# Enhancement of Antibody Production by Lysophosphatidylcholine and Alkylglycerol (43165)

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*Abstract.* **Inflammation products of normal and cancerous tissues, lysophosphatidylcholine and dodecylglycerol, were tested for their adjuvant effect on the antibody response. Mice treated with these agents and immunized with sheep erythrocytes simultaneously or at 3 days posttreatment developed a greatly enhanced antibody production as demonstrated by the Jerne plaque assay. Mice immunized at 3 days postadministration of agents did not significantly produce enhanced antibody-secreting cells as compared with those of mice simultaneously immunized. Since the mechanism of macrophage activation by lysophospholipids requires contribution of B and T cells, BALB/*c-nu/nu* mice treated with these agents and subsequently immunized with sheep erythrocytes did not produce antibodies. However, conditioned medium of *in vitro*-treated BALB/c-*nu/nu* B cells efficiently transmitted a signal to untreated BALB/c +/+ T cells for enhanced macrophage ingestion activity. This observation suggests that lysophospholipid-activated macrophages and T cells efficiently transmitted antigenic signal to the antibody-producing B cell population. Therefore, we conclude that these lipid metabolites have dual beneficial effects for the host by enhancing phagocytosis and antibody production. Thus, lysophosphatidylcholine and dodecylglycerol have potential practical application as adjuvants that could be administered separately or in combination with antigens.** [P.S.E.B.M. 1991, Vol 196]

Microbial infection induces inflammation, which attracts phagocytic cells and activates their ability to ingest invading microbes and dead cells. The inflamed lesions of normal and cancerous tissues release decomposed products of membranous lipids, lysophospholipids, and alkylglycerols, which are potent macrophage-activating agents. We previously reported that administration of various lysophospholipids and alkylglycerols to mice activates macrophages for fragment crystallizable (Fc)-mediated ingestion activity (1–3). *In vitro* treatment of macrophages alone with lysophosphatidylcholine (Lyso-Pc) or dodecylglycerol (DDG) was unable to enhance ingestion activity (1–4). Thus, involvement of nonadherent cells in the activation of macrophages was suspected. When a mixture of macrophages and nonadherent cells (B and T) was treated with 20 μg of Lyso-Pc/ml or 50 ng of DDG/ml, a greatly enhanced Fc-mediated ingestion was observed at 3 to 8 hr posttreatment (2). Such an *in vitro* treatment led to 85% of the macrophages to ingest target cells via Fc receptors.

Analyses of the contributory role of Lyso-Pc- or DDG-treated nonadherent cells in macrophages activation revealed that nonadherent cells transmit a factor(s) for the ultimate activation of Fc-mediated ingestion activity of macrophages. This developmental process for Fc-mediated ingestion activity of macrophages was studied by reconstitution analysis of adherent cells with enriched B and T cells previously treated with Lyso-Pc or DDG. The data conclusively indicate that treated B cells collaborate with T cells in a stepwise fashion for the exchange of a signaling factor(s) for macrophage activation. Specifically, Lyso-Pc- or DDG-treated B cells initiate the macrophage activation process by releasing and transmitting a signaling factor (pro-macrophage activating factor; PMAF) to T cells. In turn, the T cells modify the factor or supply a new

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factor (macrophage-activating factor; MAF) capable of the ultimate activation of macrophages for ingestion capacity (4, 5). Since both B and T cells are required for activation of macrophages, a defined T lymphocyte-deficient BALB/c-*nu/nu* mouse provided a highly pure B cell population free of T cells. Thus, a combination of BALB/c-*nu/nu* B cells with T cells of BALB/c +/+ mice provides a powerful tool to demonstrate the essential requirements of lymphocyte cell types and functions of lysophospholipid-activated macrophages. Since macrophages are antigen-presenting cells, such activated macrophages should efficiently transmit antigenic signals to the antibody-producing B cell population. Accordingly, the present communication reports the use of B cells from BALB/c-*nu/nu* mice to demonstrate macrophage activation mechanism by interspecies-specific signal transmission and the subsequent effect on the antibody response.

## Materials and Methods

**Materials.** Inbred BALB/c and BALB/c-*nu/nu* mice 6 to 12 weeks of age were obtained from The Jackson Laboratory, Bar Harbor, ME. The mice were housed in accredited animal quarters (American Association for Accreditation of Laboratory Animal Care) provided with temperature and light controls. Mice were fed Purina Mouse Chow and water *ad libitum*.

**Chemicals and Reagents.** Lyso-Pc and 1-*O*-dodecyl-rac-glycerol (DDG) were purchased from Sigma Chemical Co., St. Louis, MO. These reagents were greater than or equal to 99% pure; 0.5% precursor of lysophospholipids and no other compounds such as lipopolysaccharide were detectable. In addition to this information, we have tested for lipopolysaccharide in the final preparation of Lyso-Pc or DDG solution using the Limulus amebocyte lysate assay. No endotoxin was detectable in Lyso-Pc or DDG samples and media. The Lyso-Pc and DDG were dissolved in pyrogen-free saline, sterilized by filtration, and stored at −20°C until use. After thawing, appropriate dilutions of the reagents were made in RPMI 1640 medium just before use. Tissue culture plates (24 wells, 16-mm diameter) and 12-mm glass coverslips were used.

**Cell Harvesting.** *Macrophages.* The peritoneal cells of BALB/c and BALB/c-*nu/nu* were prepared according to the procedure described by Cohn and Benson (6) and Griffin and Silverstein (7). Cells were harvested by injecting 4–5 ml of cold (4°C) phosphate-buffered saline (PBS) free of $Ca^{2+}$ and $Mg^{2+}$ supplemented with 5–10 units of heparin/ml. The cells were then washed three times in cold PBS without heparin and resuspended in 5 ml of RPMI 1640 medium with 10% heat-inactivated (56°C, 30 min) γ-globulin-free fetal calf serum (FCS; Gibco Laboratories, Grand Island, NY). The desired number ($1–2 \times 10^7$/ml) of peritoneal cells was determined using a Thoma pipette and improved Neubauer hemacytometer. One-milliliter aliquots of the cells were layered onto a 12-mm glass coverslip (Bellco, Vineland, NJ) that had been placed in the 16-mm diameter wells of tissue culture plates (Costar, Cambridge, MA). The plates were incubated at 37°C in a humidified 5% $CO_2$ incubator for 30 min to effect macrophage adherence. Coverslips were removed, immersed with gentle agitation in warm RPMI 1640 (37°C) to dislodge nonadherent cells, and placed in fresh tissue culture wells containing 1 ml of RPMI 1640 medium/well.

*Splenic lymphocytes.* Spleen cells from BALB/c and BALB/c-*nu/nu* mice were collected and processed as described by Tyan and Ness (8). Using the plunger of a sterile 5-ml plastic syringe, spleens were gently pressed through a sterile Nickel-Chromium (Nichrome) stainless steel mesh screen cup (A. H. Thomas Co., Philadelphia, PA) into a sterile petri dish containing 15 ml of cold PBS (4°C). A sterile plastic syringe without the needle was used to mix the medium with the cells in a tilted petri dish. The cells were washed twice, treated with tris(hydroxymethyl)aminomethane-buffered ammonium chloride to lyse erythrocytes, and then washed three times in PBS. The splenic macrophages were removed by adherence to plastic petri dishes.

*Fractionation of splenic cells.* T lymphocytes of BALB/c mice were obtained by passing the cells over nylon-wool columns as described by Julius *et al.* (9). Plastic syringes (20 ml) were packed with 1.0 g of nylon-wool. Each column was autoclaved and washed with 30 ml of PBS and followed by 30 ml of PBS with 5% FCS. The wet columns were then incubated for 1 hr at 37°C. Before loading the cells, each column was flushed with 30 ml of warm (37°C) PBS with 5% FCS. Two milliliters of cell suspension ($1.5 \times 10^8$ cells/ml) were loaded in the column and incubated for 45 min in a humidified 5% $CO_2$ incubator. About 85% of the effluent cells (nylon-wool-nonadherent cells) were T lymphocytes as determined by immunofluorescence using a fluorescein-labeled monoclonal antibody specific for the Thy 1.2 alloantigen. Highly pure B lymphocytes from BALB/c-*nu/nu* mice were obtainable without a fractionation procedure.

**Production of PMAF and MAF.** BALB/c-*nu/nu* splenic nonadherent cells (B cells) were treated with 0.1 μg of DDG or 20 μg of Lyso-Pc/ml in medium with 10% FCS for 30 min. After washing in PBS, the cells were cultured in fresh medium with 10% FCS for 5 hr. After centrifugation, the supernatant (conditioned medium) was called PMAF. The PMAF was used to culture untreated BALB/c +/+ T lymphocytes for 4 hr to obtain MAF. After centrifugation, the MAF was stored at −20°C until use.

**Ingestion Assay.** Washed sheep erythrocytes (E) were coated with subagglutinating dilutions of rabbit anti-E immunoglobulin (Ig) G. After washing, EIgG

conjugate was added to 3-hr MAF-pretreated macrophages and incubated for 1 hr to allow ingestion in RPMI 1640 medium without FCS. Noningested erythrocytes were lysed by immersing the coverslips in a hypotonic solution (1:5 PBS) for 5 to 10 sec. The macrophages were fixed with methanol (95%), air dried, and stained with Giemsa stain. Ingestion was quantified microscopically. The data were expressed as the ingestion index according to Bianco *et al.* (10).

**Antibody Assay.** The basic assay as described by Jerne *et al.* (11) was used to quantitate the number of antibody-producing cells in the spleens of Lyso-Pc- or DDG-treated mice. Mice were treated with these agents and immunized with sheep E ($2 \times 10^8$ cells/mouse) simultaneously or at 3 days posttreatment. Spleen cells were harvested on the fifth day after sheep E administration. After washing, the cell number was adjusted to $4 \times 10^7$ cells/ml. The desired aliquots (10, 25, and 50 $\mu$l) were admixed with 500 $\mu$l of molten agar (Sea Plaque agarose; FCM Corp., Rockland, ME) and 50 $\mu$l of 20% E in small tubes at 37°C. The mixture was poured immediately onto an agarose precoated slide (75 × 20 mm) and spread evenly. After several minutes, the slides were covered with PBS to prevent drying and then incubated for 2 hr. PBS was drained from slides and the slides were flooded with a 1/10 dilution of guinea pig complement and reincubated for an additional 1 hr. The number of plaques produced by IgM antibody (direct plaques) was quantified microscopically.

## Results

**Lyso-Pc Dose Effect on *In Vivo* Enhancement of Antibody Production to Sheep E Antigen.** Lysophospholipids and alkylglycerols are potent macrophage-activating agents for Fc-mediated ingestion activity (1–3, 12). Since these agents are able to augment the first major function of macrophages, the question arose as to whether these agents could also have an influence on the second major function of macrophages: antigen processing and transmitting a signal(s) to specific T cells. Accordingly, mice simultaneously received injections of E and various doses of Lyso-Pc. On the fifth day postinjection, the number of plaques produced by IgM antibody was quantified microscopically. As illustrated in Figure 1, plaque-forming cells (PFC) increased as the concentration of Lyso-Pc increased from 10 $\mu$g to 20 $\mu$g of Lyso-Pc/mouse. At a Lyso-Pc dose of 20 $\mu$g/mouse, PFC reached a peak of $15 \times 10^4$. However, at a Lyso-Pc dose of 30 $\mu$g/mouse, PFC production began to decrease. The data clearly show that Lyso-Pc has an excellent adjuvant activity for antibody production. The most effective dose for adjuvant activity is 20 $\mu$g/mouse. This optimal dose on antibody production showed a direct correlation with the dose required for optimal Fc-mediated ingestion activity (1, 2).

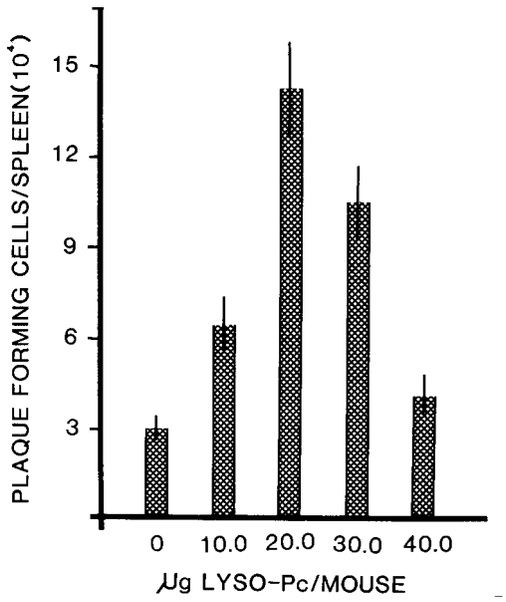


**Figure 1.** Kinetics of antibody production to sheep E antigen in mice treated with various doses of Lyso-Pc. Erythrocytes and Lyso-Pc were simultaneously injected intraperitoneally into mice. The number of direct plaques (IgM antibody) was quantified microscopically on the fifth day after E injection. There were four mice per treatment group. The number of PFC is expressed as the mean value, and the error bars indicate SD (±SE, $n = 12$).

**DDG Dose Effect on *In Vivo* Enhancement of Antibody Production to Sheep E Antigen.** Inflamed cancerous tissues release degradation products of alkylphospholipids (alkyl-lysophospholipids and alkylglycerols) (13). DDG, one of the alkylglycerols, stimulates macrophages for Fc-mediated ingestion activity (4). Since lysophospholipids are able to stimulate both phagocytosis and enhancement of antibody response, we tested the effect of alkylglycerol on antibody response. When mice were given injections simultaneous of E and various doses of DDG, a significantly enhanced antibody production was observed on the fifth day after E injection. As shown in Figure 2, antibody production increased as the concentration of DDG increased from 10 ng up to 100 ng of DDG/mouse. At a DDG dose of 100 ng/mouse, antibody production reached a maximal PFC of about $15.0 \times 10^4$. However, at DDG doses higher than 500 ng/mouse, PFC began to decrease. The data indicate that the most effective dose for adjuvant activity is 0.1 $\mu$g of DDG/mouse. This dose effect of DDG on antibody production showed a direct correlation with those of Fc-mediated ingestion activity (4).

**Enhancement of Antibody Production by Administration of DDG into Mice 3 Days Before Immunization with Sheep E.** DDG was administered to mice 3 days before immunization with E. One group of mice received a booster injection (0.1 $\mu$g of DDG/mouse) simultaneously with the immunization injection 3 days after DDG injection. The number of antibody-produc-

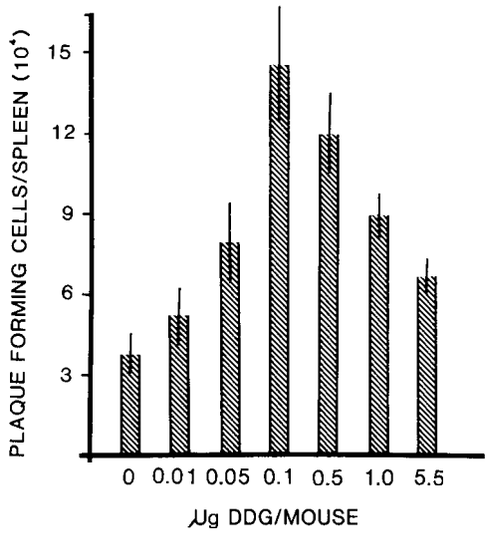


**Figure 2.** Kinetics of antibody production to sheep E antigen in mice treated with various doses of DDG. E and DDG were simultaneously injected intraperitoneally into mice. The number of direct plaques (IgM antibody) was quantified microscopically on the fifth day after E injection. There were four mice per treatment group. The number of PFC is expressed as the mean value, and the error bars indicate SD (± SE, $n$ = 12).

ing cells was quantified microscopically on the fifth day after E injection. As shown in Figure 3, the data indicate that mice immunized with E at 3 days postadministration of DDG produced a slightly larger number of PFC as compared with those of mice simultaneously immunized. In contrast, a booster injection of DDG given simultaneously with the immunizing agent 3 days after primary treatment did not enhance antibody production, but rather abrogated its production. This observation may be due, in part, to the sensitivity of activated B and T cells and macrophages to receptor-mediated extracellular cytolytic effect of DDG. The data suggest that DDG or Lyso-Pc is a potential adjuvant that could be administered separately or in combination with antigens.

**Contributory Role of T Cells in the Activation of Macrophages by Lyso-Pc or DDG for Ingestion Activity and Augmentation of Antibody Production.** As reported previously (3), ingestion activity of Lyso-Pc- or DDG-activated macrophage is specific for IgG-coated target cells. The signaling factor for the macrophage activation and ingestion capacity is derived from B cells treated with these agents. The Lyso-Pc- or DDG-treated B cells release a factor (PMAF) that sensitizes T cells to modify the factor to yield MAF (4, 5). To further substantiate this observation, T cell-deficient BALB/c-*nu/nu* mice were treated with 20 μg of Lyso-Pc or 0.1 μg of DDG/mouse. On the fifth day posttreatment, peritoneal cells were harvested and processed for ingestion activity. The results indicated that *in vivo* treatment of B cells and macrophages in the absence of T cells with Lyso-Pc or DDG produced no significant ingestion activity of IgG-coated target cells (data not shown). Similarly, T cell-deficient BALB/c-*nu/nu* mice treated with DDG or Lyso-Pc and simultaneously immunized with E did not produce antibody (Table I). On the other hand, BALB/c +/+ mice similarly treated produced a greatly enhanced antibody production to E antigen (Table I). The observation supported our previous *in vitro* findings that the induction for enhanced ingestion activity of Lyso-Pc- or DDG-treated peritoneal cells requires the contribution of T cells (5).

**Effect of Conditioned Medium of Lyso-Pc- or DDG-Treated Nonadherent BALB/c-*nu/nu* Splenic Cells Admixed with BALB/c +/+ T Cells on Macrophage (BALB/c or BALB/c-*nu/nu*) Activation for**

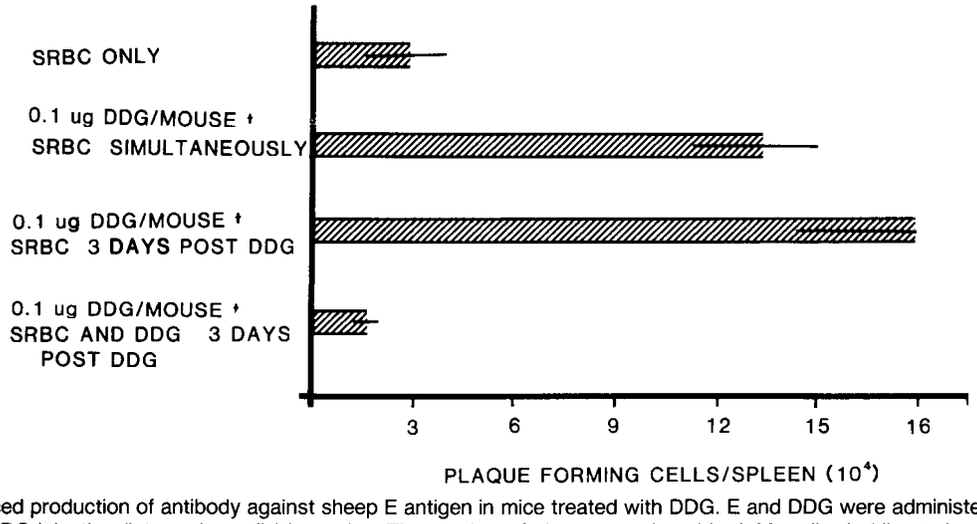


**Figure 3.** Enhanced production of antibody against sheep E antigen in mice treated with DDG. E and DDG were administered simultaneously for 3 days after DDG injection (intraperitoneally) into mice. The number of plaques produced by IgM antibody (direct plaques) was quantified microscopically. There were four mice per treatment group. The number of PFC is expressed as the mean value, and the error bars indicate SD (±SE, $n$ = 12). SRBC, sheep red blood cell.

**Table I.** Requirement for T cells for Adjuvant Activity of Lyso-Pc and DDG on Antibody Response[a]

| Mouse strain | Treatment agent | Dose ($\mu$g/mouse) | Antibody response to sheep E[b] (PFC $\times 10^4$) |
|---|---|---|---|
| BALB/c +/+ | Sham | | 2.9 ± 0.8[c] |
| | DDG | 0.1 | 16.7 ± 1.5 |
| | Lyso-Pc | 20.0 | 15.1 ± 1.2 |
| BALB/c-*nu/nu* | Sham | | 0 |
| | DDG | 0.1 | 0 |
| | Lyso-Pc | 20.0 | 0 |

[a] Mice were given DDG or Lyso-Pc injection simultaneously with the immunizing agent (sheep E).
[b] Spleen cells were harvested on the fifth day after injection.
[c] The number of PFC is expressed as the mean value ± SE, $n = 12$ (four mice per dose). The data shown are representative of two separate experiments.

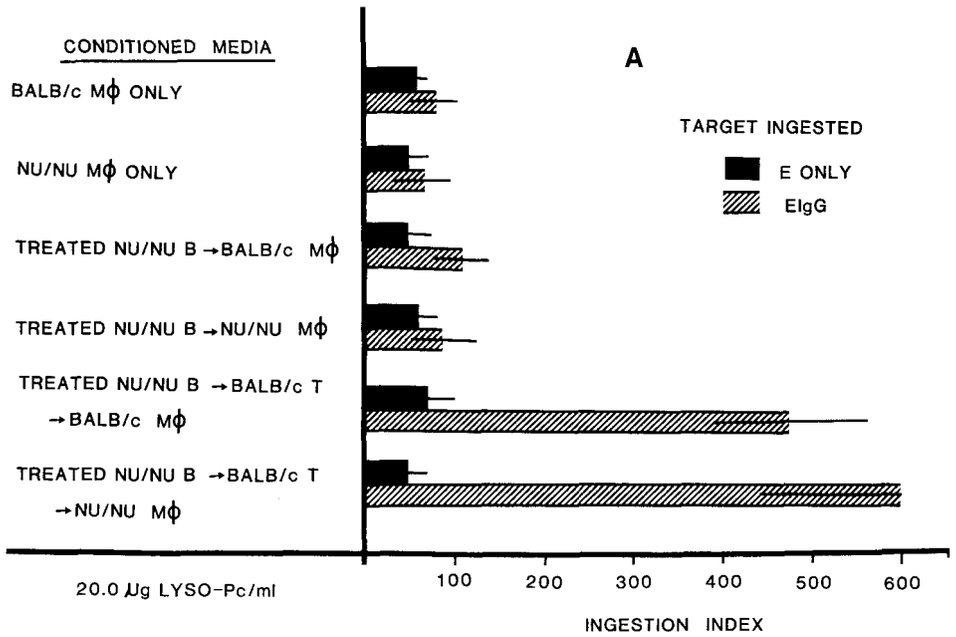


**Figure 4.** Signal transmission among *nu/nu* (BALB/c-*nu/nu*) B cells and BALB/c (BALB/c +/+)-nonadherent (T cells) and adherent cell types for the activation of macrophages (Mϕ). Stepwise cultivation by transferring culture medium of Lyso-Pc-treated BALB/c-*nu/nu* B cells to untreated BALB/c +/+ T cells and macrophages. BALB/c-*nu/nu* splenic B cells were treated with 20 $\mu$g of Lyso-Pc/ml for 30 min. After washing, the cells were incubated for 5 hr in RPMI 1640 medium containing 10% FCS. The resultant conditioned medium was used to culture untreated BALB/c +/+ T cells for 4 hr. The conditioned medium from the sensitized T cells was used to pretreat macrophages for 3 hr before ingestion assay. The ingestion index is expressed as the mean value, and the error bars indicate SD (±SE, $n = 6$). The data shown are representative of two separate experiments.

**Ingestion Activity.** Development of enhanced macrophage ingestion activity after treatment with lysophospholipids requires co-cultivation of both B and T cells (2, 3). Accordingly, conditioned medium (culture medium) of Lyso-Pc- or DDG-treated BALB/c-*nu/nu* B cells was used to culture T cells of BALB/c +/+ mice. The resultant conditioned medium was tested by admixing it with BALB/c-*nu/nu*- and BALB/c +/+-adherent cells for the development of macrophage ingestion. As shown in Figures 4 and 5, the results indicate that 3-hr incubation of BALB/c +/+- or BALB/c-*nu/nu*-adherent cells with conditioned medium of untreated BALB/c-*nu/nu* B cells produced no significant Fc-mediated ingestion activity. Three-hour incubation of BALB/c-*nu/nu*- or BALB/c +/+-adherent cells with the conditioned medium of the treated BALB/c-*nu/nu* B cells (PMAF) produced no significant Fc-mediated ingestion activity. In contrast, 3-hr incubation of BALB/c +/+ or BALB/c-*nu/nu*-adherent cells with stepwise prepared condition medium of treated BALB/c-*nu/nu* B cells and untreated T cells of BALB/c +/+ mice (Figs. 4 and 5) produced a greatly enhanced Fc-mediated ingestion activity. This observation demonstrated that the stepwise condition medium contain MAF and that treated BALB/c-*nu/nu* B cells are capable of producing PMAF, which can be converted by BALB/c +/+ T cells to MAF. At a MAF dilution of 1/50, BALB/c-*nu/nu*-adherent cells reached higher inges-

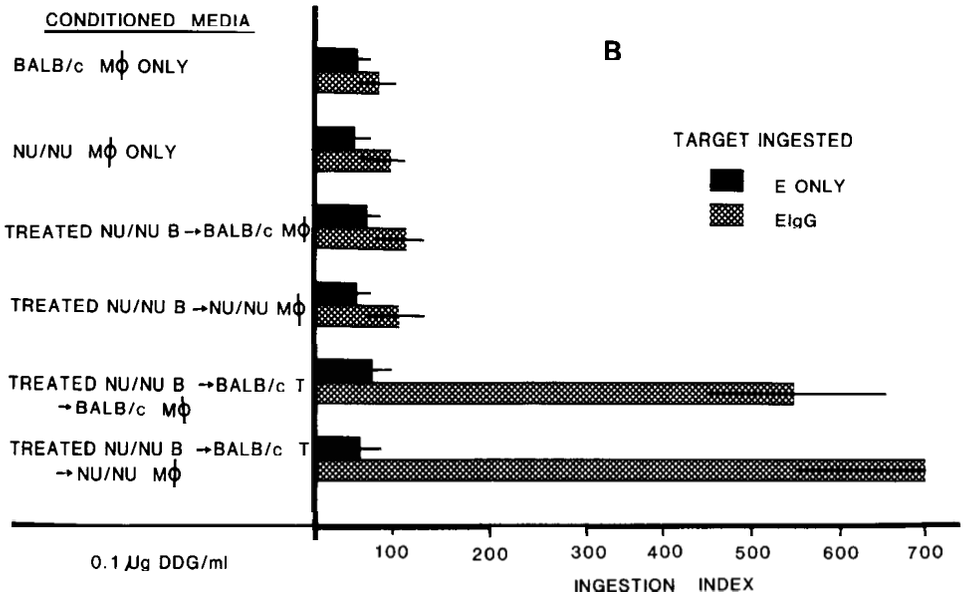


**Figure 5.** Signal transmission among *nu/nu* (BALB/c-*nu/nu*) B and BALB/c (BALB/c +/+) T cells and adherent cells types for the activation of macrophages (Mϕ). BALB/c-*nu/nu* splenic B cells were treated with dodecylglycerol (0.1 μg of DDG/ml) for 30-min. Stepwise cultivation using conditioned medium, and ingestion indices of macrophages were performed as in Figure 4. The ingestion index is expressed as the mean value of triplicate samples, and the error bars indicate SD (±SE, $n = 6$). The data shown are representative of two separate experiments.

tion indices of 600 and 700 (Figs. 4 and 5) as compared with those of the BALB/c +/+-adherent cells ingestion indices of 480 and 550 (Figs. 4 and 5). The data suggest that Lyso-Pc- or DDG-primed BALB/c-*nu/nu* B cells produced a PMAF that interacted specifically with BALB/c +/+ T cells to produce MAF capable of activation of BALB/c +/+ and BALB/c-*nu/nu* macrophages for Fc-mediated ingestion activity.

## Discussion

Lyso-Pc and DDG are inflammation products of normal and cancerous tissues. Their release during inflammation process sets into motion a series of events that leads to enhancement of the host defense mechanism. Our accumulated evidence indicates that administration of these lipid metabolites to mice results in the potentiation of the host immune system (1–4). Doses of 100 ng of DDG and 20 μg of Lyso-Pc/mouse are the most effective dosages for macrophage activation. Our findings suggest that macrophage activation by these agents requires collaboration of B and T cells and that Lyso-Pc- or DDG-primed B cells transmit a signal to T cells, and in turn, the sensitized T cells release a factor that ultimately activates macrophages. This pathway of signal transmission has been established *in vitro* by stepwise transfer of conditioned media among these cell types (5).

This mechanism has been tested by utilizing the T cell-deficient BALB/c-*nu/nu* mouse and its B cells and macrophages in combination with T cells from the BALB/c +/+ mouse. Stepwise preparation of conditioned medium of BALB/c-*nu/nu* B cells to BALB/c +/+ T cells resulted in the production of a large amount of MAF. This observation also implied that B cells and macrophages of BALB/c-*nu/nu* mouse are capable of responding to the process of macrophage activation if the B cell conditioned medium was used to culture untreated BALB/c +/+ T cells. Therefore, signal transmission from B to macrophages through T cells is an essential process in the mechanism for macrophage activation by lysophospholipids (5). Although macrophages are antigen-presenting cells, enhanced production of antibody in BALB/c-*nu/nu* mice treated with Lyso-Pc or DDG and subsequently immunized with sheep E could not be established. Hence, regardless of administration of lipid metabolites of inflammation, T cell-deficient BALB/c-*nu/nu* mice were unable to respond to T cell-dependent antigenic challenge. In contrast, BALB/c +/+ mice immunized simultaneously or 3 days after administration of lipid metabolites produced a large number of antibody-secreting cells. Thus, the Lyso-Pc- or DDG-activated macrophages efficiently ingested and processed the immunogen (E) and transmitted antigenic signal(s) to B and T cells, which resulted in the augmentation of antibody production.

It is concluded that the adjuvant effect of lysophospholipids on antibody response is a coordinated reaction of B cells, T cells, and macrophages, communicating either directly or via antigen-specific and nonspecific factor(s). This initial finding provides important leads for the study of mechanism whereby inflammatory product(s) related to microbial cell surface antigen(s) eventually stimulates macrophage activation and antibody production. Thus, inflammation products

have beneficial effects on host resistance by augmenting phagocytosis and antibody production. Therefore, Lyso-Pc or DDG is a potential adjuvant that could be administered separately or in combination with antigen.

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