Clin. exp. Immunol. (1990) **79**, 307–313

Activation process of macrophages after *in vitro* treatment of mouse lymphocytes with dodecylglycerol

S. HOMMA* & N. YAMAMOTO *Department of Microbiology and Immunology, Hahnemann University School of Medicine, Philadelphia, PA, USA*

(Accepted for publication 20 September 1989)

SUMMARY

Alkylglycerols, inflammation products of cancerous membrane lipids, efficiently activate macrophages. A brief *in vitro* treatment (30 min) of peritoneal cells (mixture of non-adherent and adherent cells) with a small amount (50 ng/ml) of synthetic dodecylglycerol (DDG) resulted in greatly enhanced Fc-receptor-mediated ingestion activity of macrophages. However, treatment of adherent cells (macrophages) alone with DDG produced no significant enhancement of macrophage ingestion activity, implying that macrophage activation requires a contribution of non-adherent cells. DDG-treated non-adherent cells were found to generate a macrophage-activating signal factor. Studies with a serum free–0·1% egg albumin-supplemented RPMI 1640 medium revealed that a serum factor is essential for macrophage activation process. Time course analysis of stepwise transfers of conditioned media of DDG-treated or untreated B cells and T cells revealed that DDG-treated B cells rapidly transmit a factor to untreated T cells which yield the ultimate macrophage-activating factor. This signal transmission among these cells for the macrophage activation process is too rapid to allow time for synthesis of inducible gene products. Thus, we hypothesized that a serum factor is modified by the pre-existing function of DDG-treated B cells and further modified by the pre-existing function of untreated T cells to yield macrophage-activating factor. This hypothesis was confirmed by the demonstration that DDG-treated splenic non-adherent cell ghosts modify a serum factor to yield macrophage-activating factor.

Keywords inflammation dodecylglycerol macrophage activation serum factor macrophage-activating signal factor

INTRODUCTION

Inflamed tissues release lysophospholipids which are degradation products of membraneous phospholipids. Administration of lysophospholipids to mice stimulates macrophages to ingest target cells via Fc receptor but not C3b receptor (Ngwenya & Yamamoto, 1985, 1986; Yamamoto & Ngwenya, 1987) and generate superoxide (Yamamoto *et al.*, 1987). *In vitro* treatment of a mixture of adherent (macrophage) and non-adherent (B and T) cells with lysophosphatidylcholine (lyso-Pc) or other lysophospholipids for a few hours produces markedly enhanced Fc-receptor-mediated ingestion activities of macrophages (Ngwenya & Yamamoto, 1986; Yamamoto & Ngwenya, 1987). However, incubation of macrophage alone with lyso-Pc results in no enhanced ingestion activity, implying a contribution of non-adherent cells to stimulation of macrophages (Ngwenya & Yamamoto, 1986; Yamamoto & Ngwenya, 1987). Therefore, *in vivo* macrophage activation by lysophospholipids was reproduced *in vitro* by lysophospholipid treatment of mixed adherent and non-adherent cell culture (Ngwenya & Yamamoto, 1986; Yamamoto & Ngwenya, 1987). This *in vitro* model system provides a mechanism to study inflammation products of various lipids, other factors and conditions required for effective activation of macrophages.

Inflammation of cancerous tissues (e.g. melanoma and bladder cancer) induced by local administration of bacterial cells (e.g. BCG) or their constituents can result in regression of local as well as metastasized tumours suggesting development of specific immunity against cancerous cells (Nauts, Swift & Coley, 1946; Zbar & Tanaka, 1971; Rapp, 1976; Old, 1988). Inflammation of normal tissues, however, has no significant effect on tumour growth in the same hosts. Although inflamed non-cancerous tissues produce lysophospholipids such as lyso-Pc which activate macrophages (Ngwenya & Yamamoto, 1985, 1986; Yamamoto & Ngwenya, 1987), lyso-Pc-activated macrophages are not sufficient to affect tumor growth. Inflamed cancerous tissues produce alkyl-lysophospholipids and alkyl-

* On leave from 1st Department of Internal Medicine, The Jikei University, School of Medicine, Tokyo, Japan.

Correspondence: Dr N. Yamamoto, Department of Microbiology and Immunology, Hahnemann University School of Medicine, Broad and Vine Streets, Philadelphia, PA 19102, USA.

glycerols as well as lysophospholipids because cancerous cells contain alkylphospholipids and monalkyldiacylglycerols (Snyder & Wood, 1969; Haward, Morris & Bailey, 1972; Yamamoto & Ngwenya, 1987; Yamamoto *et al.*, 1987). We previously reported that alkylglycerols and alkyl-lysophospholipids are potent macrophage-activating agents (Yamamoto & Ngwenya, 1987; Yamamoto *et al.*, 1988). One of the alkylglycerols, dodecylglycerol (DDG), is the most potent macrophage stimulator we have encountered (Yamamoto & Ngwenya, 1987; Yamamoto *et al.*, 1987, 1988). In our *in vivo* studies, administration of very small doses (less than 100 ng/mouse) to mice are sufficient to induce a greatly enhanced macrophage ingestion activity (Yamamoto *et al.*, 1988). In *in vitro* studies, a brief treatment of mouse resident peritoneal cells with a small amount of DDG (50 ng/ml) produced a markedly elevated ingestion activity of macrophages (Yamamoto & Ngwenya, 1987; Yamamoto *et al.*, 1987).

Participation of non-adherent (B and T) cells is essential for the DDG-primed activation process of macrophages (Yamamoto & Ngwenya, 1987; Yamamoto *et al.*, 1988). Mouse peritoneal cells (mixture of non-adherent cells) were treated with DDG for 30 min and then washed to remove DDG as well as non-adherent cells. The remaining adherent cells were cultured for 2–4 h resulting in a greatly enhanced Fc-mediated ingestion activity of macrophages (Yamamoto *et al.*, 1988). This observation suggests that a rapid signal communication from non-adherent to adherent cells occurs during the 30-min treatment period. Incubation of untreated adherent cells with DDG-treated non-adherent cells in a medium containing 10% fetal calf serum (FCS medium) developed ingestion activity of macrophages (Yamamoto *et al.*, 1988). When 0·1% egg albumin (EA) was substituted for 10% FCS in the medium for development of macrophage ingestion capacity, a greatly reduced ingestion activity was observed. Therefore, we assume that there is a contributory role of serum factor(s) necessary for the activation of macrophages.

Here we present evidence that a serum factor is rapidly modified by DDG-treated B cells and untreated T cells in a stepwise fashion, being converted to the macrophage-activating signal factor.

MATERIALS AND METHODS

Animals

Female BALB/c mice, 7–12 weeks of age, were obtained from the Jackson Laboratories, Bar Harbor, ME. Mice were fed Purina mouse chow and water *ad libitum*.

Chemicals

Ether analogue of monoglyceride, rac-SN-1(3)-DDG, was synthesized by the method of Baumann & Mangold (1964) by E. Gustow and R. A. Pieringer, Temple University.

Culture media

RPMI-1640 media supplemented with 10% heat-decomplemented (56°C, 30 min) FCS (Flow Laboratories, Rockville, MD) or 0·1% EA (Sigma Chemical Company, St Louis, MO) were used.

Isolation and enumeration of peritoneal cells and macrophages

The peritoneal cells were prepared according to the procedure described by Cohn & Benson (1965) and Griffin & Silverstein (1974). Briefly, cells were harvested by injecting 6–8 ml cold (4°C) calcium- and magnesium-free phosphate-buffered saline (PBS) supplemented with 5–10 U heparin/ml. The cells were then washed three times in cold PBS without heparin and resuspended in RPMI 1640 medium with 10% FCS (FCS medium) or with 0·1% EA (EA medium). The desired number ($1–2 \times 10^6$/ml) of peritoneal cells was determined. Aliquots (1 ml) of the cells were layered onto 12-mm glass coverslips (Bellco, Vineland, NJ) which 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 5% CO_2 incubator for 30 min to allow macrophage adherence. Coverslips were immersed in warm RPMI 1640 (37°C) with gentle agitation to dislodge non-adherent cells and placed in fresh tissue culture wells. The percentage of macrophages in adherent cells were determined and identified as macrophages by phagocytic and morphological criteria (Cohn & Benson, 1965; Griffin & Silverstein, 1974). These criteria were assessed by latex particle (0·81 μm) ingestion and Giemsa staining, respectively. About 96% of the adhering cells from untreated mice were macrophages by these criteria.

In vitro *treatment of peritoneal cells*

Resident peritoneal cells were harvested from BALB/c mice, processed and quantified as described previously (Yamamoto & Ngwenya, 1987). Peritoneal cells (a mixture of non-adherent cells and macrophages) were incubated at 37°C in a humidified 5% CO_2 incubator for 30 min to allow macrophage adherence. The peritoneal cells or adherent (macrophage) cells alone were incubated with the indicated concentrations of DDG in FCS medium or EA medium. After 30 min incubation, DDG and the non-adherent cells were removed and the adherent cells were washed three times in Dulbecco's PBS and further cultured in FCS medium or EA medium for 3 h prior to ingestion assay. Cultivation, incubation and DDG treatment were all performed at 37°C in a humidified 5% CO_2 incubator.

Splenic lymphocyte collection

Spleen cells from BALB/c mice were collected and processed as described by Tyan & Ness (1971). Briefly, 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 ice-cold PBS. 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 two times, 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 were obtained by passing the cells over nylon wool columns as described by Julius, Simpson & Herzenberg (1973). Briefly, 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 (PBS–FCS). The wet columns were then incubated for 1 h at 37°C. Prior to loading the cells, each column was flashed with 30 ml of

warm PBS–FCS. Four millilitres of PBS–FCS containing $1{\cdot}5 \times 10^8$ cells 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 non-adherent cells) were T lymphocytes as determined by immunofluorescence using a fluorescein-labelled monoclonal antibody specific for the Thy 1.2 alloantigen (New England Nuclear, Boston, MA). The binding capacity of B cells to nylon wool was used as a method to obtain enriched B cell population as described by Trizio & Cudkowicz (1974). The B cells were collected by squeezing the nylon wool. The B cell population was further depleted of contaminating T cells by treatment with anti-Thy 1.2 antiserum and guinea pig complement (GIBCO, Grand Island, NY). The enriched B cell population lacked T cells as determined by immunofluorescence.

Preparation of conditioned media of B and T cells

Mixed non-adherent (B and T) cells ($0{\cdot}5$–1×10^6 cells/ml) were treated with 50 ng DDG/ml of FCS medium for 30 min. The treated non-adherent cells were washed to remove residual DDG and cultured in FCS medium for 2 h. The resultant conditioned medium of DDG-treated non-adherent cells was added to the adherent cells and incubated for 3 h and then assayed for ingestion activity.

Alternatively, the fractionated B or T cells were treated with 50 ng DDG/ml of FCS medium for 30 min. After being washed, the treated B or T cells were resuspended in FCS medium and incubated for various periods (15, 30, 60 or 120 min). The supernatant (conditioned medium) was used as a medium for culturing treated or untreated T or B cells for various periods. The resultant conditioned media ($0{\cdot}5$ ml/well) were then added to macrophages and incubated for 3 h prior to ingestion assay.

Preparation of splenic non-adherent cell ghosts

Splenic non-adherent cells (10^6/ml) were suspended in 1:10 PBS (10-fold dilution of PBS with water) and allowed to stand for 30 min at room temperature. The resultant cell ghosts were washed twice by centrifugation (3000 rev/min, 10 min) in $1 \times$ PBS. Microscopic analysis revealed that this procedure is an appropriate method to prepare non-adherent cell ghosts. These cell ghosts were treated with DDG (50 ng/ml) for 30 min, washed with PBS and incubated in FCS medium for 2 h. The resultant conditioned medium was added to peritoneal macrophages for ingestion assay.

Ingestion assay

The ingestion of sheep erythrocytes coated with IgG (EIgG) in the absence of complement was determined according to Bianco, Griffin & Silverstein (1975). Briefly, washed erythrocytes (Rockland, Gilbertsville, PA) were coated with subagglutinating dilutions of purified rabbit anti-erythrocyte IgG fraction (Cordis Laboratories, Miami, FL). For Fc-receptor-mediated ingestion assay, a $0{\cdot}5$ ml of $0{\cdot}5$% suspension of EIgG in RPMI 1640 medium without FCS was overlaid on each macrophage-coated (monolayer) coverslip and cultured at 37°C, in a humidified 5% CO_2 incubator for 1 h. Non-internalized erythrocytes were lysed by immersing the coverslips in a hypotonic solution (1:5 PBS) for 5–10 sec. The macrophages were fixed with methanol, air dried and stained with Giemsa stain, and ingestion was quantified microscopically. The data are expressed as the ingestion index (Bianco *et al.*, 1975), calculated as: ingestion index = % of macrophages with ingested erythrocytes × average number of erythrocytes ingested per macrophage.

RESULTS

In vitro *treatment of peritoneal cells with DDG and effect of a serum-free medium on macrophage activation process*

We demonstrated that a signal factor(s) for macrophage activation is transmitted from non-adherent cells to adherent cells (Yamamoto *et al.*, 1988). To identify such a signal factor, a chemically defined serum-free medium is required for cultivation of these cells. When peritoneal cells were treated with 50 ng DDG/ml in serum-free EA medium for 30 min and washed with PBS to remove residual DDG and nonadherent cells, a 3-h cultivation of the adherent cells in EA medium produced a low level of increased macrophage ingestion (Fig. 1). When various doses of DDG were tested for *in vitro* activation of macrophages, about the same concentration (50 ng/ml) of DDG in both FCS and EA media stimulated macrophages most efficiently. Although a DDG dose of about 50 ng/ml developed the maximal ingestion activity of macrophages in both FCS and EA media, the maximal ingestion activity of macrophages in DDG-treated peritoneal cells in FCS medium is far greater than the maximal ingestion activity of macrophages in DDG-treated peritoneal cells in EA medium (Fig. 1). This suggests that a factor(s) contained in serum is required for activation of macrophages.

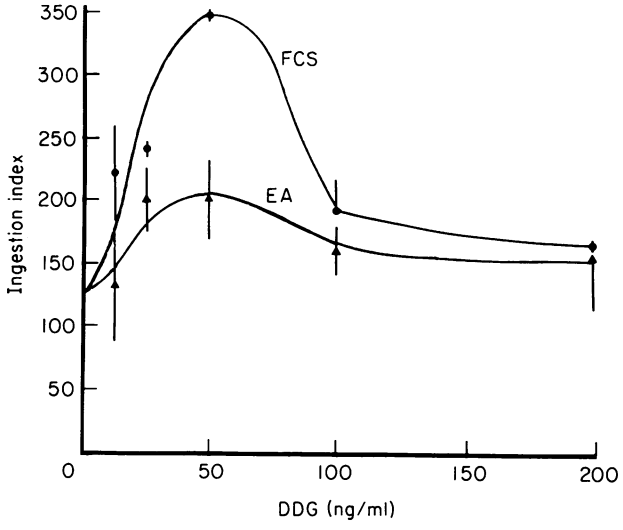

Fig. 1. *In vitro* dose effect of dodecylglycerol (DDG) on macrophage activation by treatment of peritoneal cells in media containing fetal calf serum (FCS) or egg albumin (EA). Peritoneal cells were harvested and incubated to allow macrophage adherence for 30 min in RPMI 1640 medium containing 10% FCS (FCS medium) or 0·1% EA (EA medium). These peritoneal cells were treated with various doses of DDG for 30 min. After removing DDG and non-adherent cells by washing in phosphate-buffered saline (PBS) the adherent cells were cultured for 3 h in fresh FCS medium and EA medium, respectively, prior to Fc-receptor-mediated ingestion assay. Ingestion activities were expressed as ingestion indices. Bars are s.d.

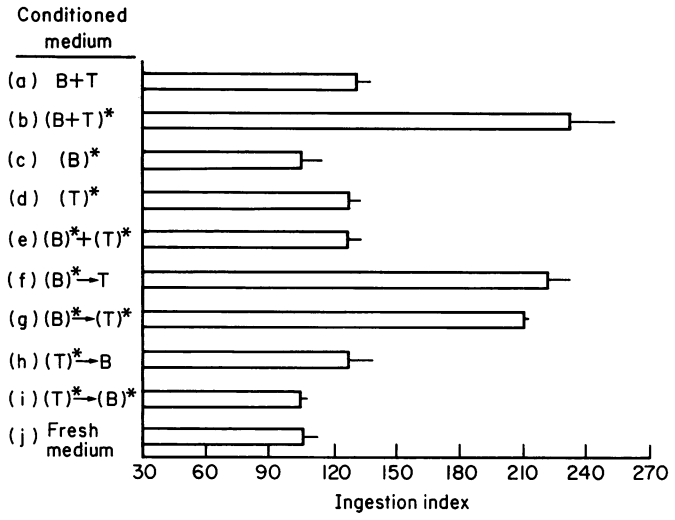

Fig. 2. Signal transmission among dodecylglycerol (DDG) treated B and T cells for macrophage activation. Enriched splenic B and T cells were prepared using nylon column method. B and T cells (10^6/ml) were individually (c–i) or mixedly (b) treated with 50 ng DDG/ml in RPMI 1640 medium containing 10% fetal calf serum (FCS medium) for 30 min. After washing, the cells were suspended in FCS medium and cultured for 2 h. The resultant conditioned media were used as media for cultivation of untreated adherent cells for 3 h prior to macrophage ingestion assay. Also, conditioned medium of treated B and that of treated T cells were mixed together 1:1 and used for cultivation of untreated adherent cells for macrophage ingestion assay (e). The conditioned medium of treated B or T cells was added to treated or untreated T or B cells and further cultured for 2 h (f–i). The resultant conditioned media were used for 3 h incubation of untreated adherent cells. Fc-receptor-mediated ingestion activity was determined for activation of macrophages and expressed as ingestion indices. * Cells were treated with DDG. Bars are s.d.

Transmission of signalling factor between non-adherent and adherent cells

Since signal transmission from non-adherent cells to macrophages can occur during a brief DDG treatment period of peritoneal cells, a conditioned medium of the DDG-treated non-adherent cells should be used to culture adherent cells for macrophage activation. Splenic non-adherent (B and T) cells were treated with 50 ng DDG/ml in FCS medium for 30 min, washed with PBS and cultured in FCS medium for 2 h. When the resultant conditioned medium was added to adherent cells and cultured for 3 h, a greatly enhanced ingestion activity of macrophages was observed (Fig. 2b). However, when conditioned medium from untreated non-adherent cells was admixed with adherent cells, no significantly enhanced ingestion activity of macrophages was observed (Fig. 2a). Therefore, we conclude that treated non-adherent cells release a signal(s) for macrophage to develop ingestion activity.

When individually DDG-treated B or T cells were cultured in FCS medium for 2 h, the resulting conditioned medium of each cell type was unable to activate macrophages (Fig. 2c and d). This result suggests that both non-adherent (B and T) cells are involved in transferring macrophage activating signal to macrophages. Cultivation of adherent cells with a mixture of the treated B cell conditioned medium and the treated T cell conditioned medium produced no significant activation of macrophage (Fig. 2e). This finding strongly suggests that a stepwise signal transmission among non-adherent (B and T) cells must have occurred. Accordingly, a conditioned medium of DDG-treated B cells was admixed with DDG-treated or untreated T cells to culture for 2 h. The resultant conditioned media efficiently activated macrophages as shown in Fig. 2f and g. However, when conditioned medium of DDG-treated T cells was added to DDG-treated or untreated B cells for 2 h cultivation, the resultant conditioned media were unable to

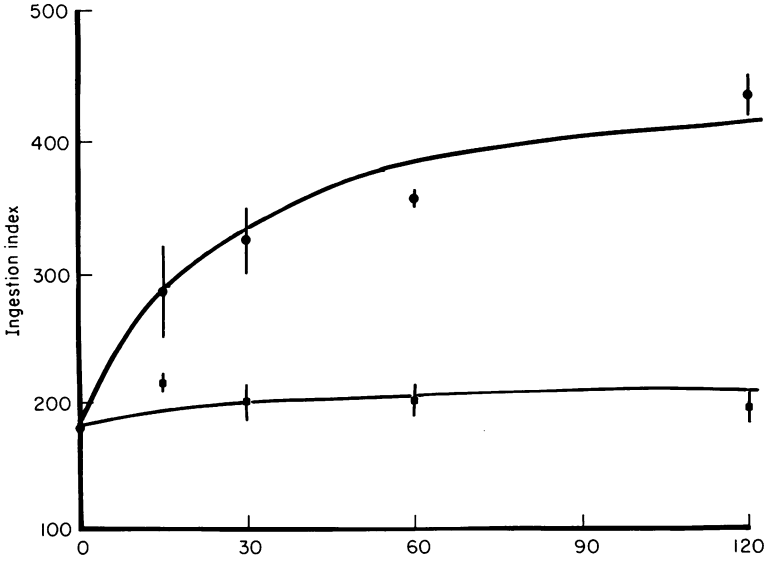

Fig. 3. Time required for production of macrophage activating signal factor from dodecylglycerol (DDG) treated B cells and untreated T cells. Mouse splenic B cells (10^6/ml) were treated with 50 ng DDG/ml in RPMI 1640 medium containing 10% fetal calf serum (FCS medium) at 37°C. After washing, the cells were incubated in FCS medium for 15, 30, 60 and 120 min at 37°C. Untreated B cells were incubated for corresponding periods. Conditioned media of untreated (■) or treated (●) B cells were used as a medium for incubation of untreated T cells (10^6/ml). Incubation periods of T cells correspond as those of B cells. These timed conditioned media were used as media for 3 h cultivation of untreated peritoneal adherent cells for Fc-receptor-mediated macrophage ingestion assay. Bars are s.d.

Table 1. Time required for maximal production of macrophage-stimulating signal from dodecylglycerol (DDG) treated B cells and untreated or treated T cells

Conditioned medium					Ingestion index
Period of cultivation (min)					
B	15→	T	15→	Mϕ	148± 6
B	60⟶	T		Mϕ	148±13
B	15→	T	60⟶	Mϕ	145± 3
B	60⟶	T		Mϕ	150±21
B*	15→	T	15→	Mϕ	212± 3
B*	60⟶	T	15→	Mϕ	228± 4
B*	15→	T	60⟶	Mϕ	368± 7
B*	60⟶	T	60⟶	Mϕ	373±32
B*	15→	T*	15→	Mϕ	210±35
B*	60⟶	T*	15→	Mϕ	218±47
B*	15→	T*	60⟶	Mϕ	231± 1
B*	60⟶	T*	60⟶	Mϕ	300± 3

Enriched splenic B and T cells were prepared using nylon wool column method. B cells (10^6/ml) and T cells (10^6/ml) were treated with 50 ng DDG/ml in fetal calf serum (FCS) medium for 30 min at 37°C and washed in phosphate-buffered saline (PBS). The treated B cells were incubated in FCS medium for either 15 mins or 60 min at 37°C. The B cell conditioned media were used for cultivation of treated or untreated T cells for 15 min or 60 min at 37°C. Similarly, untreated B cell conditioned media were used for cultivation of untreated T cells for 15 min or 60 min. The resultant conditioned media were used for 3 h cultivation of untreated adherent cells prior to macrophage ingestion assay.

* Treated with DDG.

Mϕ, macrophages.

produce a significant activation of macrophages, as shown in Fig. 2h and i.

These observations confirmed our previous finding (Yamamoto *et al.*, 1988) that DDG-treated B cells produce a signalling factor which may stimulate untreated T cells to produce the macrophage-activating signal factor. Production of the initial signalling factor, designated proactivating factor, requires serum in the medium for cultivation of the DDG-treated B cells.

Time required for signal transmission among non-adherent (B and T) cells

B cells were treated with 50 ng DDG/ml in FCS medium for 30 min, washed to remove DDG and cultured in fresh FCS medium for 15, 30, 60 or 120 min. The resultant conditioned media were used for culturing untreated T cells for corresponding periods. As shown in Fig. 3, a 2-h cultivation of each cell type (B and T cells) produced the highest macrophage activating signal factor. Thirty minutes of cultivation of these cell types produced about a half of the highest level of the macrophage activating signal factor. Even 15 min cultivation of each cell type (B and T) produced a significant amount of macrophage activation (at least 30% of the maximum activity), suggesting that the signal transmission results from pre-existing functions in these cells rather than *de novo* synthesized process.

Since a 15-min cultivation of each cell type (B and T) produced a significant amount of macrophage activating signal factor, cultivation of one of the cell types (B or T) for a longer period might produce the maximal level of macrophage-activating signal factor. As shown in Table 1, 15 min cultivation of DDG-treated B cells in FCS medium followed by 60 min cultivation of untreated T cells in the same FCS medium produced the maximal level of macrophage activating signal factor, which is identical to 60 min cultivation of each cell type. In contrast, 60 min cultivation of DDG-treated B cells in FCS medium and followed by 15 min cultivation of untreated T cells in the same FCS culture medium produced a macrophage stimulating activity similar to that of 15 min cultivation of each cell type (Table 1).

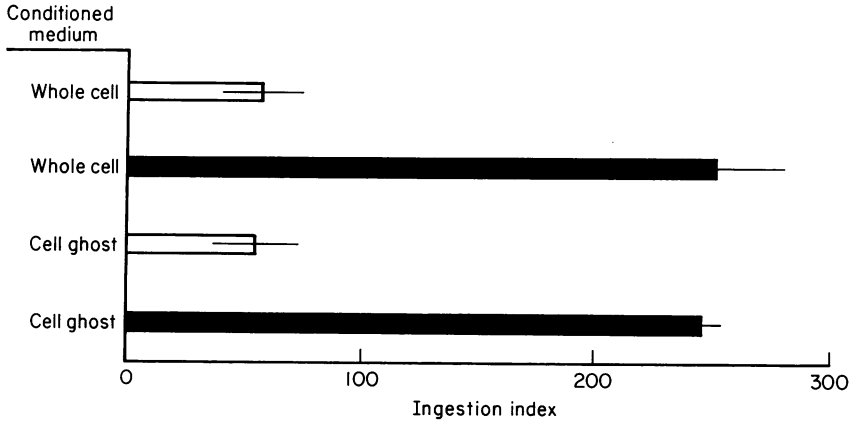

Fig. 4. Activation of macrophages by the conditioned medium of dodecylglycerol (DDG) treated non-adherent cell ghosts. Mouse splenic non-adherent cells (2×10^6/ml) were treated with 1:10 phosphate-buffered saline (PBS) for 30 min at room temperature for preparation of cell ghosts. The non-adherent cell ghosts were treated with 50 ng DDG/ml in RPMI 1640 medium containing 10% fetal calf serum (FCS medium) for 30 min at 37°C and washed with PBS. The treated or untreated non-adherent cell ghosts were incubated in FCS medium for 2 h. The resultant conditioned media were used for 3 h cultivation of untreated adherent cells prior to Fc-receptor-mediated macrophage ingestion assay. Conditioned media of untreated or treated whole non-adherent cells were also used for 3 h cultivation of untreated adherent cells for macrophage ingestion assay. (■), Treated with DDG; (□), not treated. Bars are s.d.

Therefore, DDG-treated B cells rapidly produce a signal factor, proactivating factor, and a brief (15 min) cultivation of treated B cells appears to accumulate a significant amount of the proactivating factor in the culture media. The maximal production of the macrophage-activating factor seems to require a longer time of 1 h or more for cultivation of untreated T cells in the proactivating factor containing conditioned medium. However, appearance of these signal factors begins even before 15 min cultivation of each cell type. This remarkably rapid signal transmission implies that these signalling factors are processed by pre-existing cellular functions of non-adherent cells because synthesis of inducible gene products would require a longer period of time. Therefore, a serum factor could be modified by a pre-existing enzyme(s) of B cells after DDG treatment and further modified by a pre-existing enzyme(s) of untreated T cells. Since DDG treatment of cells (e.g. B cells) results in increase of lipid membrane fluidity (Yamamoto *et al.*, 1987), membrane enzymes may be readily accessible to the serum factor to interact.

Macrophage-activating signal factor was produced by incubating DDG-treated B and T cell ghosts in FCS medium

We prepared enucleated cell ghosts of non-adherent (B and T) cells by hypotonic shock in 1:10 PBS. The splenic non-adherent cell ghosts were treated with DDG (50 ng/ml) for 30 min, washed in PBS and incubated in FCS medium for 2 h. After removal of the non-adherent cell ghosts, the conditioned medium was used for cultivation of peritoneal adherent cells (macrophages). As shown in Fig. 4, a greatly enhanced Fc-receptor-mediated ingestion activity of macrophages was observed. Therefore, it is unequivocally concluded that the serum factor is rapidly modified by the pre-existing membrane enzymes of B and T cells to yield a macrophage-activating factor.

DISCUSSION

A brief DDG treatment (30 min) of a mixture of peritoneal non-adherent (B and T) and adherent cells resulted in a greatly enhanced Fc-mediated ingestion activity of macrophages. This observation suggests that a signal factor(s) for macrophage activation is rapidly transmitted from non-adherent cells to adherent cells during the 30-min treatment period. Since cultivation of adherent cells with a mixture of treated B cell conditioned medium and treated T cell conditioned medium produced no significant activation of macrophages, the enzymes to modify the serum factor are not released into the culture medium from the B and T lymphocytes and stepwise signal transmission among non-adherent cells must occur. From these observations, it seems reasonable to assume that cellular enzymes capable of interacting with a serum factor are localized in plasma membrane. Therefore, modification of the serum factor by B and T cells require complexing with cell membranes before the enzymatic action takes place. It has been demonstrated that some serum proteins efficiently bind to the lymphocyte cell surface (Galbraith & Galbraith, 1980; Constans, Oksman & Viau, 1981; Machii *et al.*, 1986). Since DDG treatment of cells increases membrane fluidity (Weber, 1985; Yamamoto & Ngwenya, 1987), mobilization of membraneous constituents could increase enzymatic activity which accelerates modification of the serum factor to yield proactivating signal factor. However, modification of the serum factor by T cells does not require DDG treatment of T cells (see Table 1), suggesting that the enzyme of T cells seems to be localized on membrane surface and is readily accessible to proactivating signal factor for its conversion to ultimate macrophage activating signal factor. Therefore, this rapid developmental process of macrophage ingestion activity suggests that the serum factor is rapidly modified by the pre-existing enzymes of B and T cells in a step-wise fashion to yield the macrophage activating factor. This hypothesis is supported by the finding that DDG treated nonadherent (B and T) cell ghosts were able to convert the serum factor into a macrophage activating factor. Thus, the serum factor is essential for macrophage activation process as a precursor or substrate for development of macrophage activating signal factor. In a preliminary study (Homma *et al.*, 1988), this serum factor was found to be a vitamin D_3-binding protein (group-specific component, Gc) as demonstrated by complete inhibition of macrophage activation with anti-Gc treated serum.

However, DDG was able to activate macrophages when peritoneal cells were treated with 50 ng DDG/ml in a serum-free EA medium and cultured in EA medium though the level of macrophage ingestion was very low. This low level of activity could result from a small amount of serum factor complexed to the lymphoid cell membranes. Several investigators (Constans *et al.*, 1981; Petrini, Emerson & Glabraith, 1983a; Petrini, *et al.*, 1983b; Nel *et al.*, 1985; Machii *et al.*, 1986) demonstrated lymphocyte cell membrane-bound serum Gc protein. If non-adherent lymphocytes are treated with DDG and cultured in EA medium, such proteins could be converted to macrophage-activating signal factor.

ACKNOWLEDGMENTS

This investigation was supported in part by a National Science Foundation grant no. DMB-8511651 to N.Y.

REFERENCES

BAUMANN, W.J. & MANGOLD, H.K. (1964) Reaction of aliphatic methansulfonates. I. Synthesis of long chain glycerol-(1) ethers. *J. org. Chem.* **29,** 3022.

BIANCO, C., GRIFFIN, F.M. & SILVERSTEIN, S.C. (1975) Studies of the macrophage complement receptors. Alteration of receptor function upon macrophage activation. *J. exp. Med.* **141,** 1278.

COHN, Z.A. & BENSON, B. (1965) The differentiation of mononuclear phagocytes, morphology, cytochemistry, and biochemistry. *J. exp. Med.* **121,** 153.

CONSTANS, J., OKSMAN, S. & VIAU, M. (1981) Binding of the apo and holo form of the serum vitamin D binding protein to human lymphocyte cytoplasma and membrane by indirect immunofluorescence. *Immunol. Lett.* **3,** 159.

GALBRAITH, G.M.P. & GALBRAITH, R.M. (1980) Metabolic and cytoskeltal modulation of transferrin receptor mobility in mitogen activated human lymphocytes. *Clin. exp. Immunol.* **42,** 285.

GRIFFIN, F.M. & SILVERSTEIN, S.C. (1974) Segmental response of the macrophages plasma membrane to a phagocytic stimulus. *J. exp. Med.* **139,** 323.

HAWARD, B.V., MORRIS, H.P. & BAILEY, J.M. (1972) Ether lipid, α-glycerol phosphate dehydrogenase and growth rate in tumors and cultured cells. *Cancer Res.* **32,** 1533.

HOMMA, S., ST. CLAIRE, D.A., NGWENYA, B.Z. & YAMAMOTO, N. (1988) A serum factor regulates the activation mechanism of macrophages

during inflammation processes. *Abstr. Annu. Meet. Am. Soc. Microbiol.* **88,** 121.

JULIUS, M.H., SIMPSON, E. & HERZENBERG, L.A. (1973) A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* **3,** 645.

MACHII, T., KIMURA, H., UEDA, E. CHUJO, T., MORITA, T., KATAGIRI, S., TAGAWA, S. & KITANI, T. (1986) Distribution of Gc protein (vitamin D binding protein) on the surfaces of normal human lymphocytes and leukemic lymphocytes. *Acta haematol.* **75,** 26.

NAUTS, H.J., SWIFT, W.E. & COLEY B.L. (1946) The treatment of malignant tumors by bacterial toxins, as developed by the late William B. Coley, M.D., reviewed in the light of modern research. *Cancer Res.* **6,** 505.

NEL, A.E., NAVAILLES, M., EMERSON, D.L., GOLDSCHMIDT-CLERMONT, P., PATHAK, S.K., TSANG, K. & GALBRAITH, R.M. (1985) Altered configuration of Gc on the plasma membrane of transformed and malignant human B lymphocytes. *Clin. Immunol. Immunopathol.* **37,** 191.

NGWENYA, B.Z. & YAMAMOTO, N. (1985) Activation of peritoneal macrophages by lysophosphatidylcholine. *Biochim. biophys. Acta,* **839,** 9.

NGWENYA, B.Z. & YAMAMOTO, N. (1986) Effects of inflammation products on immune systems: lysophosphatidylcholine stimulates macrophages. *Cancer Immunol. Immunother.* **21,** 174.

OLD, L.J. (1988) Tumor necrosis factor. *Sci. Am.* **258,** 59.

PETRINI, M., EMERSON, D.L. & GALBRAITH, R.M. (1983a) Linkage between surface immunoglobulin and cytoskeleton of B lymphocytes many involve Gc protein. *Nature,* **306,** 73.

PETRINI, M., GALBRAITH, R.M., WERNER, P.A.M., EMERSON, D.L. & ARNAUD, P. (1983b) Gc (vitamin D binding protein) binds to cytoplasm of all human lymphocytes and is expressed on B-cell membranes. *Clin. Immunol. Immunopathol.* **31,** 282.

RAPP, H.J. (1976) Immunotherapy of experimental cancer with BCG. In *Tumor Virus Infections and Immunity* (ed. by R. L. Crowell, H. Friedman & J. E. Prier) p. 261. University Park Press, Baltimore.

SNYDER, F. & WOOD, R. (1969) Alkyl and alk-1-enyl ethers of glycerol in lipids from normal and neoplastic human tissues. *Cancer Res.* **29,** 251.

TRIZIO, D. & CUDKOWICZ, G. (1974) Separation of T and B lymphocytes by nylon wool columns: evaluation of efficacy by functional assays *in vivo*. *J. Immunol.* **113,** 1093.

TYAN, M.L. & NESS, D.B. (1971) Mouse blood leukocytes: *in vitro* primary and secondary responses to two synthetic polypeptide. *J. Immunol.* **106,** 289.

WEBER, N. (1985) Metabolism of orally administered rac-1-0-[1′-^{14}C] dodecylglycerol and nutritional effects of dietary rac-1-0-dodecylglycerol in mice. *J. Lipid Res.* **26,** 1412.

YAMAMOTO, N. & NGWENYA, B.Z. (1987) Activation of macrophages by lysophospholipids and ether derivatives of neutral lipids and phospholipids. *Cancer Res.* **47,** 2008.

YAMAMOTO, N. NGWENYA, B.Z., SERY, T.W. & PIERINGER, P.A. (1987) Activation of macrophages by ether analogs of lysophospholipids. *Cancer Immunol. Immunother.* **25,** 185.

YAMAMOTO, N. ST. CLAIRE, D.A. JR, HOMMA, S. & NGWENYA, B.Z. (1988) Activation of mouse macrophages by alkylglycerols, inflammation products of cancerous tissues. *Cancer Res.* **48,** 6044.

ZBAR, B. & TANAKA, T. (1971) Immunotherapy of cancer: regression of tumors after intralesional injection of living *Mycobacterium bovis*. *Science,* **172,** 271.