EFFECT OF A LYSOLECITHIN ANALOGUE ON NONSPECIFIC RESISTANCE TO INFECTION OF MICE

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SUMMARY The effect of racemic 1-octadecyl-2-methoxy-sn-glycero-3-phosphorylcholine (ET-18-OCH₃) on the nonspecific resistance of mice to infection with *Salmonella typhimurium* was investigated. Two *S. typhimurium* strains with different virulence were studied and no effect was observed in either case at concentrations of ET-18-OCH₃ up to 100 µg/mouse. However, a concentration of 500 µg/mouse caused decreased resistance to *S. typhimurium*, correlating with a depression of carbon clearance. Treatment of macrophages with ET-18-OCH₃ *in vitro* inhibited phagosome-lysosome fusion, but had no effect on zymosan-induced luminol-dependent chemiluminescence. The relationship between the adjuvant and nonspecific anti-infectious activity of ET-18-OCH₃ and other compounds is discussed.

INTRODUCTION

The phospholipid 2-lysophosphatidylcholine (LPC) is an important intermediate in the continuous and rapid phospholipid turnover of eukaryotic cellular membranes (Stein and Stein, 1966). Although the intracellular concentration of LPC is stringently controlled by at least three enzymes, it can cause transient changes in phagocytes. Accumulation of this potentially harmful compound has been observed after its injection into mice, or after incubation of macrophages *in vitro*, with immunological adjuvants like Freund's complete adjuvant. It was therefore postulated that the increased formation of LPC by an adjuvant-activated phospholipase A of macrophages might explain the mechanism of adjuvant activity in general (Modolell *et al.*, 1979; Munder *et al.*, 1970, 1979, 1981 and 1983). If this is true LPC should act as an adjuvant itself, and this was found to be so. However, since LPC is rapidly metabolised, synthetic compounds that are slowly metabolised like 1-octadecyl-2-methoxy-sn-glycero-3-phosphorylcholine (ET-18-OCH₃) were used to induce an increase in the immunological reactivity of the organism to a variety of soluble and particulate antigens. It was also observed that alky-lysophospholipids like ET-18-OCH₃ caused a remarkable antitumour response *in vivo* and *in vitro*, presumably

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mediated in part by macrophages (Munder *et al.*, 1983). Because of the important role that macrophages play in resistance to bacterial infection, we investigated the influence of LPC-analogues on this natural defence system. This communication describes the effect of ET-18-OCH₃ on the susceptibility of C3H/f and Balb/c mice to infection with *Salmonella typhimurium*.

MATERIALS AND METHODS

Mice. Two- to three-month-old male C3H/f and Balb/c mice, raised at the Max-Planck Institut für Immunbiologie, were used in the experiments.

Lysolecithin analogue. Racemic 1-octadecyl-2-methoxy-sn-glycero-3-phosphorylcholine (ET-18-OCH₃) (Medmark, Grünwald-München, FRG) was used. It was dissolved in phosphate-buffered saline (PBS) at pH 7.2 and kept frozen at -20°C until used.

Bacterial strains. *S. typhimurium* strains SF1826 and SF10223 (both with the O antigenic factors 1, 4, 5, 12_i, 12_j; lysotype LT20) were used. Strain SF1826 is very virulent in mice and strain SF10223 is relatively avirulent. Both strains have been described before (Blumenstock and Jann, 1981; von Jeney, Günther and Jann, 1977).

The bacteria were grown in Standard Broth I (Merck, Darmstadt, FRG) at 37°C overnight, diluted 1 in 10 with fresh broth and incubated until the late logarithmic phase (OD₆₀₀ 0.8–0.9 after about 4 h). The organisms were counted in a Neubauer chamber, appropriate dilutions were made and the number of viable bacteria was determined by plating on agar. The mice were infected intraperitoneally (i.p.) after i.p. injection of PBS (controls) or ET-18-OCH₃. Deaths of the animals were recorded daily for 4 weeks after injection. LD50 values were determined by the method of Reed and Muench (1938).

Cultivation of bone marrow macrophages. Macrophages were obtained from bone marrow of (Balb/c × C57B1)F₁ mice, essentially as described by Modolell *et al.* (1979). In short, 4 × 10⁶ bone marrow cells were cultured in Teflon bags containing 60 ml of Dulbecco's modification of Eagle's medium, 10 ml of fetal calf serum, 5 ml of horse serum and 30 ml of cell-culture supernate containing colony-stimulating factor.

Determination of the number of bacteria in organs. Mice were anaesthetised with ether and blood was obtained by cutting the axillary vessels. After bleeding the animals, the spleen, one kidney and the brain were removed aseptically, washed in sterile saline and homogenised in 1 ml of saline. The number of bacteria was determined by plating serial dilutions on Loeb agar (Difco); colonies were counted after overnight incubation at 37°C. The results were expressed as cfu/organ or /ml of blood.

Carbon clearance test. This was performed in Balb/c mice according to the method of Biozzi, Benacerraf and Halpern (1953) with uncentrifuged carbon suspension (C11/1431a; Pelikan Werke, Hannover, FRG) diluted with PBS. Since the treatment with different doses of ET-18-OCH₃ did not lead to changes in the body and organ weights, the phagocytic index (K) and not the corrected phagocytic index was determined.

Phagosome-lysosome fusion. This was investigated by the acridine orange method of Hart and Young (1975). Briefly, 2 × 10⁶ unelicited peritoneal cells from Balb/c mice in Eagle's medium, containing penicillin 100 IU/ml and streptomycin 100 µg/ml were distributed into 35-mm plastic plates (Falcon 3001, Division of Becton-Dickinson, Oxnard, CA) and incubated in a CO₂ incubator. After 2 h the nonadherent cells were removed by washing twice with warm PBS and the adherent cells were cultivated further in Eagle's medium (2 ml/plate) containing fetal calf serum 1% and either ET-18-OCH₃ or dextran sulphate (sodium salt, mol. wt 5 × 10⁵; Pharmacia, Uppsala, Sweden) in an atmosphere containing CO₂ 10%. After 24 h the medium was decanted and fresh medium containing acridine orange 5 µg/ml (Merck, Darmstadt, FRG), but without antibiotics or serum, was added for 10 min. The macrophage monolayers were washed twice with warm Eagle's medium and a suspension of *Saccharomyces cerevisiae* in medium without serum and antibiotics was added and the plates were incubated further in a CO₂ incubator. After different periods plates were washed with cold PBS. The monolayers were covered with a thin cover slip and examined with a Zeiss fluorescence microscope with epi-illumination. Observation of fluorescent rims around the ingested yeasts and of coloured cells in the phagosomes was regarded as a sign of phagosome-lysosome fusion.

Chemiluminescence. After cultivation for 8–11 days, 3×10^5 bone marrow macrophages were incubated for 24 h in an atmosphere of CO₂ 10% in Packard scintillation vials containing 5 ml of modified Eagle's medium to which had been added 20 mM HEPES, sodium bicarbonate 3.7 g/L, penicillin 100 IU/ml, streptomycin 100 µg/ml, and fetal calf serum 1%, with or without ET-18-OCH₃. The macrophages were then washed twice with warm PBS and resuspended in 2.5 ml of modified Eagle's medium without serum or sodium bicarbonate. Phenol red and 10 µl of Luminol solution (C. Roth, Karlsruhe, FRG) (20 mg in 1 ml of dimethyl sulphoxide) were added to each sample. Before measuring the chemiluminescence response, the vials were kept in the dark in a modified Packard scintillation counter which operated at 37°C in the out-of-coincidence mode. At the end of this period the background chemiluminescence was measured. At time zero, opsonised zymosan 50 µl was added and the chemiluminescence of each sample was measured at intervals of 6 min for 1 h. The net chemiluminescence response was measured as counts/min/ 3×10^5 cells. For opsonisation, 200 mg of zymosan (Sigma, Munich) were washed in PBS and incubated in 4 ml of fresh rat serum at 37°C for 30 min. The mixture was then centrifuged and the opsonised zymosan was washed four times with PBS at 4°C. The final pellet was resuspended in 4 ml of PBS and kept frozen in small portions at -70°C until use.

RESULTS

Protective activity of ET-18-OCH₃

The LD₅₀ values of the two *Salmonella* strains for the mice used are presented in table I. They are in agreement with those reported by von Jeney *et al.* (1977).

Growth of the bacterial strains used was not inhibited by ET-18-OCH₃ at a concentration of 1 mg/ml. Deep rough mutants with lipopolysaccharide chemotypes Rd₂ and Re (Jann and Westphal, 1975) are killed by lysolecithin and its analogues but smooth forms are not (H.U. Weltzien, personal communication).

TABLE I
LD₅₀ values for the two *S. typhimurium* strains used for infection of mice

Mouse strain	LD ₅₀ (bacterial numbers) for <i>S. typhimurium</i> strain	
	SF 1826	SF 10223
C3H/f	2×10^4	6×10^5 *
Balb/c	10	1.5×10^3

*Data from Blumenstock (1979).

C3H/f mice were treated with 10 or 100 µg of ET-18-OCH₃ before and after infection with 10 LD₅₀ of *S. typhimurium* SF1826. The animals died between day 5 and day 10, the same period of time as control animals treated i.p. with PBS. Similar results were obtained by infection with 10 LD₅₀ (60 bacteria/animal) of *S. typhimurium* SF1826 after pretreatment of Balb/c mice with 200 µg of ET-18-OCH₃ and by infection with 10 LD₅₀ (1.5×10^4 bacteria/animal) of *S. typhimurium* SF10223 after pretreatment with 10 and 100 µg of ET-18-OCH₃. This lack of protection was confirmed by infecting C3H/f mice with 2×10^5 *S. typhimurium* SF1826 and treating after 1 day with PBS or with 100 µg of ET-18-OCH₃. Numbers of bacteria in the spleens of the mice were determined daily and found to be identical in all cases.

Decrease of resistance to infection by ET-18-OCH₃

To test whether a higher dose of ET-18-OCH₃ would protect mice from infection with *S. typhimurium*, we treated C3H/f mice with 500 µg (0.1 LD₅₀) of the lysolecithin analogue. Unexpectedly, these mice died earlier than controls that were not pretreated after infection with *S. typhimurium* SF1826. As shown in fig. 1, the earlier ET-18-OCH₃ was injected before bacterial challenge, the longer the mice survived. Death of most mice thus treated occurred 20–30 h after infection. Intraperitoneal injection of 500 µg of ET-18-OCH₃ on the third day after infection resulted in the death of all animals within the following 24 h (data not shown).

Because multiplying *S. typhimurium* release endotoxin in the host, the death of the animals after such a short time may have been caused by endotoxic shock, provided that ET-18-OCH₃ can induce endotoxin hypersensitivity. However, as shown in table II, ET-18-OCH₃ had no influence on the lethal effect of endotoxin.

Preliminary experiments showed that the number of bacteria present 12 h after

FIG. 1.—Survival of C3H/f mice infected i.p. with 10 LD₅₀ (A) and 1 LD₅₀ (B) of *S. typhimurium* SF 1826. Animals were treated i.p. with PBS 1 day before infection (●), or with 500 µg of ET-18-OCH₃ at the time of infection (○), 1 day before infection (×), 2 days before infection (▲) or 4 days before infection (□); n = number of mice in the group.

TABLE II

Toxicity of lipopolysaccharide in the absence and presence of ET-18-OCH₃

Lipopolysaccharide (μ g)	ET-18-OCH ₃ (μ g)	Number dead*	Lethality (%)
400	0	5	83
200	0	3	50
100	0	0	0
0	500	0	0
100	500	0	0

* Groups of 6 C3H/f mice were given i.p. injections of lipopolysaccharide from *S. typhimurium* SF 1826 1 day after the injection of PBS or ET-18-OCH₃.

infection in the organs of mice that had been treated with 500 μ g of ET-18-OCH₃ on the day before infection, was similar to the numbers observed in untreated controls. However, 24 h after infection many of the mice that had been pretreated with ET-18-OCH₃ looked severely ill or were dying. Therefore we compared the numbers of bacteria in spleen, brain, kidneys and blood 15, 18 and 21 h after infection of pretreated and control mice (fig. 2). The numbers of bacteria in the organs of the control animals did not differ significantly during this short period of observation. In contrast, the number of bacteria in the organs of mice that had been treated with 500 μ g of ET-18-OCH₃ on the day before infection increased rapidly. After 21 h the viable counts were about a hundredfold higher than those of the control animals. The most striking differences were seen in blood. While no, or only a few salmonellae could be found in

FIG. 2.—Number of bacteria in organs of C3H/f mice infected with 10 LD₅₀ of *S. typhimurium* SF 1826 1 day after i.p. injection of 500 μ g of ET-18-OCH₃ (●) or with 0.5 ml of PBS (○). Each circle represents the result from a single animal.

FIG. 3.—Effect of ET-18-OCH₃ on the carbon clearance in Balb/c mice after i.p. injection of 500 µg (●) and 100 µg of ET-18-OCH₃ (○). Each point represents the mean value $\pm$ SD from 5–7 mice. The solid and dotted horizontal lines represent mean value and standard deviation for the clearance coefficient in 15 untreated animals.

the blood of untreated animals within 24 h of infection, $> 10^7$ bacteria/ml could be detected in pretreated mice.

Effect of ET-18-OCH₃ on carbon clearance

Intraperitoneal injection of ET-18-OCH₃ caused a depression of the phagocytic capacity of the reticuloendothelial system during the following 2 days (fig. 3). This was more marked after the injection of 500 µg than after the injection of 100 µg of ET-18-OCH₃.

Effect of ET-18-OCH₃ on phagosome-lysosome fusion

Phagolysosome formation in monolayers of Balb/c macrophages was detected with *S. cerevisiae* and acridine orange according to the method of Hart and Young (1975). The results obtained with and without pretreatment of the macrophages with ET-18-OCH₃ are shown in fig. 4. The inhibition of the fusion of phagosomes (indicated by *S. cerevisiae*) and lysosomes (stained with acridine orange) was significant. Similar results were obtained with dextran sulphate, a known inhibitor of phagosome-lysosome fusion (Hahn, 1974; Goren, Swendsen and Henson, 1980).

Effect of ET-18-OCH₃ on luminol dependent chemiluminescence

The phagocytic capacity of macrophages can be measured by luminol dependent chemiluminescence, as shown previously (Allen and Loose, 1976; Schleupner and

FIG. 4.—Kinetics of phagosome-lysosome fusion in Balb/c peritoneal macrophages cultivated for 24 h in medium with 1% fetal calf serum (●), in the same medium with ET-18-OCH₃ 0.5 µg/ml (□) or with 1.5 µg/ml (■). The values obtained after injection of dextran sulphate 10 µg/ml (Δ) under the same conditions are also shown. Each point represents a mean value from 5–7 experiments.

Glasgow, 1978). We studied the chemiluminescence response of macrophages derived from bone marrow during the phagocytosis of opsonised zymosan (Blumenstock and Jann, 1981). Pretreatment of macrophages overnight with 0.5 µg of ET-18-OCH₃/ml did not change the chemiluminescence response. When the concentration of ET-18-OCH₃ was increased to 1.5 µg/ml, a slightly decreased response was observed. Because serum was absent in these experiments, the observed decrease may be due to damage of the macrophages at this concentration.

DISCUSSION

Moderate doses (200 µg/mouse) of the lysolecithin analogue ET-18-OCH₃ had no influence on the infection of inbred mice with *S. typhimurium* and a higher dose (500 µg/mouse) significantly decreased the resistance of the animals to infection. Injection of 500 µg of ET-18-OCH₃ per mouse in the absence of infection did not affect the animals. The effect of ET-18-OCH₃ was maximal within the first day after injection and was only marginal 4 days after injection.

Bacterial counts in the organs of mice, particularly in blood and spleen, were significantly increased within about 15 h after i.p. injection of ET-18-OCH₃. When the carbon clearance from the blood stream of uninfected mice was measured at various times, a decrease was observed 24 h after injection of ET-18-OCH₃. Normal clearance was restored after 3 days.

These results indicate that ET-18-OCH₃ influenced the reticuloendothelial system of the mice such that they became more susceptible to infection as has been demonstrated for other adjuvants (Stein and Stein, 1966; Friedman and Moon, 1977).

The possibility that the effect was due to sensitisation of the animals to bacterial lipopolysaccharide could be ruled out.

It thus seems possible that ET-18-OCH₃ acted on the macrophages so that they could not cope with the infecting *S. typhimurium*. When luminol-dependent chemiluminescence induced by opsonised zymosan was used to monitor the general oxidative state and metabolism of peritoneal macrophages, no difference was found between the extent and kinetics of chemiluminescence of resident peritoneal macrophages (Blumenstock and Jann, 1981) and of those from mice treated with ET-18-OCH₃ one day before the test. Exposure of macrophage monolayers to ET-18-OCH₃ (complexed to normal serum) *in vitro* also failed to influence the luminol-dependent zymosan-induced chemiluminescence. This suggests that the general metabolism of murine peritoneal macrophages is not impaired by ET-18-OCH₃.

S. typhimurium are facultative intracellular parasites, which are able to survive inside macrophages. Although the mechanism of the intracellular survival is not well understood, one way of avoiding the host defence is the prevention of phagosome-lysosome fusion. Since ET-18-OCH₃ was found to reduce phagolysosome formation in peritoneal mouse macrophages, this effect may render the animals more susceptible to infection. In fact, ET-18-OCH₃ might mechanistically transform the *S. typhimurium* into a true intracellular parasite, such as mycobacteria which are known to prevent phagolysosome formation (Armstrong and Hart, 1971; Allan and Loose, 1976; Goren *et al.*, 1980).

ET-18-OCH₃ also has an effect on tumour growth in mice (Munder *et al.*, 1981). The tumoricidal activity of the lysolecithin analogue may be explained by extracellular killing of the tumour cells either by macrophages or by polymorphonuclear leukocytes. In these experiments, ET-18-OCH₃ exerted adjuvant activity, which seems to be in contradiction to the inhibition of phagolysosome formation postulated to cause the increased sensitivity of the mice to infection with *S. typhimurium*. It is however known that agents that interfere with the fusion of phagosomes with lysosomes of phagocytic cells, such as dextran sulphate, also exert macrophage activating activity and thus act as adjuvants (Biozzi *et al.*, 1953; Diamantstein *et al.*, 1971; Hahn, 1974; Hart and Young, 1975; Arnold, Miller and Weltzien, 1979; Modolell *et al.*, 1979). Thus, the results described in this communication indicate that the effect of ET-18-OCH₃ on phagocytic cells may result in increased sensitivity to infection or protection, depending on the mechanism dominating the host defence.

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