers the generation process of the macrophage activating factor.

Inflammation metabolites of cancerous membrane lipids, alkylglycerols, are potent macrophage stimulating agents (3, 7). Although both lysophospholipids and alkylglycerols can induce the same mechanism of macrophage activation, cancerous lipid metabolites (i.e., alkylglycerols) are far more (roughly

400-fold) effective than lysophospholipids in terms of the minimal doses required for macrophage activation. Since the majority of lysophospholipid molecules is bound by albumin in serum (22–24), dose effects of lysophospholipids and alkylglycerols on nonadherent cells in EA medium were compared. The extent of macrophage activation obtained by treatment with 50 ng DDG/ml in EA medium is equivalent to that with 1 μg lyso-Pc/ml in EA medium (Figures 1 and 2). Thus, about 20-fold lower molar concentration of DDG than that of lyso-Pc is required to achieve the same extent of macrophage activation in a serum-free medium. These results support the therapeutic efficacy of intratumor *Mycobacterium* injection (6) that can generate alkylglycerols.

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