Activation of Peritoneal Macrophages by Orally Administered Ether Analogues of Lysophospholipids (43230)

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Abstract. **Oral administration of dodecylglycerol, inflammatory product of cancerous tissues, and the alkyl lysophospholipid derivative, 1-O-octadecyl-2-O-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3-choline), greatly activated mouse peritoneal macrophages. The activation was dose related and was assessed as increased Fc-mediated ingestion of red blood cells, superoxide production, chemiluminescence activity, and incorporation of radioactive thymidine and leucine. Furthermore, the data show that dodecylglycerol or ET-18-OCH_3-choline was capable of inducing equally high levels of macrophage activation and cytotoxic action on tumor cells, just as occurs with intraperitoneal administration. Dodecylglycerol appeared to activate the macrophages at a relatively lower dose (5 μg/mouse) than ET-18-OCH_3-choline (15 μg/mouse). The optimal oral doses required to activate macrophages for ingestion and cytotoxic activities were relatively higher than previously observed when these agents were administered intraperitoneally. Thus, the dose difference provided crucial information for correlating oral dosages with *in vivo* concentration of these agents as bioassayed by macrophage activation. These observations have extended and further support our earlier findings that these agents are effective immunopotentiators and thus could therapeutically be used to activate macrophages for cytotoxic effects on tumor cells via the oral route.**
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Inflammatory response involves a ternary cellular complex in the induction of immune response (1). One of the mechanisms whereby inflammation promptly and efficiently induces protective immunity is via the production of lysophospholipids and alkyl-lysophospholipids (2, 3). These lipid metabolites of normal cells (lysophospholipids) and cancerous tissues (alkyl lysophospholipids) have been shown to be potent immunopotentiators (4, 5).

We recently reported that intraperitoneal administration of various lysophospholipids and alkyl glycerols to mice activate macrophages for enhanced Fc-mediated ingestion (6–9) and adjuvant (1) activities. Intraperitoneal administration of 20 μg of lysophosphatidylcholine (Lyso-Pc) and 0.1 μg of dodecylglycerol (DDG; a synthetic alkyl glycerol)/mouse produce highly activated splenic and peritoneal macrophages. Specifically, the DDG treatment of mice induces an enhancement of Fc-mediated ingestion activity of macrophage and/or metabolic capability measured by the increase in superoxide production (5). Therefore, these agents could be considered as activators of the metabolic capabilities of macrophages.

The mechanism whereby lysophospholipids activate macrophages has been elucidated at least at the cellular level (9). Lyso-Pc- or DDG-treated B cells release and transmit a pro-macrophage-activating factor to T cells. The factor-sensitized T cells modify pro-macrophage-activating factor or excrete a *de novo* macrophage-activating factor which is capable of the ultimate activation of macrophages for more prompt and efficient ingestion capacity and subsequent augmentation of antibody production (1, 9).

Accumulated evidence strongly indicate that dodecylglycerol and lysophosphatidylcholine are potent *in vivo* as well as *in vitro* macrophage-activating agents (4, 6–8, 10). Potentially, this is of paramount clinical importance since the lysophospholipid- or alkyl glyc-

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erol-induced macrophage activation can lead to death of microbial agents and tumor cells (4, 11–14). These potential practical applications prompted investigators to study the metabolic fate of orally administered dodecylglycerol. Their evidence indicate that dodecylglycerol is converted to alkyl diacylglycerols which are subsequently incorporated into different organs (15). On this basis, we hypothesize that oral administration of alkyl lysophospholipids should activate macrophages for ingestion just as occurs with intraperitoneal administered alkyl lysophospholipids. The available data of the *in vivo* retention and metabolism of dodecylglycerol may provide the basis for correlating orally administered doses of DDG with our established intraperitoneal dose of DDG which is capable of inducing activation of macrophages. Therefore, the present communication reports the effects of orally administered ether analogues of lysophospholipids on the activation of macrophages and their subsequent cytotoxic action on tumor cells.

Materials and Methods

Animals. Inbred BALB/c mice (6–12 weeks of age) were obtained from The Jackson Laboratory, Bar Habor, ME. The mice were housed in accredited animal quarters (American Association for Accreditation of Laboratory Animal Care). Mice were fed Purina Mouse Chow and water *ad libitum.*

Chemicals and Reagents. Ether analogue of monoglyceride, 1-*O*-dodecyl-*rac*-glycerol (dodecylglycerol), was purchased from Sigma Chemical Co., St. Louis, MO. 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3-choline) was purchased from Calbiochem Corp., La Jolla, CA. The purity of these agents was ≥99% pure; 0.5% precursors of lysophospholipids and no other compounds such as lipopolysaccharide were detectable. The reagents were dissolved in dimethyl sulfoxide (1 mg/50 μl), and then diluted more than 1000-fold in pyrogen-free saline to desired concentration. The reagents were sterilized by filtration and stored at −20°C until use. After thawing, desired dilutions of the reagents were made in RPMI 1640 medium just before use.

Tumor Cell Line. The human retinoblastoma tumor cell line Y-79 was purchased from the American Type Culture Collection, Rockville, MD. The cells were grown in suspension in 50-ml Falcon plastic flasks, cultured in RPMI 1640 medium supplemented with 15% fetal calf serum and 50 mg of gentamicin/ml, and split for subculturing twice weekly. The cell aggregates were allowed to settle to the bottom of the tube, the excess supernatant was removed and discarded, and the aggregates were pipetted gently and subcultured at a ratio of 1:4 for continuous proliferation. Before being used, tumor cells were washed three times in phosphate-buffered saline (PBS), and trypan blue exclusion test was used for determining the number of viable cells.

Macrophage Stimulation, Harvesting, and Culturing. Mice were administered the desired doses of ET-18-OCH_3-choline and DDG orally using a 1-ml tuberculin syringe fitted with an animal intubation needle with spherical ball tip (Thomas Scientific, Philadelphia, PA). Seven days after primary administration of agents, a second dose was administered in a similar manner. On designated days after primary or secondary administration of agents, mice were killed by CO_2. Peritoneal cells were harvested and processed according to the procedure described by Cohen and Benson (16) and Griffin and Silverstein (17). Briefly, cells were harvested by injecting 4–5 ml of cold (4°C) PBS free of Ca^{2+} and Mg^{2+} supplemented with 5 to 10 units of heparin/ml. The cells were 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 (Gibco Laboratories, Grand Island, NY). The desired number of peritoneal cells (2×10^6/ml) was determined using a Thoma pipette and an improved Neubauer hemacytometer. The cells were then distributed in 100-μl aliquots into round bottomed 96-well plates (Corning Glass Works, King of Prussia, PA), and the plates were incubated for 2 hr at 37°C, in a humidified 5% CO_2 incubator.

Radioisotope Incorporation Assay. Peritoneal cells were harvested from orally treated mice and processed as described above. After removal of nonadherent cells, the macrophages were pulsed with 0.5 μCi of [^{3}H] thymidine (sp act, 68 Ci/nmol) or L-[^{3}H]leucine (sp act, 108 Ci/nmol) (ICN Biomedical, Inc., Irvine, CA)/well and incubated for 3 hr. Cells were harvested with a semiautomated cell harvester (Skatron Inc., Sterling, VA) on filter paper, and the filters transferred to scintillation vials into which 4 ml of scintillation fluid were added. Radioactivity was measured in a liquid scintillation counter (Packard Instruments Co., Inc., Downers Grove, IL). The test was performed in quadruplicates and the results were expressed as net counts per minute.

Superoxide Generation in Stimulated Macrophages. Mice were orally administered the desired doses of DDG 2 and 4 days preharvest of the peritoneal macrophages. The peritoneal cells of both groups were harvested on the same day. The rapid microassay method for measuring superoxide production described by Pick and Mizel (18) was employed. After washing, the cells were placed in microplates containing RPMI 1640 medium supplemented with 10% fetal calf serum and incubated at 37°C in a humidified 5% CO_2 incubator for 30 min to allow adherence. The nonadherent cells were removed by washing with warm PBS. Macrophages at a concentration of 3×10^5/ml were mixed with cytochrome *c* and incubated for 10 min. After addition of phorbol 12-myristate 13-acetate (PMA)

(Sigma Chemical Co.), the superoxide-generating activity of the macrophages was determined spectrophotometrically by reading the optical densities of the wells at 550 nm with a microplate reader (Bio-Tek Instruments Inc., Winooski, VT). The data were expressed as nanomoles of superoxide produced per milligram of macrophage protein.

Chemiluminescence Assay. Peritoneal macrophages from mice administered ET-18-OCH_3-choline orally were collected and washed as described previously. Cells were used at a concentration of 1×10^7/ml. Reagents were pipetted into duplicate cuvettes (test and control samples) in the following order: PBS-gelatin with Ca^{2+} and Mg^{2+} ions, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) in 10^{-5} *M* dimethyl sulfoxide, macrophages, and PMA. The contents of the cuvette were mixed gently and conveyed to the reading position and light emission recorded as mV using the luminometer coupled to a display monitor and printer (LKB-Wallac 1251 Luminometer, Turku, Finland).

Ingestion Assay. Washed sheep erythrocytes were coated with subagglutinating dilutions of rabbit antierythrocyte immunoglobulin G(IgG). After washing, erythrocyte IgG conjugate was added to orally activated macrophages and incubated for 1 hr to allow ingestion in RPMI 1640 medium without fetal calf serum. Noningested erythrocytes were lysed by immersing the coverslips in a hypotonic solution (1:5 PBS) for 5 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.* (19): Ingestion index = % macrophages with ingested erythrocytes × average number of erythrocytes ingested per macrophage.

Cytotoxic Assay. Macrophages from mice (treated and untreated) were co-cultured with equal concentrations of the retinoblastoma tumor cells (2×10^5/well) and incubated overnight under the same conditions described above. Three hours before harvesting, the cells were pulsed with 50 μl (0.5 μCi) of either [^{3}H] thymidine (sp act, 68 Ci/nmol) or L-[^{3}H]leucine (sp act, 108 Ci/nmol) (ICN Biomedical, Inc.)/well and incubated for an additional 3 hr. Radioactivity was measured and expressed as described above.

Results

Stimulation of Peritoneal Macrophages by Oral Administration of ET-18-OCH_3-Choline. Previous reports by several investigators (20–23) indicate that macrophages can respond to growth factors, such as colony-stimulating factor and granulocyte-macrophage colony-stimulating factor in a number of ways, including initiation of DNA synthesis. DNA synthesis was measured by incorporation of [^{3}H]thymidine by the macrophages. We previously reported macrophage activation by lysophospholipids *in vitro* and via intraperitoneal administration (1, 8). To further substantiate our finding and broaden the potential practical application of lysophospholipids, we tested the effects of the ether analogues of lysophospholipids on macrophage activation after oral administration to mice.

As illustrated in Figure 1, the dose effects of ET-18-OCH_3-choline on *in vivo* activation of the macrophages clearly indicates a dose-dependent activation. There was no significant activation of macrophages by lower doses (5–10 μg/mouse) of ET-18-OCH_3-choline used. Between 15- and 20-μg/mouse doses, there was a very high increase in activity as measured by [^{3}H] thymidine incorporation. A similar macrophage activation activity (result not shown) was seen with orally administered dodecylglycerol.

Superoxide-Generating Activity of Peritoneal Macrophages Activated by Oral Administration of Dodecylglycerol. Superoxide production is one of the parameters associated with cytocidal action of macrophages against microbial agents, notably bacteria. Therefore, we studied this parameter to ascertain the cytocidal capacity of macrophages activated via the oral

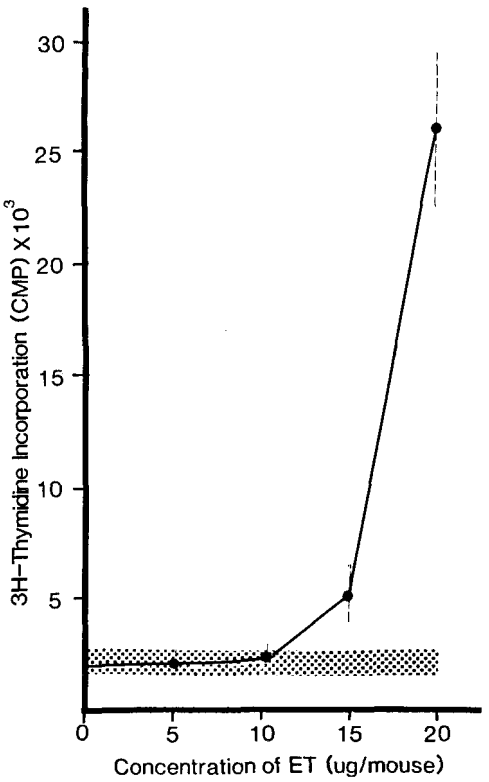

Figure 1. Dose effects of ET-18-OCH_3-choline on *in vivo* activation of mouse peritoneal macrophages. Macrophages were collected 3 days after challenge oral treatment of mice with the appropriate doses of ET-18-OCH_3-choline and incubated overnight at 37°C and in the presence of 5% CO_2 in a regular incubator. Three hours before harvesting, cells were pulsed with 0.5 μCi of [^{3}H]thymidine/well and counting was done with a scintillating counter. Results are expressed as mean cpm of quadruplicate samples ± SD. Data shown are representative of three separate experiments.

route. Table I shows the time kinetics after oral administration of DDG. By the fourth day after administration, there was a maximal increase in superoxide production at 5 μg of DDG/mouse. As the DDG doses increased to 20 μg/mouse, the superoxide activity declined, perhaps due to toxic effect of this high dose of DDG. The results appear to support the proposition that orally administered DDG activates macrophage metabolic activity for cytocidal action.

Chemiluminescence Activity of Macrophages Activated by Orally Administered ET-18-OCH_3-Choline. Chemiluminescence activity, one of the recent additions to the armamentarium for the study of phagocytosis and opsonization actions of formed elements of the blood, was studied as an adjunct parameter to substantiate the macrophage activation ability of orally administered ET-18-OCH_3-choline.

Peritoneal cells were harvested on the third or fourth day after primary oral administraton of 10, 15, and 20 μg of ET-18-OCH_3-choline/mouse. The result indicated that chemiluminescence response to PMA stimulus was not significantly different from untreated macrophages (data not shown). Subsequently, mice were treated with the same doses, and 7 days after primary oral treatment, identical booster dose regimens were administered. Three days after challenge, macrophage chemiluminescence activity was assayed.

As shown in Table II, macrophages from booster-stimulated mice produced a 4-fold rise in chemiluminescence response to PMA stimulus at 15 μg of ET-18-OCH_3-choline/mouse. However, macrophages from mice treated with 20 μg/mouse showed a decreased chemiluminescence response to the stimulant. There was also an increase in chemiluminescence when DDG was given, although not as striking as with the former (data not shown). This finding obviously gives an insight into phagocytosis, a major mechanism by which the immune system protects the body against bacterial infection (24). Therefore, these agents could be administered orally as well as intraperitoneally to augment the immune system.

Table I. Effect of Orally Administered DDG on Superoxide-Generating Activity of Mouse Peritoneal Macrophages[a]

Days after oral treatment	Dose of DDG (μg/mouse)	Superoxide production (nmol O_2^-/mg macrophage protein)
	Control[b]	0.02 ± 0.00
	0	0.49 ± 0.13[c]
2	5	2.95 ± 0.57
	20	1.90 ± 0.29
	Control	0.04 ± 0.01
	0	0.68 ± 0.33
4	5	4.46 ± 0.56
	20	2.08 ± 0.73

[a] Peritoneal macrophages were harvested on the same day from groups of mice pretreated 2 and 4 days with the appropriate doses of the compound.
[b] Control refers to mean values of activated cells. Cells were assayed in the presence of superoxide dismutase (300 units/ml).
[c] Results are expressed as mean nanomoles of superoxide (0_2^-) produced per milligram of macrophage protein of triplicate samples ±SD. Data are representative of two separate experiments.

Table II. Chemiluminescence Activity of Peritoneal Macrophages[a] following Oral Treatment of Mice with ET-18-OCH_3-Choline

Dose of ET-18-OCH_3-choline (μg/mouse)	Chemiluminescence activity (mV)
0	1.8 ± 0.4[b]
10	1.7 ± 0.2
15	7.7 ± 0.3
20	3.5 ± 0.2

[a] Peritoneal macrophages were harvested and processed with luminol, freshly prepared PBS-gelatin, and PMA and chemiluminescence activity was determined.
[b] Results are expressed as mean peak values (mV) ± SD. Data shown are representative of three separate experiments.

Ingestion Activity of Mouse Peritoneal Macrophages Activated by Orally Administered DDG. Like other findings already reported concerning ingestion action of macrophages after peritoneal administration of the lysophospholipids (4, 8), oral treatment of mice with ET-18-OCH_3-choline and dodecylglycerol showed that these agents are efficient activators of macrophages. Therefore, we tested the direct phagocytic capacity of macrophages derived from orally treated mice.

As illustrated in Figure 2, ingestion activity of peritoneal macrophages increased as the concentration of DDG increased up to 20 μg/mouse. However, the dose kinetics showed no significant difference in ingestion indices of the three doses used on the fourth day after oral treatment. As reported earlier, ingestion activity of macrophages from lysophospholipid-treated mice appears to be specific for the Fc receptors but not C3b receptors (4, 6, 8). Therefore, the ingestion of these specific immunoglobulin-opsonized target cells indicate that activation of macrophages via intraperitoneal injection of DDG is reproduced by oral administration of DDG.

Development of Ingestion Mechanism of Macrophages Derived from Mice Orally Administered with Dodecylglycerol. Since orally administered dodecylglycerol is converted to alkyl diacylglycerols (14), a time course analysis for the development of phagocytic activity was studied. As illustrated in Figure 3, ingestion activity of macrophages from orally treated mice exhibited increased ingestion of IgG-coated target cells as early as Day 1 after treatment. All doses (5, 10, and 20 μg/mouse) were able to efficiently activate macrophages for enhanced Fc-mediated ingestion activity. The 20-μg dose, however, lags behind the smaller doses

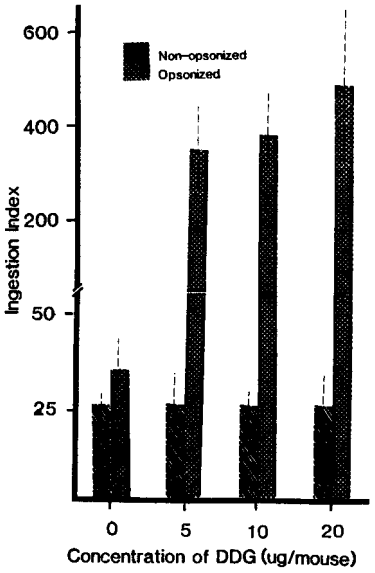

Figure 2. Ingestion activity of mouse peritoneal macrophages after 5 days of oral administration of DDG. Peritoneal macrophages were harvested one single day after 5 days of pretreatment and incubated *in vitro* for 1 hr prior to the ingestion assay. Each value of the ingestion index represents the mean ± SD for two separate experiments performed in duplicate, 500 macrophages having been counted on each coverslip.

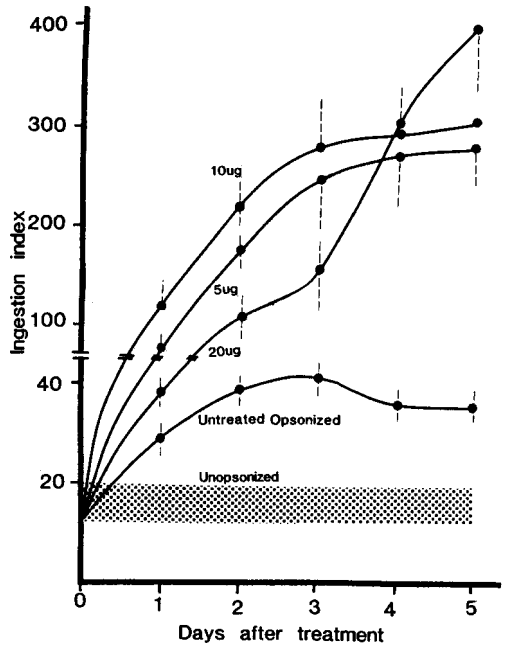

Figure 3. Time course analysis of ingestion activity of peritoneal macrophages derived from oral administration of various doses of DDG. Macrophages were harvested one single day after 1–5 days of pretreatment and incubated *in vitro* for 1 hr prior to ingestion assay. Each value of ingestion index represents the mean ± SD for two separate experiments performed in duplicate, 500 macrophages having been counted on each coverslip.

until after the third day when there was a sharp rise in activation which was sustained to the fifth day. This observation may suggest toxic effect of DDG at this high dose, but as the toxic effect wanes, the macrophages recover and respond appropriately by Day 4 posttreatment. Therefore, the result suggest that orally administered DDG is capable of activating macrophages within 2 days for prompt ingestion of cytocidal activity.

Cytotoxic Action of Lysophospholipids on Retinoblastoma Cell Line. To further substantiate that macrophages can be activated to develop cytotoxic activity by oral administration of the lysophospholipids, their activating capability was measured by their ability to inhibit uptake of radioisotope by retinoblastoma tumor cells. Figure 4 shows that activated macrophages were indeed cytotoxic to the tumor cells by inhibiting uptake of radioactive thymidine or leucine by the tumor cells. When the macrophages were treated *in vitro* with dodecylglycerol (Fig. 4A), there was more than 60% inhibition of [^{3}H]thymidine incorporation by the tumor cells. Similarly, macrophages from mice treated orally with the dodecylglycerol and ET-18-OCH_3-choline (Fig. 4B) produced about 50 and 80% inhibition of [^{3}H] thymidine uptake, respectively. These results are in agreement with those by earlier workers (11, 21, 22) who demonstrated that macrophages activated by these agents were capable of inhibiting radioisotope uptake in tumor cells.

Discussion

Dodecylglycerol and ET-18-OCH_3-choline are inflammatory products of cancerous membrane lipids and alkyl lysophospholipid derivative, respectively. These lysophospholipids stimulate macrophages *in vitro* as well as *in vivo* for enhanced Fc-mediated ingestion and antibody production (4, 7, 8). A single intraperitoneal injection of these agents into mice at very low doses (0.1–2.5 μg/mouse) efficiently induce Fc-mediated ingestion of macrophages for an extended period of at least 7 days (4, 6–8). Similarly, *in vitro* DDG- and ET-18-OCH_3-choline-treated macrophages in the presence of B and T cells develop ingestion activity with Fc receptor specificity.

Oral administration of these agents significantly potentiated the host immune system. The treatment activated peritoneal macrophages for a greatly enhanced [^{3}H]thymidine incorporation (Fig. 1), superoxide and chemiluminescence activities (Tables I and II), and Fc-mediated ingestion of IgG-coated target cells (Figs. 2 and 3) as well as inhibition of uptake of [^{3}H] thymidine or L-leucine by retinoblastoma tumor cells (Fig. 4). In most cases, the response of the activated macrophages was dose dependent. The optimal oral macrophage-activating doses were between 15 and 20 μg for ET-18-OCH_3-choline and 5 and 20 μg/mouse

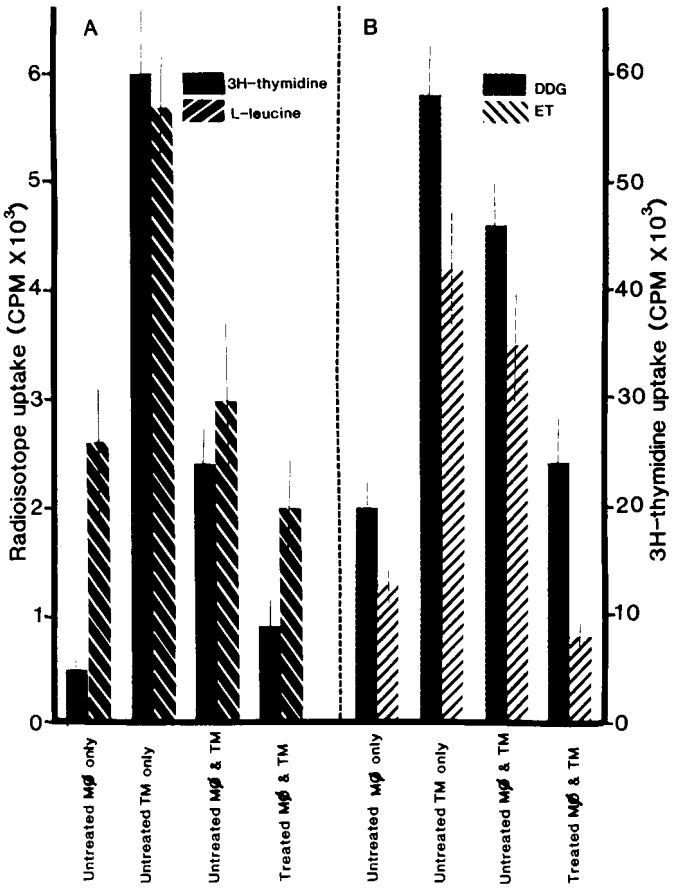

Figure 4. Cytotoxic effect of activated mouse peritoneal macrophages on retinoblastoma tumor cells by DDG and ET-18-OCH_3-choline (ET). Tumor cells (TM) and macrophages (Mϕ) were co-incubated at 37°C and in the presence of 5% CO_2 overnight, and radioisotope uptake was determined after 3-hr pulsing. (A) Macrophages were treated *in vitro* with dodecylglycerol (0.5 μg/ml), co-cultured with tumor cells, and pulsed with [^{3}H]thymidine or L-leucine. (B) Macrophages were from mice treated orally with dodecylglycerol or ET-18-OCH_3-choline (15 μg/mouse), co-cultured with tumor cells, and pulsed with [^{3}H]thymidine. Counts per minute values represent [^{3}H]thymidine or L-leucine uptake by macrophages and/or tumor cells. Results are expressed as mean cpm of quadruplicate samples ± SD. Data shown are representative of three separate experiments.

for DDG. With respect to DDG, the 5-μg/mouse dose produced equally comparable activation activity to the larger doses. These doses are about 10 times the corresponding optimal activating doses found when the macrophages were treated *in vitro* (0.5 μg/ml for DDG and 1.25–2.50 μg/ml for ET-18-OCH_3-choline). In a previous study in which peritoneal cells were treated intraperitoneally with DDG, the optimal dose for ingestion activity was found to be 0.1 μg/mouse (5) and this dose correlates with *in vivo* concentration attained after oral administration of the agents as bioassayed by macrophage activation. In fact, both dodecylglycerol and ET-18-OCH_3-choline have been shown to be well absorbed from the gastrointestinal tract (15, 27). As a substrate, DDG is rapidly absorbed from the intestine and incorporated into ether glycerolipids of various organs and tissues in high proportions. Since the activation mechanism and ingestion receptor specificity of macrophages were reproduced *in vitro* as well as *in vivo* (intraperitoneal and oral), possibly all of these chemicals could have a similar action mechanism to trigger activation of macrophages. This is in spite of the fact that the alkyl glycerols are structurally quite different from the other ether derivatives of lysophospholipids.

The present results support and substantiate previous findings of lysophospholipid-activated macrophages via routes other than oral, and the consequent cytocidal action of these activated macrophages (11, 12, 22, 24, 26, 28). The advantage of these agents is their relative ease of administration via the oral route with equally good immunopotentiating effects.

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