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Anti-neoplastic Action of Peritoneal Macrophages Following Oral Administration of Ether Analogues of Lysophospholipids

Benjamin Z. Ngwenya, Nicholas P. Fiavey and Mary M. Mogashoa

Inflamed lesions of normal and cancerous tissues induce activation of phospholipase A in plasma membranes resulting in the release of various decomposed products of membranous lipids. Oral administration in mice of dodecylglycerol (DDG), a synthetic alkylglycerol, and an alkyl ether analogueof lysophospholipids, 1-0-octadecyl-2-0-methyl-rac-glycero-3-phosphocholine (ET-18-OCH_3-choline) efficiently activated peritoneal macrophages for enhanced Fc-mediated (fragment crystallisable) ingestion of red blood cells and direct cytotoxic action on retinoblastoma tumour cells. The activated macrophages not only inhibited tumour cell growth, but also markedly induced cytolysis of tumour cells. The antitumour capability of the macrophages was substantiated by luminol-enhanced chemiluminescence. These findings suggest that dodecylglycerol and ET-18-OCH_3-choline administered orally retain their ability to induce a high level of macrophage activation and tumour cytotoxicity, just as occurs with intraperitoneal administration. Thus, these compounds have potential practical application in chemotherapy and immunotherapy of the tumour, which could be accomplished by simple oral rather than parenteral administration.
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INTRODUCTION

INFLAMMATION INDUCED by immunological reactions, chemical agents and microbial infections results in the activation of phospholipase A in cell plasma membranes. Subsequently, the enzyme decomposes membranous phospholipids to yield various lysophospholipids which attract and activate phagocytic cells [1, 2] to avidly combat the insulting agent.

Recent reports have indicated that intraperitoneal administration of various lysophospholipids in mice activate macrophages for enhanced Fc-mediated (fragment crystallisable) ingestion [3–5], antibody production [6] and anti-tumour [7] activities. Administration of 20 μg lysophosphatidylcholine (Lyso-Pc) or 0.1 μg dodecylglycerol (DDG; a synthetic alkylglycerol) per mouse, for example, produced highly activated splenic and peritoneal macrophages. Specifically, this intraperitoneal DDG treatment of mice induced an enhancement of Fc-mediated ingestion activity of macrophages and/or metabolic capability measured by an increase in superoxide production [7]. Thus, these agents could be considered as activators of the metabolic capabilities of macrophages, which enables them to readily ingest and kill foreign agents. In fact, we have demonstrated that Lyso-Pc- and DDG-treated macrophages acquire tumoricidal activity [7] and appear to efficiently transmit antigenic stimulus to B and T cells, resulting in the augmentation of antibody production [6].

We reported that lyso-Pc- or DDG-treated B cells release and transmit a promacrophage activating factor (PMAF) to T cells. The factor-sensitised T cells probably modify PMAF 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 [6, 8].

Our accumulated evidence strongly indicates that dodecylglycerol and lysophosphatidylcholine are potent *in vivo* as well as *in vitro* macrophage-activating agents. Clinically, these findings are of great importance since the lysophospholipid- or alkylglycerol-induced macrophage activation can lead to death of microbial agents and some tumour cells [7]. Furthermore, the alkyl-lysophospholipid derivative, 1-0-octadecyl-2-0-methyl-rac-glycero-3-phosphocholine (ET-18-OCH_3-choline), is known to be cytotoxic to some cancer cells [9–13], and the alkylglycerols have been reported to have bacteriostatic and bactericidal activity [14, 15]. These potential practical applications prompted investigators to study the metabolic fate of orally administered dodecylglycerol. Accordingly, it has been established that dodecylglycerol is converted to alkyldiacylglycerols which are subsequently incorporated into different organs [16]. On this basis, we hypothesise that oral administration of alkyl-lysophospholipids should efficiently activate macrophages for enhanced ingestion and cytotoxic capability just as occurs with intraperitoneally administered alkyl-lysophospholipids. The present study reports the effects of orally administered ether analogues of lysophospholipids on the activation of macrophages and their subsequent cytotoxicity to retinoblastoma tumour cells.

MATERIALS AND METHODS

Animals

Inbred BALB/c mice, 6–12 weeks of age were obtained from the Jackson Laboratories, Bar Harbour, Maine. 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*.

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Chemicals and reagents

Ether analogue of monoglyceride, 1-0-dodecyl-rac-glycerol (DDG) was purchased from Sigma, ET-18-OCH_3-choline was purchased from Calbiochem Corporation, La Jolla. The purity of these agents was greater than or equal to 99% pure; 0.5% precursors of lysophospholipids and no other compounds such as lipopolysaccharide were detectable. The reagents were dissolved in dimethyl sulphoxide (DMSO; 1 mg/50 μl) and then diluted more than 1000-fold in pyrogen-free saline to the desired concentration. The reagents were sterilised 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.

Macrophage stimulation, collection 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 animal intubation needle with spherical ball tip (Thomas Scientific, Philadelphia). Seven days after primary administration of agents, a second injection was administered in a similar manner. In all experiments, mice were not fasted prior to oral treatment. On designated days after secondary or primary administration of agents, mice were killed (3–6 mice/dose) by CO_2. Peritoneal cells were harvested, pooled and processed according to the procedure described by Cohn and Benson [17] and Griffin and Silverstein [18]. Cells were harvested by injecting 4–5 ml cold (4°C) phosphate-buffered saline (PBS) free of Ca^{2+} and Mg^{2+}, supplemented with 5–10 U heparin/ml. The cells were washed three times in cold PBS without heparin, resuspended in 5 ml RPMI-1640 medium with 10% heat-inactivated (56°C, 30 min) gammaglobulin-free fetal calf serum (FCS) (Gibco). The desired number of peritoneal cells (2×10^6/ml) was determined using a Thomas pipette and improved Neubauer haemacytometer.

Cultivation of retinoblastoma tumour cells

The human retinoblastoma tumour cell line Y-79 was purchased from the American Type Culture Collection. The cells were grown in suspension in 50 ml Falcon plastic flasks, cultured in RPMI-1640 medium supplemented with 15% FCS and 50 mg 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 pipetted gently and subcultured at a ratio of 1:4 for continuous proliferation. Prior to use, tumour cells were washed three times in PBS, and Trypan Blue exclusion test was used for determining the number of viable cells.

Cytotoxic assay

Radioisotope uptake assay. Peritoneal macrophages in 100 μl aliquots were distributed into round-bottom 96-well plates (Corning) and co-cultured with equal concentrations of retinoblastoma tumour cells (2×10^5/well) in log growth (45–50% confluent), and the plates were incubated at 37°C in a humidified 5% CO_2 incubator. Three hours before harvesting, the cells were pulsed with 50 μl (18.5 KBq) of [^{3}H]thymidine (specific activity, 2.516 TBq/nmol) (ICN Biomedicals, Irvine) per well and incubated for an additional 3 h. Cells were harvested with a semi-automatic cell harvester (Skatron) on filter paper, and the filters transferred to scintillation vials into which 4 ml scintillation fluid was added. Each test was done in quadruplicate. Radioactivity was measured in a liquid scintillation counter and the data expressed as net count per minute (CPM):

$$CPM_{\text{tumour cells}} = CPM_{\substack{\text{treated macrophages} \\ \text{and tumour cells}}} - CPM_{\text{treated macrophages.}}$$

Radioisotope release assay. Tumour cell ($1–2 \times 10^6$ cells/ml) cultures in log growth (45–50% confluent) were labelled with 18.5 KBq of [^{3}H]thymidine (specific activity 2.516 TBq/nmol) for 3–4 h in appropriate medium at 37°C, in a humidified 5% CO_2 incubator. After incubation the radiolabelled tumour cell culture was washed free of unincorporated thymidine. The cells were reincubated in fresh medium for additional 3 h prior to thymidine release assay. Before co-culturing the prelabelled tumour cells with activated macrophages (1:5 ratio) for 18 h, the supernatant containing spontaneously released [^{3}H]thymidine was aspirated. This process decreased spontaneous release of label during the assay. At the end of the reincubation period, the tubes were centrifuged and 1 ml aliquots of supernatant culture were removed. The radioisotope released was measured and the results were expressed as above.

Chemiluminescence assay

Peritoneal macrophages from mice orally administered DDG and ET-18-OCH_3-choline were collected and washed as previously described. 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 (800 μl), luminol in dimethylsulphoxide (DMSO), 10^{-5} mol/l (20 μl), macrophages (100 μl) and phorbol-myristate-acetate (PMA) (10 μl). The contents of the cuvette were mixed gently and conveyed to the measurement position, and light emission was recorded in mV using the luminometer coupled to a display monitor and printer (LKB-Wallac 1251 Luminometer).

Ingestion assay

Washed sheep erythrocytes (E) were coated with subagglutinating dilutions of rabbit anti-E immunoglobulin G (IgG). After washing, ELgG conjugate was added to orally activated macrophages and incubated for 1 h to allow ingestion in RPMI-1640 medium without FCS. Non-ingested erythrocytes were lysed by immersing the coverslips in a hypotonic solution (1:5 PBS) for 5–10 s. 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.

RESULTS

Time-course analysis of the development of ingestion activity of macrophages derived from mice orally treated with dodecylglycerol

In order to determine the time required to activate macrophages in mice orally treated with various doses of DDG, a time-course analysis was deemed necessary. As illustrated in Fig. 1, macrophages from orally treated mice exhibited increased ingestion of IgG-coated sheep erythrocytes as early as 2 days after treatment and continued to increase, with increased duration up to 5 days after treatment. The maximum ingestion index was obtained at 5 days after treatment with 5 μg DDG/mouse. The kinetic study indicates that significant activation of macrophages for Fc-mediated ingestion activity was time-dependent.

The effect of orally administered ET-18-OCH_3-choline on phagocytic activity of mouse peritoneal macrophages

Since activated macrophages for phagocytosis also undergo metabolic activation, we studied the effect of orally administered

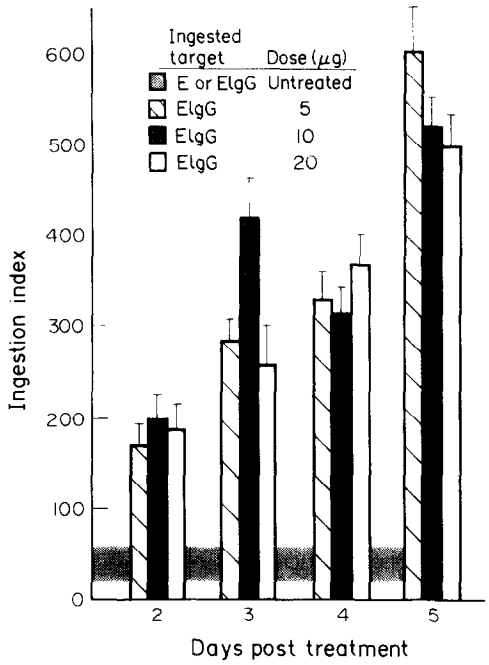

Fig. 1. Ingestion of opsonised (immunoglobulin G: IgG) sheep erythrocytes (E) by mouse peritoneal macrophages activated by orally administered dodecylglycerol. Peritoneal cells were harvested 1 single day after 5 days pretreatment and incubated 1 h prior to ingestion assay. Each value represents the mean (S.D.) for two separate experiments performed in duplicate.

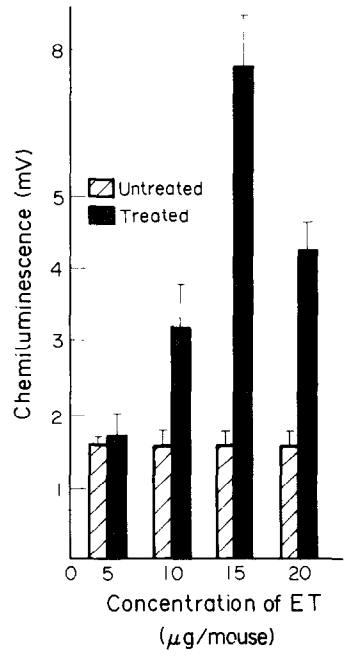

Fig. 2. Chemiluminescence activity of mouse peritoneal macrophages after oral treatment with ET-18-OCH_3-choline. Results are expressed as mean peak values (S.D.) (mV). Data shown are representative of three separate experiments.

ET-18-OCH_3-choline on macrophage metabolic capability as measured by luminol-enhanced chemiluminescence (CL) response to PMA [20, 21]. Macrophages from mice receiving primary treatment showed a slight increase in CL activity (data not shown), while macrophages from mice that received a second treatment produced a greatly enhanced CL activity. As illustrated in Fig.2, the maximal CL activity was obtained with 15 μg/mouse. At a dose of 10 μg/mouse, CL activity of the treated macrophages was significantly different from sham control. Macrophages from mice treated with 20 μg/mouse showed a lower CL response to the stimulant compared with the response from those given the 15 μg/mouse dose. Therefore, ET-18-OCH_3-choline could be considered as an activator of the metabolic capabilities of macrophages.

Cytocidal effect of ET-18-OCH_3-choline on human retinoblastoma tumour cell line

The alkyl-lysophospholipid derivative ET-18-OCH_3-choline is known to have cytocidal activity on some tumour cells [1, 8, 22, 23]. Accordingly, we tested its direct cytocidal capacity on human retinoblastoma cell line Y-79 *in vitro*. As illustrated in Fig. 3, treatment of tumour cells with various doses (5, 10, 20 and 40 μg/ml) of ET-18-OCH_3-choline significantly reduced the viability of the tumour cells, resulting in 75–95% cytocidal activity for doses more than or equal to 20 μg/mouse within 24 h after treatment. By the second day after treatment, the 40 μg/ml dose had attained 100% cytocidal activity, whereas at the lower doses (5, 10 and 20 μg/ml) ET-18-OCH_3-choline sustained the cytocidal activity up to 3 days of culture. It appears, however, that the tumoricidal efficacy of this agent is dose-dependent.

Anti-tumour activity of macrophages derived from mice orally treated with ET-18-OCH_3-choline and dodecylglycerol

Since ET-18-OCH_3-choline and DDG are able to augment macrophage function, the question arose as to whether such activated macrophages could also have direct cytotoxic capacity on tumour cells. Accordingly, we tested their direct cytotoxic capacity on human retinoblastoma cell line Y-79 *in vitro*.

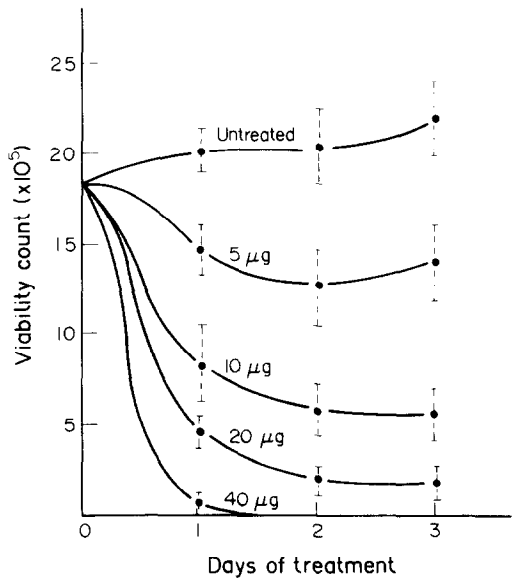

Fig. 3. Direct tumoricidal activity of ET-18-OCH_3-choline after *in vitro* treatment of retinoblastoma tumour cells. Viability count was determined using a haemacytometer and Trypan Blue exclusion test, and the counts are expressed as means of four values (S.D.).

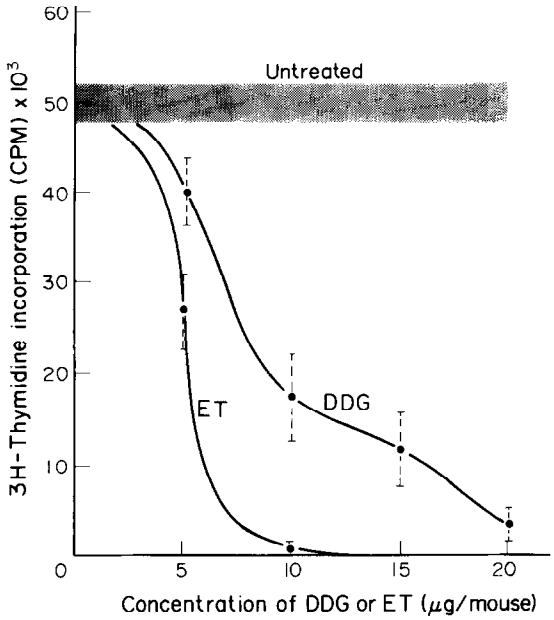

Fig. 4. Cytotoxic effect of orally activated mouse peritoneal macrophages on retinoblastoma tumour cells. Mouse peritoneal macrophages were harvested 4 days after oral treatment with various doses of dodecylglycerol (DDG) and ET-18-OCH$_3$-choline (ET). Tumour cells and macrophages were co-cultured for 18 h and pulsed for 3 h. Results are expressed as mean counts per minute (CPM) of quadruplicate samples (S.D.). Data shown are representative of three separate experiments.

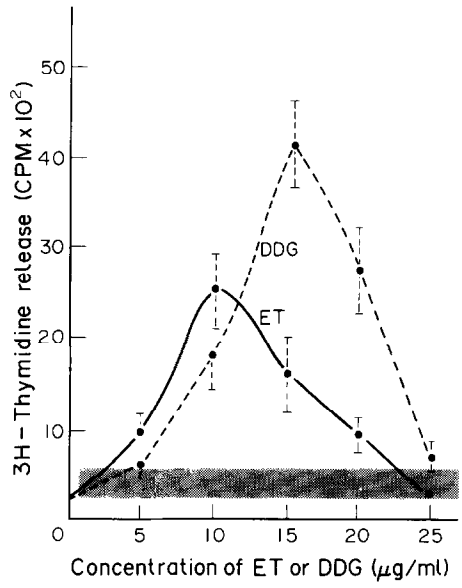

Fig. 5. Dose effect on tumoricidal activity of orally activated mouse peritoneal macrophages. Macrophages were harvested 4 days after treatment with dodecylglycerol (DDG) and ET-18-OCH$_3$-choline (ET). Retinoblastoma cells were prelabelled with 18.5 KBq [^{3}H]-thymidine for 3 h and co-cultured with activated macrophages (1:5 ratio) for 18 h. Results are expressed as mean CPM of triplicate samples (S.D.). Data are representative of two separate experiments.

As illustrated in Fig. 4, macrophages derived from either ET-18-OCH-$_3$-choline- or DDG-treated mice greatly inhibited the radioisotope uptake by the tumour cells. The oral dose required to activate macrophages for 100% antitumour activity is, however, much less for ET-18-OCH$_3$-choline (about 10 μg/mouse) than that for DDG (> 20 μg/mouse). A similar inhibition of [^{3}H]leucine uptake by retinoblastoma cells in the presence of macrophages derived from orally treated mice was also observed (data not shown). Therefore, macrophages activated by oral administration of these agents responded efficiently against human retinoblastoma cells.

Dose kinetic analysis of the development of cytotoxic activity of macrophages to retinoblastoma tumour cells

Macrophage cytotoxic activity against tumour cells is effected via two mechanisms: tumour inhibition of growth or cytostasis and actual cytolysis. Either parameter can be measured to quantify macrophage–tumour cell interactions. Since we established that macrophages from orally treated mice induced a marked tumour cytostasis, it was desirable to test for the actual cytolytic capability of such activated macrophages. As shown in Fig. 5, release of [^{3}H]thymidine from prelabelled tumour cells interacting with activated macrophages increased as the concentration of DDG or ET-18-OCH$_3$-choline increased up to 15 and 10 μg/mouse, respectively. Although absolute values for release of [^{3}H]thymidine by the tumour cells lysed by macrophages derived from mice treated with ET-18-OCH$_3$-choline was lower than that from DDG treated mice, the optimal effective dose for cytolysis is lower for ET-18-OCH$_3$-choline (10 μg/mouse) than DDG (15 μg/mouse). For DDG doses above 15 μg/mouse, [^{3}H]thymidine release drastically decreased, whereas at doses higher than 10 μg ET-18-OCH$_3$-choline/mouse, [^{3}H]thymidine release gradually declined. The decline in macrophage cytotoxic action may be attributed to the toxicity of the high doses of these agents on the macrophage activation mechanism *vis-à-vis* macrophage cytotoxic activity.

Time-course analysis for development of cytotoxic activity of macrophages derived from mice treated with dodecylglycerol

Activated macrophage-induced tumour cytolysis was not only dose-dependent but also varied with duration of administration of the activating compounds to mice. Consequently, the time kinetics for development of the macrophage activation mechanism for tumour cytolysis was analysed. Release of [^{3}H]thymidine by prelabelled tumour cells signified the extent of activated macrophage–tumour cell interaction. As seen in Fig. 6, macrophages from the orally treated mice exhibited about a 2-fold increase in [^{3}H]thymidine release at 2 days after treatment. The cytolytic activity increased with time, reaching maximal cytolysis on day 3 after treatment. A significant level of cytotoxicity by activated macrophages was still apparent 4 days after treatment. Thus, these observations establish that orally administered DDG or ET-18-OCH$_3$-choline is tumoricidal and that optimal cytolysis was attained 3 days after treatment.

DISCUSSION

Our recent publications indicate that *in vitro* treatment of peritoneal macrophages with alkyl-lysophospholipids and alkylglycerols, and intraperitoneal and oral administration of these compounds to mice, greatly activate macrophages for enhanced Fc-receptor-mediated ingestion activity, antitumour and antibody responses [3–7, 24, 25]. The present data indicate that oral administration of dodecylglycerol (DDG) or ET-18-OCH$_3$-choline to mice results in a greatly enhanced activation of peritoneal macrophages that produces not only significant cytostasis but also cytolysis of retinoblastoma tumour cells. Since

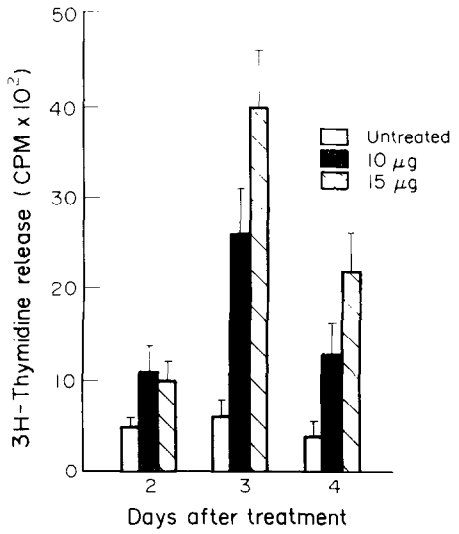

Fig. 6. Time kinetic analysis for development of cytotoxic action of peritoneal macrophages from mice orally treated with dodecylglycerol. Macrophages were harvested 1 single day after 4 days of treatment. Radioisotope release from retinoblastoma labelled cells co-cultured with the treated macrophages was determined and the results are expressed as in Fig. 5.

inhibition of radioisotope uptake alone has been shown not to be an adequate measure of tumoristatic and/or tumoricidal activity [26], nevertheless these results, combined with the release of radioisotope by the tumour cells following their interaction with lysophospholipid-activated macrophages, strongly suggest that these compounds have a significant effect on DNA synthesis by the tumour cells.

It is established that cells in culture, activated and non-activated macrophages, may be cytostatic to other cells through a process known as "contact inhibition of cell growth" [27]. On this basis, therefore, it appears that tumour cytolysis is a more interpretable endpoint for measuring activated macrophage cytotoxic activity. It should also be mentioned, however, that the measurement of tumour cytolysis by activated macrophages in the absence of antibody generally requires about 16–24 h to become evident. Accordingly, our maximal toxicity was observed at about 18 h of macrophage–tumour cell interaction. It should be emphasised that the observed cytotoxicity endpoint at 18 h may underestimate the extent of macrophage–tumour interaction. It is conceivable, however, that tumour cell death may have occurred much earlier than actually revealed by maximal release of [^{3}H]thymidine. These findings confirm our proposition that orally administered alkylglycerol and lysophospholipids, which have been shown by other investigators [16, 28] to be well absorbed from the intestines and incorporated into glycerolipids of their target organs, retain their ability to induce activation and subsequent tumoricidal activity of macrophages.

The difference in macrophage activation process between these two closely related compounds may be due, in part, to the way each compound is absorbed and metabolised. DDG may be converted by a monoglyceride kinase to alkylglycerophosphates, and is further metabolised to yield phosphatidic acid, which inhibits CDP-diglyceride synthesising enzyme resulting in blockage of lipotechoic acid synthesis [15, 29]. On the other hand, ET-18-OCH_3-choline, an alkyl phospholipid cannot enter the deacylation–acylation cycle of cellular phospholipids since lysophospholipase cannot split the alkyl bond, and the acylation is blocked, resulting in a stable compound. These differences in metabolic processes may result in prolonged macrophage activation *vis-à-vis* efficient tumoricidal activity. The present data confirm and further extend previous findings on lysophospholipid-activated macrophages via routes other than oral, and the subsequent antitumour action of these activated macrophages [10, 12, 26]. The fact that these can be accomplished by simple oral rather than parenteral administration with equally good results may provide a new and relatively simple approach to antitumour therapy.

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Cell Proliferation Kinetics of Human Gastric Carcinoma: An Immunohistochemical Study With a Monoclonal Antibody Against DNA Polymerase-α

Kiyoshi Saitoh, Tomoki Chiba and Kyoichi Nakamura

Cell proliferation kinetics of human gastric carcinoma were studied immunohistochemically using a monoclonal antibody against DNA polymerase-α (Pol-α). The distribution patterns and percentages of proliferative cells were examined in cases with various histological types of gastric carcinoma and compared with those of normal epithelium of the gastric foveolae. Pol-α-positive epithelial cells were localised at the isthmus of the normal foveola, while Pol-α-positive cancer cells were distributed irregularly in the cancer nests. The percentage of Pol-α-positive cells (%PPC) was significantly higher in the carcinoma [mean (S.D.) 41.6 (12.9)%] than in the normal foveola [24.8 (6.4)%] ($P < 0.01$). Also, the intestinal-type carcinoma showed a relatively higher %PPC [44.9 (12.0)%] than the diffuse type [36.2 (15.1)%] ($P < 0.05$), and the %PPC of signet ring cell carcinoma was extremely low [7.3 (2.2)%] ($P < 0.01$). Pol-α-positive cancer cells were observed most abundantly in the lamina propria of the mucosa. They decreased in number with the depth of cancer infiltration down to the subserosa.
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INTRODUCTION

HUMAN GASTRIC carcinomas show varied proliferation patterns and growth rates. It is important to estimate proliferative activities of the carcinomas to predict the degree of malignancy of each case. For this purpose cell proliferation kinetics of human gastric carcinoma have been studied by detection of S-phase cells with either tritiated thymidine ([^{3}H]TdR) [1–3] or bromodeoxyuridine (BrdU) [4], and some interesting information has been presented. It was reported that the intestinal-type carcinoma showed a higher labelling index than the diffuse-type carcinoma and that the signet ring cell carcinoma had a low labelling index and might be out of cell cycle [1, 2]. The cases with high labelling indices of BrdU were reported to have a poorer prognosis because of a high degree of lymph node metastasis [4]. However, the labelling method using [^{3}H]TdR or BrdU has many restrictions when performed on human tissues, and the findings of these studies were still not sufficient to determine the relation between the proliferation patterns of the carcinoma and the clinicopathological characteristics, such as histological subtype, depth of infiltration, size of carcinoma, age and sex of the patients, and positivity or negativity of lymph-node metastasis.

DNA polymerase-α (Pol-α) is a key enzyme which catalyses ribonucleoside triphosphate-dependent DNA synthesis in cooperation with DNA primase [5, 6], and the cells having Pol-α activities in the nuclei are supposed to be in cell cycle. Recently, monoclonal antibodies against Pol-α were produced which enabled us to detect proliferating cells immunohistochemically on pathological specimens [7–9]. As a result, several studies have been performed on the human cancer tissues indicating

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