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Photodynamic Immunopotentiation: *in vitro* Activation of Macrophages by Treatment of Mouse Peritoneal Cells with Haematoporphyrin Derivative and Light

Nobuto Yamamoto, Sadamu Homma, Theodore W. Sery, Larry A. Donoso and J. Kenneth Hoober

Peritoneal macrophages treated *in vivo* with haematoporphyrin derivative (HPD) exhibited significant enhancement of Fc receptor mediated ingestion activity. To examine this process more rigorously, we studied photodynamic activation of macrophages by exposure *in vitro* of mouse peritoneal cell cultures (containing macrophages and B and T-lymphocytes) to HPD and red fluorescent light. A short (10 s) exposure of peritoneal cells in medium containing 0.03 ng HPD/ml produced the maximal level of ingestion activity of macrophages. A singlet oxygen quencher, DABCO, inhibited the effect of HPD. Photodynamic treatment of macrophages alone did not activate the cells and activation was only observed when macrophages were mixed with photodynamically treated non-adherent cells (B and T-lymphocytes). These results imply that activation of macrophage is a consequence of peroxidation of lymphocyte membrane lipids by photodynamically generated singlet oxygen.
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INTRODUCTION

RECENT STUDIES of porphyrin photosensitisation have focussed on the mechanism of action of haematoporphyrin derivative (HPD) in cancer photodynamic therapy (PDT). Kessel [1, 2] and Kessel and Chou [3], using murine leukaemia L1210 cells in culture, showed that phototoxicity of HPD depends on cellular uptake of the most hydrophobic components. Lesions in various organelles (e.g. mitochondrial, lysosomal, nuclear and endoplasmic reticulum) generated by PDT have been described [4]. It is now generally accepted that cell membranes are the principal sites of HPD-induced photodamage [4, 5]. For example, membrane rupture of chinese hamster ovary cells exposed to PDT correlates directly with survivability, as measured by colony formation [6]. The principal target moieties in the cell membranes seem to be phospholipids. Their photodynamic modification appears to be peroxidation of unsaturated fatty acyl groups [7, 8] and ultimately results in membrane rupture and cell death.

The aim of HPD-PDT is to obtain selective destruction of cancer cells. HPD-PDT has been used on many patients with encouraging results [9, 10]. Cure requires total elimination of the cancer cells in the targeted tissues as well as in metastasised cancer cells. It would be advantageous if the metastasised cancer

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cells could be eliminated immunologically rather than through chemotherapy. However, recent studies have shown that PDT can induce a state of immunosuppression [11–14]. Administration of anticancer chemotherapeutic drugs also often induces immunosuppression [15]. Since lymphocytes are excellent sensors for foreign agents and chemicals, high therapeutic doses of anticancer drugs and haematoporphyrin derivatives are very likely to be detrimental to lymphocytes.

Several investigations on photodamage of cell membranes dealt primarily with sensitised photoperoxidation of unsaturated lipids [7, 8]. Peroxidation of membrane lipids is likely to cause an increase in the fluidity of membrane lipids due to increased polarity and degradation of peroxidation products of lipids [16–18]. As we demonstrated previously [19–23], a low concentration of exogenously given lipid degradation products increases membrane fluidity, resulting in *in vivo* and *in vitro* activation of macrophages for enhanced Fc receptor mediated ingestion activity. As a continuation of this work, we found that much smaller doses of haematoporphyrin than used in previous studies in fact stimulate lymphocytes and activate macrophages, resulting in immunologically beneficial effects to the hosts. Therefore, photoperoxidation of membranous lipids may also cause activation of macrophages.

PDT-damaged cancerous cells can be efficiently phagocytised by activated macrophages, leading to development of immunity against a cancer cell antigen. Because macrophages are antigen presenting cells, activation of macrophages is a primary step of immunopotentiation. Therefore, photodynamic immunopotentiation with HPD may improve the probability of cure of cancer by HPD-PDT. In the present communication, we report macrophage activation by photodynamic action.

MATERIALS AND METHODS

Animals

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

Chemicals

Photofrin II (Photofrin Medical, Raritan, New Jersey) was used as the source of HPD. Haematoporphyrin was purchased from Porphyrin Products, Logan, Utah. 1,4-diazabicyclo[2.2.2]octane (DABCO) was from Aldlich Chemical (Milwaukee, Wisconsin).

In vivo treatment of mice and macrophage collection and cultivation

Mice were injected intraperitoneally with increasing doses of HPD and maintained with normal ambient light environment. Peritoneal cells (mixture of macrophages and B and T lymphocytes) were harvested 4 days postadministration, the time when ingestion activity of macrophages reached a greatly elevated level with other macrophage stimulating agents [20–22]. Age-matched sham control mice were injected with equal volumes (0.3 ml) of sterile pyrogen-free saline. The peritoneal cells were prepared according to the procedure described by Cohn and Benson [24] and Griffin and Silverstein [25]. Briefly, cells were harvested by injecting into the peritoneum 5–7 ml ice-cold 0.01 mol/l sodium phosphate buffer (pH 7.1) containing 0.9% NaCl (PBS) and 5–10 U heparin/ml, with a tuberculin needle attached to a 10 ml syringe. The abdomen was massaged for several seconds and the peritoneal fluid was then withdrawn with a 21 gauge needle attached to a 10 ml syringe. The cells were then washed 3 times with cold PBS without heparin and resuspended in phenol red free Medium 199 with 10% fetal calf serum (gamma-globulin free), which had been treated at 56°C for 30 min to inactivate complement (FCS medium 199). Cell density was adjusted to the desired number ($1–2 \times 10^5$/ml) [21]. Aliquots (1 ml) of the cells were laid onto 12 mm glass coverslips (Bellco, Vineland, New Jersey) which had been placed in 16 mm diameter wells of tissue culture plates (Costar, Cambridge, Massachusetts). The plates were incubated at 37°C in a humidified 5% Co_2 incubator for 30 min to allow macrophages to adhere to the coverslips and then washed with PBS to remove non-adherent cells (B and T lymphocytes).

Enumeration of adherent macrophages

Macrophages from HPD treated and untreated control mice were identified by morphological criteria and ability to ingest latex particles (0.81 μm in diameter) [26]. About 96% of the adhering cells from treated and untreated mice were macrophages by the above criteria.

In vitro treatment of mixed peritoneal cells

Resident peritoneal cells were harvested from untreated mice, processed and quantified as described previously [20–22]. After 30 min incubation at 37°C in a humidified 5% CO_2 incubator to allow macrophage adherence to the coverslips in culture wells, various concentrations of HPD or haematoporphyrin were added to these peritoneal cell cultures in the dark. The peritoneal cells in culture wells were incubated in the dark for 30 min at 37°C or first exposed to red fluorescent light (Sylvania F15T8R, 0.5 Wm^{-2}) for 5–15 s prior to 30 min incubation in the dark. The non-adherent cells and the drugs were then removed and the adherent cells (at least 96% macrophages) were washed 3 times with PBS. Incubation in FCS medium 199 was then continued 3 h for development of ingestion activity.

Phagocytosis assay

Phagocytosis of sheep erythrocytes coated with immunoglobulin G (IgG) was determined as described by Bianco *et al.* [27]. Briefly, washed erythrocytes (Rockland, Gilbertsville, Pennsylvania) were coated with subagglutinating dilutions of purified rabbit anti-erythrocyte, IgG fraction (Cordis Laboratories, Miami, FL). For the Fc-receptor mediated phagocytosis assay, 0.5 ml of a 0.5% (cell volume/volume) suspension of the above conjugates 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-internalised erythrocytes were lysed by immersing the coverslips in hypotonic solution (PBS diluted 5 fold with water) for 5–10 s. The macrophages were fixed with methanol, air dried and stained with Giemsa stain; the number of phagocytised erthyrocytes per cell was determined microscopically; 500 macrophages were counted for each data point. The data are expressed as the ingestion index [27], which is defined as the percent of macrophages with ingested erythrocytes × average number of erythrocytes ingested per macrophage.

RESULTS

Effect of administration of HPD on in vivo activation of peritoneal macrophages

HPD was administered to mice and analysed for Fc receptor mediated ingestion activity of peritoneal macrophages at 4 days

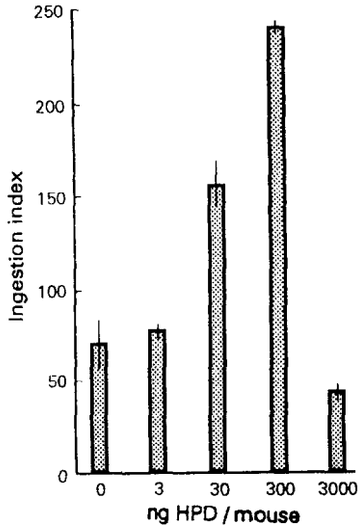

Fig. 1. Dose effect of HPD on activation of macrophages in BALB/c mice. Hatched bars are the mean values of 3 animals per analysis; vertical lines indicate S.E.

postadministration. As shown in Fig. 1, administration of small doses (30–300 ng HPD/mouse) induced a greatly increased ingestion activity of peritoneal macrophages. At a HPD dose of 300 ng/mouse, phagocytic activity of macrophages reached the maximal ingestion index of 244 as compared with an index of 70 for untreated control (Fig. 1). Higher doses (e.g. 3000 ng/mouse) suppressed phagocytic activity of macrophages below the untreated control. In these experiments the mice were exposed only to ambient daytime white fluorescent light.

Effect of in vitro treatment of mouse peritoneal cells with HPD in the dark on activity of macrophages

With *in vivo* experiments, the effect of HPD is difficult to quantitate in terms of dose level and light exposure. Therefore, mouse peritoneal cells were incubated *in vitro* with various low concentrations (0.01–10 ng/ml) of HPD for 30 min in the dark, washed to remove non-adherent cells and residual HPD and cultured for an additional 3 h in the dark. Macrophage ingestion activity was then assayed. As shown in Fig. 2a, no significant increase in ingestion activity was observed in unilluminated samples.

Photodynamic activation of peritoneal macrophages with HPD and red light

The longest wavelength absorption maximum of HPD is about 630 nm, which also is its smallest absorption peak. However, 630 nm has been selected as the optimal wavelength for HPD-PDT because of the greater tissue penetrating ability of longer wavelengths. Because red fluorescent light emits between 630–670 nm, we designed photodynamic treatment of cell cultures with this light. Photodynamic activation of macrophages was studied with various low concentrations of HPD and short exposures to red light. For the results shown in Fig. 2b, peritoneal cell cultures in FCS medium 199 containing 0–10 ng HPD/ml were exposed to light for 5 s, incubated 30 min in the dark and then washed free of non-adherent cells and residual HPD. This treatment with 0.03–1 ng HPD/ml produced a significantly increased phagocytic activity of macrophages after a further 3 h cultivation of the macrophages. Various concentrations of HPD (0.01–600 ng/ml) with larger doses (10 or 15 s) of red fluorescent light were also employed. A 10-s light exposure was able to maximally activate macrophages at a dose of 0.03 ng HPD/ml. The highest ingestion index of 250 was observed in these samples, as shown in Fig. 2c. A 15 s light exposure also produced a maximal ingestion index of 225 at a dose of 0.03 ng HPD/ml as compared with an index for untreated control of about 100 (Fig. 2d). HPD doses higher than 300 ng/ml and red light suppressed phagocytic activity of macrophages below the untreated controls (data not shown).

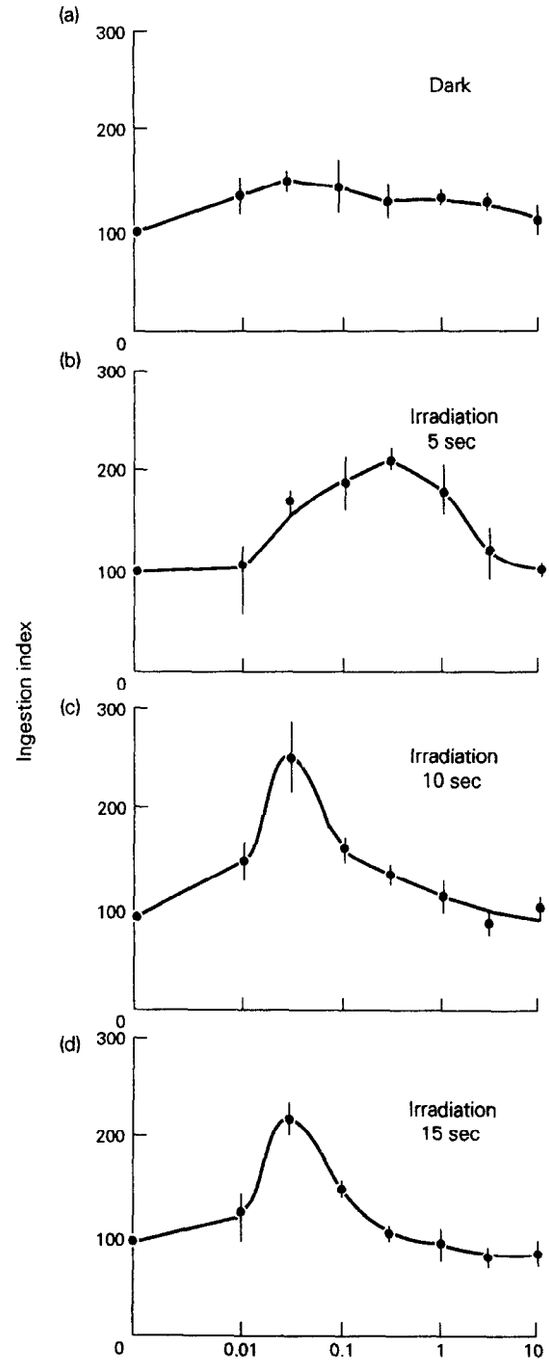

Fig. 2. Photodynamic activation of macrophages by ingestion indices at various HPD concentrations (0.01 to 10 ng/ml) at a fluence of 0.5 Wm^{-2} of red fluorescent light. (a) Treatment of mouse peritoneal cells in the dark, (b) 5, (c) 10 and (d) 15 s of exposure to light. Mean (S.E.) of triplicate assays.

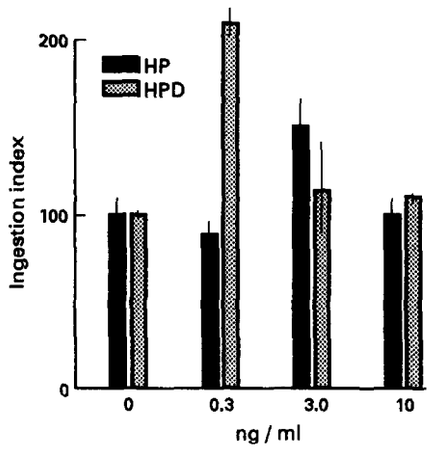

Fig. 3. Comparative concentration effect of haematoporphyrin and HPD on photodynamic activation of macrophages (5 s). Mean (S.E.) of triplicate assays.

Comparative dose effects of haematoporphyrin and HPD

Haematoporphyrin and HPD are both excellent photosensitisers. Because of the hydrophilicity of haematoporphyrin, it interacts weakly with cell membranes [8]. On the other hand, the greater hydrophobicity of HPD allows binding to cell membranes [5]. Since most photogenerated reactive species can diffuse only limited distances prior to reaction with the target molecules [29], it is likely that photosensitisers located near the membrane surface or within the membrane itself are more active than those in the medium. As shown in Fig. 3, a 5 s red fluorescent irradiation with 3 ng haematoporphyrin/ml produced macrophage activation with an ingestion index of only 150 whereas with 0.3 ng HPD/ml an ingestion index of 220 was obtained.

Effect of DABCO on photodynamic activation of macrophages using haematoporphyrin and HPD

To identify the type of oxidative mechanism involved in macrophage activation, photodynamic treatment of peritoneal cells was performed in the presence of DABCO, a known singlet oxygen quencher [30]. As shown in Table 1, photodynamic activities of haematoporphyrin (3 ng/ml) or HPD (0.03 ng/ml) on macrophage activation were greatly reduced by 1 mmol/l DABCO and completely inhibited by 2 mmol/l DABCO. Therefore, photodynamic activation of macrophages was most likely achieved by oxidation of lymphocytes membrane lipids with singlet oxygen, which was generated by HPD or haematoporphyrin in the presence of light.

Table 1. Inhibitory effect of DABCO on macrophage activation

Treatment of peritoneal cells	DABCO (mmol/l)	Ingestion index
Untreated	0	98 (14)
	2	110 (7)
Haematoporphyrin (3 ng/ml)	0	205 (19)
	1	124 (18)
	2	97 (12)
Haematoporphyrin derivative (0.03 ng/ml)	0	214 (17)
	1	121 (19)
	2	101 (21)

Mean (S.E.).

Table 2. Contribution of non-adherent cells to HPD-photodynamic activation of macrophages

Cells	HPD (0.03 ng/ml)	Red fluorescent light (0.5 W/m²) (s)	Ingestion index
Adherent cells			
Alone	–	10	92 (7)
Plus non-adherent cells	–	10	90 (12)
Alone	+	10	88 (11)
Plus non-adherent cells	+	10	259 (16)
Irradiated non-adherent cells added to adherent cells	–	10	90 (10)
Irradiated non-adherent cells added to adherent cells	+	10	159 (15)

Mean (S.E.) of triplicate assays.

Contribution of HPD-treated non-adherent cells to enhanced ingestion activity of macrophages

To test whether non-adherent cells contribute to macrophage activation, peritoneal macrophages were separated from non-adherent cells and treated with 0.03 ng HPD/ml in FCS medium 199 for 10 s of red light exposure followed by a 3 h incubation. No significant enhancement of ingestion activity of macrophages was observed under these conditions (Table 2). Because *in vitro* treatment of a mixture of peritoneal cells with 0.03 ng HPD/ml in FCS medium 199 and 5–15 s red light exposure greatly enhanced ingestion activity of peritoneal macrophages (Fig. 2), contribution of non-adherent cells to the activation of macrophages was considered. Accordingly, peritoneal non-adherent cells (B and T lymphocytes) were suspended in FCS medium 199 containing 0.03 ng HPD/ml and exposed to red fluorescent light for 10 s. These cells were then washed with PBS and mixed with peritoneal adherent cells (macrophages) in FCS medium 199. Ingestion activity of macrophages was greatly enhanced when measured after 3 h further cultivation (Table 2). Therefore, participation of non-adherent cells seems to be essential for the activation of macrophages for Fc receptor mediated ingestion.

DISCUSSION

Because the singlet oxygen quencher DABCO can block the photodynamic activation of macrophages, the activation of macrophages is probably triggered by generation of singlet oxygen ($^1O_2$) from irradiated HPD and haematoporphyrin. Photodynamic activation of macrophages with HPD is far more efficient than that with haematoporphyrin. HPD is relatively hydrophobic and as such interacts more readily with cell membranes [28] whereas the hydrophilicity of haematoporphyrin prevents it from interacting with membranes [5]. Because the short lifetime of $^1O_2$ allows it to diffuse only limited distances [29], the target molecules must be accessible near its source. The most likely target of oxidation within a membrane is the unsaturated fatty acyl group of phospholipids [8]. Peroxidation of unsaturated fatty acids and their consequent degradation can cause an increase in the fluidity of membranes [16–18]. We

previously reported that an increase in fluidity of B cell membranes by treatment with inflammation products of tissues (i.e. lysophospholipids and alkylglycerols) resulted in activation of macrophages [20, 21]. An increase in fluidity of lymphocyte membrane lipids after photodynamic treatment also appears to initiate activation of macrophages for enhanced Fc receptor mediated ingestion, as indicated in the present study. Cocultivation of photodynamically treated non-adherent lymphocytes was found to be required for activation of macrophages, which suggests that the primary photodynamic lesion is within lymphocyte membranes.

Photochemotherapeutic procedures with haematoporphyrin derivative (HPD-PDT) have been based on the concept that it should be possible to target the treatment to cancer tissues. In contrast to surgical removal of cancer tissues, HPD-PDT leaves killed or damaged cells in the treated lesion. In the present paper we demonstrated that a small amount of HPD plus light greatly enhances macrophage activation. We would expect that killed or damaged cancer cells could be ingested by these activated macrophages. Since macrophages are antigen presenting cells, activation of macrophages is a primary step of immunopotentiation. We point out, however, that large doses of HPD plus light causes an extensive cell death [5] but results in a decrease in macrophage activity, which consequently would lead to immunosuppression.

In vitro studies of photodynamic damage to cells [6, 13, 32] usually monitor cell death, a result that requires much higher doses of HPD and light than used in the experiments reported here. We cannot extrapolate from such studies to potential *in vivo* effects, because it is difficult to quantitate HPD concentration and intratissue light penetration. However, our studies suggest that systemic administration of even relatively low HPD doses (<300 ng/mouse, 15 μg/kg body weight) with red light exposure may produce a small number of damaged cells which can be phagocytised by activated macrophages. Therefore, further *in vivo* and *in vitro* studies of macrophage activation by HPD-PDT, such as we have described, should provide information on the appropriate dose levels for optimal therapeutic conditions.

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