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PHOSPHATIDYLINOSITOLMANNOSIDE-BASED LIPOSOMES INDUCE NO SYNTHASE IN PRIMED MOUSE PERITONEAL MACROPHAGES

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Liposomes prepared from phosphatidylinositolmannosides (extracted from BCG) and cholesterol are efficiently endocytosed by macrophages. Phagocytosis of particles or microbes modifies macrophage metabolism and in some cases, delivers potent stimulating signals to macrophages. We examined the effect of phosphatidylinositolmannoside-based liposomes on three macrophage functions especially important for host defenses : nitric oxide production, oxidative burst and TNF-α secretion. Phosphatidylinositolmannoside-based liposomes, added as empty vesicles, induced a strong NO synthase activity in mouse peritoneal macrophages primed either by interferon-γ or by trehalose dimycolate. They also induced a moderate production of TNF-α. Phosphatidylinositolmannosides conferred activating properties to pH-sensitive liposomes. In contrast, liposomes composed of phosphatidylcholine and phosphatidylserine were unable to activate primed macrophages.

Considerable attention has been paid to the potential value of liposomes as carriers for improving the delivery of drugs and DNA. Liposomes are especially suited to deliver drugs to phagocytic cells ; they have been used to target to macrophages antibiotics [1] or activating agents [2]. Addition of selected molecules to the lipid phase further increases endocytosis of liposomes by monocytes or macrophages ; for example, Fidler et al. have shown that distearoylphosphatidylserine (PS) increased the capture of liposomes both *in vivo* and *in vitro* [3]. We have shown that phosphatidylinositolmannosides (PIM), a major lipid constituant of mycobacteria [4], were also able to target liposomes towards macrophages [5].

The antiproliferative capacity of activated mouse macrophages is mainly due to their ability to produce nitric oxide (NO), the activation process corresponding to the induction of a NO synthase (NOS) which produces NO

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from L-arginine [6]. Induction of NOS activity in mouse peritoneal macrophages requires a sequence of two signals : interferon-γ (IFN-γ) is essential for the priming of macrophages and inflammatory cytokines (interleukin-1 or tumor necrosis factor-α, TNF-α) or microbial products (lipopolysaccharide, LPS or muramyldipeptide, MDP) trigger NOS expression in primed macrophages [6, 7]. It has been demonstrated that viable intracellular parasites such as *Leishmaniae* or *Mycobacteriae* can contribute to NOS induction [8, 9, 10]. By contrast, the capacity of inert particules to activate macrophages is still a matter of controversy (compared results in [8] and [9]).

A sustained production of NO by macrophages is an essential element in the host defenses to tumors, parasites and viruses, but it can be also immunosuppressive [11]. It is thus important to know if some kind of liposomes recommanded to target drugs towards macrophages could *per se* induce the expression of NOS. Similarly, it may be asked if the inclusion of some peculiar components in the lipid phase would give to liposomes the capacity to trigger the oxidative burst or TNF-α secretion ; these two significant cytotoxic activities of macrophages may exert, as nitric oxide, beneficial or detrimental effects.

MATERIALS AND METHODS

Cell cultures. Specific pathogen-free (C57Bl/6 x DBA/2)F1 mice were obtained from Iffa-Credo (l'Arbresle, France); they were maintained under barrier conditions and received sterile food and water. Peritoneal cells were processed as previously described [10]. They were placed in RPMI 1640 with HEPES and glutamine, supplemented with 5% FCS of low LPS content (<0.1 ng/ml) (Gibco, Grand Island, NY) and antibiotics

Induction of NOS. Two types of macrophages were used :

1) macrophages primed *in vivo* by trehalose dimycolate (TDM). TDM from *Mycobacterium tuberculosis*, strain Peurois, suspended in water [12] was injected i.p. (50 μg TDM/mouse), 7 days before macrophage harvesting. NOS activity is induced in TDM-primed macrophages by a short exposure (4 h) to low doses of LPS (1 to 10 ng/ml) [13]. LPS was from Sigma (L 6011) (St. Louis, MO).

2) Inflammatory macrophages were elicited by thioglycolate : 1.5 ml of thioglycolate broth (Sanofi-Pasteur Diagnostics, France) were injected i.p. 4 days before macrophage harvesting. NOS activity is induced in thioglycolate-elicited macrophages by a short exposure to IFN-γ (2 to 10 U/ml) and LPS (1 to 10 ng/ml) [14, 15]. Murine recombinant IFN-γ was kindly given by Dr G. R. Adolf (Ernst-Boehringer Institut für Arzneimittel Forschung, Vienna, Austria).

Liposomes. Cholesterol (Chol) was a gift from Pr. C. Lutton (Université Paris XI, France) ; distearoylphosphatidylcholine (PC), dipalmitoyl-phosphatidylserine (PS), oleic acid (OA) and phosphatidylethanolamine (PE) were from Sigma.

Four kinds of liposomes, selected for their ability to be endocytosed by macrophages, were prepared. 1) PC/PS liposomes were made of PC/PS/Chol, in a ratio 7/3/5 by weight : this corresponds to the optimal composition for endocytosis by macrophages as determined by Schroit and Fidler [3] , the

targetting to macrophages being due to PS. 2) Liposomes containing phosphatidyl-inositolmannosides (PIM) were made of PIM/Chol in a ratio 2/1 by weight [5]. Phosphatidylinositolmannosides were extracted from *Mycobacterium bovis* BCG as described by Lee and Ballou [4]. These phospholipids were found to be free of β-hydroxymyristic acid, a component indicative of endotoxin contamination [16]. 3) pH-sensitive-liposomes (OA) were made of PE/Chol/OA in a ratio of 7/5/3 by weight. Straubinger et al. [17] have shown that these liposomes are stable at neutral pH but instable in endosomes at pH 5. 4) liposomes (PIM/OA) were made of PE/Chol/OA/PIM in a ratio of 7/5/3/1 by weight : we have determined that addition of PIM triples the endocytosis of pH sensitive liposomes by macrophages (data not shown).
Tenfold concentrated liposome suspensions to be delivered to macrophage cultures were incubated with 8 μM polymyxin B (Calbiochem, La Jolla, CA) for 30 min at room temperature.

NOS activity, oxidative burst, TNF-α secretion and cell viability. NOS activity was measured by the accumulation in the culture medium of nitrite, one of its stable end products. Nitrite concentration was measured by using Griess reagent as previously described [18]. Production of hydrogen peroxide was measured by the rate of oxidation of phenol red (30 μM) in the presence of horseradish peroxidase [12]. TNF-α was measured by its capacity to inhibit the proliferation of L929 cells pretreated by actinomycin D [19]. Viability of macrophages was tested by their capacity to secrete lysozyme.

RESULTS

Induction of NO-synthase in TDM-primed macrophages. Macrophages elicited *in vivo* by TDM are primed and can be activated by a single activation signal. TDM-primed macrophages do not express NO synthase. After stimulation *in vitro* by LPS (3 ng/ml), NO synthase is induced and nitrites accumulate in culture medium : 18 h after LPS addition, nitrites were 36.6 μM ± 4.1 (mean of 5 independent determinations). Four types of liposomes, added as empty particules, were tested for the ability to induce NO synthase. As shown in Figure 1, the 2 types of phosphatidylinositolmannoside-containing liposomes (PIM and PIM/OA) triggered elevated levels of nitrites, whereas PC/PS and OA liposomes did not.

Induction of NO-synthase in thioglycolate-elicited macrophages. In contrast to TDM-primed macrophages, inflammatory macrophages elicited by thioglycolate broth require *in vitro* two stimuli (a priming and a activating agent) to express NO synthase. As shown in Table 1, in the presence of 2 U/ml IFN-γ (acting as a priming signal), the phosphatidylinositolmannoside-containing liposomes (PIM and PIM/OA) induced the production of NO : triggering of NO synthase was dose-dependent, the half-effect being observed with PIM or PIM/OA liposomes at a dose of 6 ± 2 μg/ml of total lipids ; PC/PS and OA liposomes were inactive. The 4 types of liposomes were inefficient in the absence of IFN-γ.

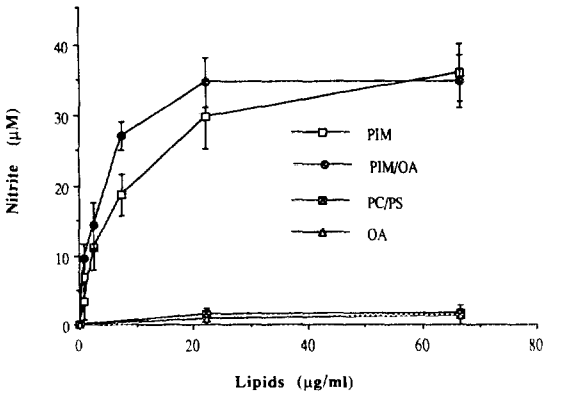

Figure 1. Induction of NO synthase expression in TDM-primed macrophages by empty liposomes : dose-response curve.
NO synthase activity was measured by the accumulation of nitrite in the culture medium 18 h after the addition of liposomes. Results are the mean ± SD of 5 independent experiments ; LPS (3 ng/ml) was used as a control and gave an accumulation of nitrite of 36.6 ± 4.1 μM. When compared by unpaired Student's t test, the effects of PIM and PIM/OA liposomes were not significantly different, except for data collected at 7.4 μg of lipids ($p = 0.01$).

Phosphatidylserine has been described as an inhibitor of nitric oxide production [20]. However, PC/PS liposomes (66.6 μg of lipids/ml) did not inhibit NO synthase induction in thioglycolate-elicited macrophages when added either at the same time as IFN-γ and LPS or 24 h before the stimuli ; the absence of inhibitory effect was observed even when suboptimal doses of stimuli were used. PC/PS liposomes did not interfere with the activating effect of PIM liposomes (2 independent experiments).

Liposomes do not trigger the oxidative burst. TDM-elicited macrophages produce high levels of hydrogen peroxide upon triggering by 0.1 μM TPA [12] ; the amount of H_2O_2 produced by 1.2 x 10^6 macrophages was sufficient to oxidize completely, in the presence of horseradish peroxidase, 30 μM phenol red in less than 60 min (maximal rate of H_2O_2 production 1.8 ± 0.02 nmol/min). In contrast, 2 h after addition of liposomes (PIM, PIM/OA or PC/PS added at a concentration of 66.6 μg of lipids/ml) the concentration of the reduced form of phenol red (measured by the absorbance at 560 nm, after NaOH addition) was strictly unchanged (2 independent experiments).

Induction of TNF-α secretion by liposomes. In contrast to TDM-primed macrophages, thioglycolate-elicited macrophages can be induced to secrete TNF-α by a treatment with LPS. As shown in Table 1, PIM/OA liposomes (66.6 μg of total lipids) triggered a secretion of TNF-α similar to the one observed after addition of 3 ng/ml LPS ($p = 0.6$) ; the other liposomes were inactive. IFN-γ increases the level of TNF-α detected after LPS addition. As shown in Table 1,

Table 1 : induction of NO synthase and TNF-α in thioglycolate-elicited macrophages

Stimuli	Nitrites (μM)	TNF-α (U/ml)
0	0.2 ± 0.4	< 0.5
LPS	1.2 ± 1.6	7.3 ± 3.4
PC/PS	1.9 ± 2.4	< 0.5
PIM	2.4 ± 1.9	1.3 ± 1.4
OA	0.5 ± 0.4	< 0.5
PIM/OA	0.7 ± 0.8	5.4 ± 5.9
IFN-γ	1.8 ± 1.9	< 0.5
IFN-γ + LPS	36.2 ± 5.0	23.0 ± 6.6
IFN-γ + PC/PS	2.6 ± 1.5	< 0.5
IFN-γ + PIM	33.3 ± 6.4	9.2 ± 2.8
IFN-γ + OA	5.6 ± 1.9	< 0.5
IFN-γ + PIM/OA	27.0 ± 4.2	41.4 ± 9.5

IFN-γ = 2 U/ml ; LPS = 3 ng/ml ; liposomes = 66.6 μg/ml of lipids.
The accumulation of nitrite in culture medium and the production of TNF-α were measured 18 h and 44 h, respectively, after the addition of activating agents. Results are presented as mean ± SD of 5 independent experiments. For nitrite accumulation, the effect of liposomes containing phosphatidylinositolmannosides (PIM or PIM/OA) is not different from the effect of LPS ($p > 0.5$, by Student's unpaired t test) ; for TNF-α production in the presence of IFN-γ, the actions of PIM/OA liposomes and LPS were significantly different ($p = 0.04$).

liposomes containing phosphatidylionositolmannosides synergized with IFN-γ, in contrast with the two other types of liposomes which were without any effect ; PIM/OA liposomes were reproducibly more active than PIM liposomes and 3 ng/ml LPS.

DISCUSSION

When they contain mannosylated phospholipids in the lipid phase, empty liposomes are able to deliver an activating signal to primed murine peritoneal macrophages. Besides PIM, lipoarabinomannans (LAM), other glycolipids of mycobacterial origin have been studied [21, 22]. LAM contain phosphatidylinositolmannosides and differ by the nature of the non-reducing ends which consist of branched arabinans (AraLAM) or linear oligomannosyl units (ManLAM). *A priori*, considering the presence of the mannose receptor on macrophages, ManLAM should be more efficient as macrophage activators. This is not the case, either for TNF-α or NO synthase induction [23, 24, 25]. However, as far as adhesion to human monocyte-derived macrophages is concerned, terminal mannose has a decisive role [26].

We have shown previously that PIM liposomes interact with the mannose receptor since they can inhibit the endocytosis of mannosylated BSA [5]. In the

present paper, we demonstrated that, similarly to some microbes, phosphatidylinositolmannoside-based liposomes, can deliver an activating signal to primed macrophages and induce NO synthase. In contrast, PIM liposomes are poor TNF-α inducers : this is in accordance with the data of [23, 24] where ManLAM are less efficient than AraLAM. It must be stressed that PC/PS liposomes, which are rapidly endocytosed by macrophages [3], do not induce TNF-α nor NO synthase. The present results contribute evidence that stimulation of the mannose receptor is an efficient means of triggering NO synthase, a significant antimicrobial activity of murine macrophages.

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