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Treatment of *Klebsiella pneumoniae* Septicemia in Normal and Leukopenic Mice by Liposome-Encapsulated Muramyl Tripeptide Phosphatidylethanolamide

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The effect of free muramyl tripeptide phosphatidylethanolamide (MTPPE) and liposome-encapsulated MTPPE (LE-MTPPE) on *Klebsiella pneumoniae* septicemia resulting from intraperitoneal bacterial inoculation was investigated in mice. When administering a single prophylactic dose at 24 h before bacterial inoculation, the percentage survival was 55% (MTPPE) or 40% (LE-MTPPE), whereas untreated control mice died. Only repeated prophylactic treatment with LE-MTPPE could further increase survival up to 85%.

In the immunocompromised host, severe infections are difficult to treat with antibiotics. One of the factors that contribute to this lack of success of antibiotic treatment is the failure of the host defense to give adequate support. In this respect it is of great importance to stimulate the nonspecific host defense, in particular, the mononuclear phagocyte system (MPS). This can be effected by the immunomodulating agent muramyl tripeptide phosphatidylethanolamide (MTPPE).

MTPPE is a lipophilic derivative of muramyl dipeptide. Muramyl peptides have various stimulating effects on macrophages (6, 7, 14, 22, 24) and have been proven to be effective in stimulating the nonspecific host defense against several bacterial infections (20, 21). In a previous study (16), we investigated the effect of MTPPE in a model of infection induced by intravenous (i.v.) inoculation of *Klebsiella pneumoniae* in mice. *K. pneumoniae* is one of the pathogens cultured from samples from infected hospitalized patients and is a serious complication in patients with malignancies (3). Since these immunocompromised patients are mostly prone to infections during a prolonged period of time, it is expected that repeated administration of immunomodulating agents is needed. Repeated administration of MTPPE has been shown to result in toxic side effects (2, 5, 25). Encapsulation of MTPPE in liposomes may therefore be of importance, since in this form a reduction in MTPPE toxicity has been demonstrated (5, 25). In addition, because of liposomal encapsulation, MTPPE is targeted to the cells of the MPS (13, 16). In the present study, we used an infection model in which *K. pneumoniae* was inoculated intraperitoneally (i.p.), resulting in the appearance of *K. pneumoniae* in the blood at regular intervals, eventually leading to septicemia. This model has a clinical relevance because immunocompromised patients are also prone to the development of septicemia from a local infection. The efficacy of several prophylactic and therapeutic treatment schedules with free MTPPE or liposome-encapsulated MTPPE (LE-MTPPE) was investigated in immunocompetent as well as leukopenic mice.

Infections were induced by i.p. inoculation of 10^3 CFU of *K. pneumoniae* ATCC 43816 capsular serotype 2 into specific-pathogen-free, 11- to 13-week-old male C57BL/Ka mice (ITRI-TNO, Rijswijk, The Netherlands). The 50% lethal dose after i.p. inoculation was less then 10 CFU (500 CFU/kg of body weight). Mice were injected with MTPPE, LE-MTPPE, placebo liposomes (a generous gift of CIBA GEIGY Ltd. Basel, Switzerland), or phosphate-buffered saline (PBS) by following various treatment schedules. Each comparison group contained 20 mice. Liposomes were prepared as described by Van Hoogevest and Frankenhauser (26). Then, mice were housed in separate cages, and every day the survival of mice was examined until 21 days after bacterial inoculation. From dead mice only *K. pneumoniae* was recovered from the blood.

In some of the mice, leukopenia was induced by i.p. injections of cyclophosphamide (Sigma Diagnostics, St. Louis, Mo.) at a first dose of 120 mg/kg at 11 days before bacterial inoculation and then at doses of 80 mg/kg every third day thereafter. To quantify the numbers of leukocytes in blood, blood samples (0.8 ml) were taken from the retroorbital plexus and were collected in polypropylene tubes containing 1 mg of dried EDTA (BDH Chemicals Ltd., Poole, England). For total leukocyte counts, blood was diluted 1:10 with Türk solution (0.1% crystal violet in 1% acetic acid), and the number of leukocytes was determined in duplicate in a Bürkers hemocytometer. The clearance capacity of these leukopenic mice was compared with that of immunocompetent mice as follows. At different intervals after i.v. inoculation with 10^4 CFU of *K. pneumoniae*, blood samples (0.8 ml) were taken as described above. Serial 10-fold dilutions were prepared and volumes of 0.2 ml of each dilution were spread onto tryptone soy agar plates (Oxoid Ltd., Basingstoke, England). All plates were incubated overnight at 26°C.

Statistical evaluation of the differences in the decrease in survival with time as well as the eventual percentage of survival between the MTPPE-treated, LE-MTPPE-treated, PBS-treated, and placebo liposome-treated groups of animals was performed by the log rank test (17). The Mann-Whitney test was used for evaluation of the differences in the numbers of leukocytes and bacteria between the LE-MTPPE-treated and PBS-treated groups of animals.

After i.p. inoculation of mice with 10^3 CFU of *K. pneumoniae*, bacteria appeared in the blood and reached levels of 10^3 to 10^4 CFU of *K. pneumoniae* per ml of blood within 4 h and 10^6 CFU per ml blood after 2 days. Eventually, all mice

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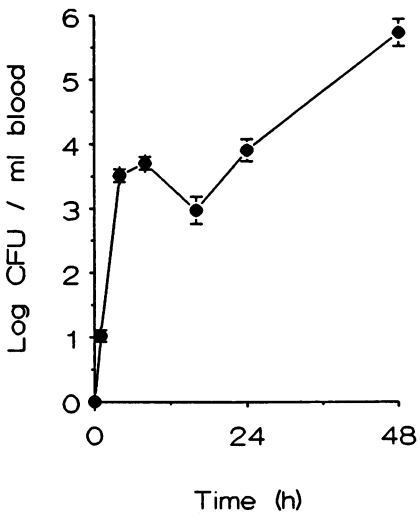

FIG. 1. Numbers of *K. pneumoniae* in the blood of mice after i.p. inoculation. At 24 h before i.p. inoculation with 10^3 CFU *K. pneumoniae*, mice were treated i.v. with PBS. Each point represents the geometric mean ± standard error of the mean for six mice.

died (by day 3) (Fig. 1). Administration of a single dose of 50 μg of free MTPPE at 24 h before bacterial inoculation resulted in maximal survival of 55%. With a dose of 100 μg of free MTPPE, the survival rate was not increased. From a twofold dose range, 6.3 μg of MTPPE per mouse was the lowest dose that resulted in a significantly increased survival rate compared with that for PBS-treated mice.

Table 1 shows that administration of 25 μg of LE-MTPPE per mouse at 24 h before bacterial inoculation resulted in the survival of 40% of the mice. A dose of 6.3 mg of placebo liposomes, which is equivalent to the amount of lipid in which 25 μg of MTPPE is encapsulated, had no effect. A dose of 25 μg of LE-MTPPE was the maximum dose that could be administered, since at higher doses the equivalent amount of placebo liposomes had toxic effects. From a twofold dose range, 3.1 μg of LE-MTPPE per mouse was the lowest dose that resulted in a significantly increased survival rate compared with that for PBS-treated mice. Administration of additional therapeutic doses of 3.1 μg of LE-MTPPE could improve the effect of administration of a single dose of 3.1 μg at 24 h before bacterial inoculation. Depending on the interval, survival was increased to 35% (three doses every 48 h) or 55% (three doses every 24 h). Repeated administration of 0.8 mg of placebo liposomes, equivalent to the amount of lipid in which 3.1 μg of MTPPE was encapsulated, had no effect.

Repeated prophylactic administration of doses of 3.1 μg of LE-MTPPE resulted in the best percent survival (Table 1). Depending on the number of doses and the interval, survival was increased to 85% (four doses every 48 h) or 75% (four doses every 24 h). Repeated administration of 0.8 mg of placebo liposomes with an interval of 48 h had no effect. Repeated administration of 0.8 mg of placebo liposomes at an interval of 24 h also resulted in a significantly increased survival (5%) compared with that for PBS-treated mice ($P = 0.04$).

Table 1 also shows that in cyclophosphamide-treated leukopenic mice infected i.p. with 10^3 CFU of *K. pneumoniae*, prophylactic administration of four doses of 3.1 μg of LE-MTPPE at an interval of 48 h (the treatment schedule which was most effective in immunocompetent mice) led to

TABLE 1. Efficacies of LE-MTPPE treatment schedules on *K. pneumoniae* septicemia in mice

Mice and time (h) of administration[a]	Dose (μg)	% Survival	Time to death (days)[b]	Significance[c]
Immunocompetent mice				
−24 h	25	40	6.9 ± 4.8	0.001
−24	12.5	20	5.8 ± 2.7	0.001
−24	6.3	10	4.3 ± 1.7	0.05
−24	3.1	5	4.4 ± 1.2	0.01
−24	0	0	3.6 ± 0.9	
−24, +24	25	30	6.7 ± 2.8	0.001
−24, +24, +72	25	45	7.2 ± 4.0	0.001
−24, +24	3.1	30	4.6 ± 1.4	0.001
−24, +24, +72	3.1	35	5.2 ± 2.3	0.001
−24, 0	3.1	45	4.5 ± 1.5	0.001
−24, 0, +24	3.1	55	4.5 ± 1.7	0.001
−72, −24	3.1	40	3.7 ± 0.9	0.001
−120, −72, −24	3.1	70	6.8 ± 3.9	0.001
−168, −120, −72, −24	3.1	85	7.0 ± 1.6	0.001
−48, −24	3.1	40	6.8 ± 2.2	0.001
−72, −48, −24	3.1	75	3.4 ± 0.5	0.001
−96, −72, −48, −24	3.1	75	4.8 ± 0.8	0.001
Leukopenic mice				
−168, −120, −72, −24	3.1	5	4.2 ± 0.9	0.001
	0	0	2.2 ± 0.4	

[a] LE-MTPPE was administered at the indicated times before (−) or after (+) bacterial inoculation (day 0).
[b] Values are means ± standard deviations on the basis of the time of bacterial inoculation (day 0).
[c] Versus PBS-treated mice.

TABLE 2. Clearance of *K. pneumoniae* from the blood of normal and leukopenic mice[a]

Mice	Log no. of *K. pneumoniae*/ml blood at the following times after i.v. bacterial inoculation[b]			
	1 min	15 min	30 min	60 min
Normal	3.80 ± 0.05	3.15 ± 0.05	3.01 ± 0.14	3.05 ± 0.10
Leukopenic	3.80 ± 0.05	3.29 ± 0.08	3.25 ± 0.14	3.06 ± 0.13

[a] Normal and leukopenic mice were inoculated i.v. with 10^4 CFU of *K. pneumoniae*.

[b] Values are geometric means ± standard errors of the mean for six mice.

an increased survival rate compared with that in PBS-treated leukopenic mice. The numbers of total leukocytes in the blood of LE-MTPPE-treated leukopenic mice did not differ from the numbers in PBS-treated leukopenic mice. They were 670 ± 25 and 687 ± 26/μl of blood (mean ± standard error of the mean for six mice), respectively. In PBS-treated immunocompetent mice, leukocyte numbers fluctuated around 3,700/μl of blood. The cyclophosphamide treatment in itself did not lead to a decreased capacity of the MPS to clear *K. pneumoniae* from the blood in comparison with the clearance capacity of the MPS of PBS-treated immunocompetent mice (Table 2).

Administration of a single dose of MTPPE, LE-MTPPE, muramyl dipeptide (MDP), or other MDP derivatives seemed to be most effective when the dose was given at 24 h before bacterial inoculation (1, 16, 20). Administration of a single dose of LE-MTPPE after bacterial inoculation did not appear to be effective (16). The results obtained with repeated administration of MDP derivatives were discrepant (4, 8, 11, 12, 19, 21). Various microorganisms were used (viruses, bacteria, or parasites), and in most studies only a few treatment schedules were compared. However, a detailed study related to the effects of prophylactic or therapeutic treatment schedules for MDP derivatives has not yet been performed.

Investigations on the efficacy of repeated administration of immunomodulating agents are of clinical relevance since immunocompromised patients are prone to infections during a prolonged period of time. Results of the present study show that the greatest therapeutic effect was obtained when all doses of LE-MTPPE were given prophylactically at an interval of 48 h. This indicates that time is needed for macrophages to become maximally activated. This may be the result of the direct stimulation by LE-MTPPE but also by products released by activated macrophages, such as interleukin-1 or tumor necrosis factor. In addition, more macrophages may be activated during this period of prophylactic administration. In future studies, the activation state and the involvement of cytokines and other cell types in the LE-MTPPE-induced resistance against *K. pneumoniae* infection will be elucidated.

In the present study, we also investigated the effect of stimulating host defenses in leukopenic mice. These mice were immunocompromised as a result of treatment with cyclophosphamide, resulting in a 82% decrease in the numbers of peripheral leukocytes. The effects of repeated prophylactic administration of LE-MTPPE in terms of the increased survival rate and increased percent survival observed in immunocompetent mice could also be found in leukopenic mice. Other investigators (9, 10, 21, 23) assessed only the effect of single doses of the MDP derivative administered at 24 h before bacterial inoculation.

Whereas in immunocompetent mice we previously found increased numbers of leukocytes in the blood as a result of LE-MTPPE treatment (16), the present study demonstrated that, in cyclophosphamide-treated leukopenic mice, the numbers of leukocytes in the blood were not increased after repeated administration of LE-MTPPE. This was due to the cyclophosphamide-induced blockade of the recruitment of leukocytes. It can be concluded that the LE-MTPPE-induced recruitment of leukocytes does not contribute substantially to the therapeutic effect of LE-MTPPE. Activation of tissue macrophages by LE-MTPPE is of major importance in the induced antibacterial resistance in leukopenic mice. In immunocompetent mice, we also found that activation of tissue macrophages is of great importance in the increased resistance against *K. pneumoniae* infections (15). Our results are in agreement with the findings of Nakajima et al. (18), who indicated that leukocyte numbers in the blood of cyclophosphamide-treated mice were not increased after treatment with the lipophilic MDP-Lys (L18).

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