FREE AND LIPOSOME-ENCAPSULATED IMMUNOMODULATORS STIMULATING THE NONSPECIFIC RESISTANCE AGAINST BACTERIAL INFECTIONS

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VRIJE EN LIPOSOMAAL-INGEKAPSELDE IMMUNOMODULATOREN TER STIMULATIE VAN DE NIET-SPECIFIEKE AFWEER TEGEN BACTERIELE INFECTIES

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Voor Johan
Voor pa en ma

LIST OF ABBREVIATIONS

CAT	catalase
CFU	colony forming unit
CSF	colony stimulating factor
^{14}C-PC	^{14}C-phosphatidylcholine
DF	deferoxamine mesylate
DMSO	dimethylsulfoxide
DPPE	dipalmitoyl phosphatidylethanolamine
FBS	fetal bovine serum
Fitc	fluorescein isothiocyanate
^{67}Ga-DF	^{67}gallium-deferoxamine
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
IFN-γ	interferon-gamma
i.p.	intraperitoneal
i.v.	intravenous
LD_{50}	50% lethal dose
LE-DMP	liposome-encapsulated dichloromethylene diphosphonate
LE-IFN-γ	liposome-encapsulated interferon-gamma
LE-MTPPE	liposome-encapsulated muramyl tripeptide phophatidylethanolamide
LPS	lipopolysaccharide
M-CSF	macrophage colony stimulating factor
MDP	muramyl dipeptide
MPS	mononuclear phagocyte system
MTPPE	muramyl tripeptide phosphatidylethanolamide
NMMA	N^G-monomethyl-arginine acetate
PBS	phosphate-buffered saline
Rho-PE	rhodamine phosphatidylethanolamine
SA	sodiumazide
SEM	standard error of the mean
SOD	superoxide dismutase
Tritc	tetramethylrhodamine isothiocyanate
TSA	tryptone soya agar

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Host defense against bacterial infections

Antimicrobial defenses in the immunocompetent host

The host possesses a variety of specific and nonspecific defense mechanisms against invasion of potentially pathogenic bacteria (115,140,183,206,219-221). The skin and mucosa form the first line of defense. The intact skin is not penetrable to most bacteria. The glandular secretions contain lactic acid and fatty acids that inhibit bacterial growth. At the mucosal level, a variety of physical and biochemical mechanisms prevent colonization of pathogenic bacteria. Antibodies found on mucosal surfaces are able to neutralize bacterial toxins and prevent attachment of bacteria to the epithelium. In the respiratory tract the movement of cilia, coughing and sneezing are important for removing bacteria. In the alimentary tract lysozyme containing saliva, low pH of gastric acid, proteolytic enzymes, and movement of intestinal contents are important. A low pH and regular voiding prevent the invasion of pathogenic bacteria in the urogenital tract as does the microflora resident in the skin, alimentary tract and the urogenital tract. In addition, the lysozyme and lactoferrin in tears and saliva display antibacterial activity.

If bacteria succeed in passing the first line of defense, the second line of defense, consisting of humoral and cellular defense mechanisms, is needed for elimination of the invaders.

Humoral antimicrobial factors can be either nonspecific (lysozyme, lactoferrin and transferrin, complement, fibronectin, interferon, C-reactive protein, and tuftsin) or specific (antibodies) (115,140,206,220,221). Lysozyme and complement are able to kill bacteria. Lactoferrin and transferrin bind to iron, thereby depriving bacteria of the iron necessary for their growth. Complement, fibronectin, C-reactive protein and antibodies are able to facilitate the uptake of bacteria by phagocytic cells (opsonization).

The cellular defense mechanism against bacteria consists of granulocytes, monocytes and macrophages, which take up bacteria and usually will kill them (115,206,220,221). In addition, after phagocytosis of bacteria by monocytes or macrophages, bacterial antigens are processed and are exhibited on the surface of the cell together with an HLA-class II molecule, and specific T- and B-cells can then be activated. Some bacterial strains are only effectively killed by the monocytes or macrophages after these cells have been activated by cytokines produced by specific T-cells.

Antimicrobial defenses in the immunocompromised host

The antimicrobial defense mechanisms not always act optimally. This decreased defense can be caused by several factors. Host defense disorders can be classified as primary (congenital) or secondary (acquired) (195). Primary immunodeficiencies are rare and manifest

TABLE 1.1 Factors impairing the host defense (195,206)

catheters, prostheses, foreign bodies	irradiation
burns	immunosuppressive treatment
protein-calorie malnutrition	splenectomy
age	infections (mostly viral)
neoplastic diseases	some chronic diseases
chemotherapeutic therapy	stress

themselves as impaired granulocyte or monocyte function, antibody or complement production or cell mediated immunity. Acquired disturbances of the host defense are more common and an overview of factors leading to this is given in Table 1.1. Catheters, prostheses, foreign bodies and burns disrupt the skin and mucous membrane (195). Protein-calorie malnutrition results in damaged skin and mucous membrane barriers with decreased lysozyme concentrations and, in addition, T-cell function is also weakened (195,206). In neonates, the humoral and cellular host defense mechanisms are immature while, in the elderly, the quality of the first line of defense is decreased, the humoral responses are reduced and the T-cell function is impaired (195,206). Neoplastic diseases are associated with impaired humoral or cellular host defenses. Chemotherapeutic therapy results in leukopenia and deranged T-cell and B-cell immune functions while irradiation affects all rapidly proliferating cells, such as epithelium cells of the gastro-intestinal tract (195,206). Immunosuppressive treatment particularly affects the cellular host defenses: cyclosporin A weakens T-cell function while monocyte and granulocyte function may be impaired by glucocorticosteroids (195,206). Splenectomy results in a decreased clearance of bacteria from the blood and an impaired humoral defense (195,206). Infections with viruses (e.g. Epstein Barr virus, cytomegalovirus, human immunodeficiency virus) or bacteria (e.g. Mycobacterium spp, Brucella spp) contribute to suppression of the cellular host defenses (195,206). In addition, some chronic diseases such as diabetes mellitus or hepatic cirrhosis are associated with an impaired second line of defense (195,206), while stress is also linked with immune dysfunction (195).

Stimulation of nonspecific antimicrobial defenses

The rationale for stimulation of nonspecific antimicrobial defenses

From clinical experience it is known that, in immunocompromised patients severe infections frequently occur and are a continuing threat to patients. Antibiotic treatment of these infections is not always successful despite the use of new and potent antibiotics (8,169,195). Several factors may contribute to the failure of antibiotic treatment. One factor is an impaired host defense system unable to provide adequate support for antibiotic therapy (8,169). For the potentiation of treatment of severe infections different approaches are available. One method is the intensification of antibiotic treatment by developing new dosing regimens or developing rational delivery systems for antibiotics. The use of antibiotic delivery systems such as liposomes should result in increased antibiotic concentrations either in the

infected tissues or intracellularly in the infected cells and a reduced toxicity of potentially toxic antibiotics.

Another approach for the potentiation of treatment of severe infections is stimulation of the nonspecific resistance of the host. Since the mononuclear phagocyte system (MPS) plays a major role in the nonspecific host defense against infection, stimulation of the MPS may be of great value. It is expected that this not only leads to an increased resistance against infections involving the MPS, but also against infections in general. Immunocompromised patients are often leukopenic, resulting in development of septicemia from a local infection at an early stage. For these patients maximal blood clearance capacity of the MPS is of great importance. Cells of the MPS can be stimulated by immunomodulators.

Stimulation of nonspecific antimicrobial defenses by immunomodulators

Immunomodulators are agents that can stimulate cells of the immune system. Experimental studies in animals have demonstrated that administration of various immunomodulators can enhance the nonspecific resistance against bacterial infections (Table 1.2). In the first studies immunomodulators of bacterial origin were used. Since these agents are potent inducers of cytokines, more recently these cytokines themselves were investigated for their capacity to enhance antibacterial resistance. To this aim models of bacterial, fungal, viral and protozoal infections were used. In most cases the percentage survival of infected animals was used as parameter for therapeutic efficacy. The immunomodulators were administered in the free form, via several routes (subcutaneously, intraperitoneally, intravenously, intranasally). Usually a single dose was given.

In this thesis the effects of a lipophilic muramyl dipeptide derivative, muramyl tripeptide phosphatidylethanolamide (MTPPE), as a representative of immunomodulators of bacterial origin, and of interferon-γ (IFN-γ), as a representative of cytokines, are investigated. It is known that these agents have the capacity to stimulate the cells of the MPS to tumorcytotoxic activity (95,196,202). In clinical studies MTPPE and IFN-γ are at present under investigation in cancer patients with respect to their capacity to stimulate the nonspecific resistance in the removal of metastases (56,70,125).

The basic molecule of MTPPE is muramyl dipeptide, which is part of the peptidoglycan of the cell wall of bacteria and is the minimum active compound in Freund's complete adjuvant. It can be synthetically produced and modifications can be made to reduce toxic side effects (53). Muramyl dipeptide and its derivatives have many effects on macrophages in terms of morphological and biochemical changes, enzyme and monokine production and macrophage-mediated tumorcytotoxicity (61,65,134,172,181). In addition, it has been shown that treatment of animals with muramyl peptides led to resistance against infections with intracellular as well as extracellular micro-organisms. Exposure of macrophages to muramyl peptides in vitro results in killing of intracellular micro-organisms. A summary of the studies of the antimicrobial resistance enhancing effects of muramyl peptides in vivo, in the immunocompetent and immunocompromised host, as well as in vitro is given in Table 1.3.

IFN-γ is a cytokine produced by activated T-lymphocytes which exerts a broad spectrum

TABLE 1.2 Immunomodulators with antimicrobial resistance enhancing properties

Immunomodulator	Micro-organism	References
bacterial origin		
lipopolysaccharide	*Candida albicans*	212[b]
	Klebsiella pneumoniae	72
lipid X	*Escherichia coli*	79
monophosphoryl lipid A	*Staphylococcus epidermidis*	23
trehalose dimycolate	*Klebsiella pneumoniae*	126
	Toxoplasma gondii	121
	Trypanosoma cruzi	121
muramyl peptides	various micro-organisms	Table 1.3
cytokines		
interleukin-1	*Candida albicans*	16[b]
	Escherichia coli	38
	Listeria monocytogenes	39,40,93
	Klebsiella pneumoniae	159
	Plasmodium berghei	38
	Pseudomonas aeruginosa	159,207[c]
	Salmonella typhimurium	139
	Streptococcus pneumoniae	207[c]
	Toxoplasma gondii	25
	herpes simplex virus	93
	cytomegalovirus	208
	Sendai virus	93
interleukin-2	*Candida albicans*	212[ab]
	Escherichia coli	83
	Listeria monocytogenes	83
	Klebsiella pneumoniae	94
	Mycobacterium avium	91[b]
	Toxoplasma gondii	190
	herpes simplex virus	214
interleukin-4	*Leishmania major*	17[ab]
	Listeria monocytogenes	44[ab]
interleukin-6	*Listeria monocytogenes*	44[ab]
colony stimulating factor (CSF)		
G-CSF	*Candida albicans*	131,181[ab]
	Escherichia coli	131
	Listeria monocytogenes	186
	Pseudomonas aeruginosa	131
	Serratia marcescens	131
	Staphylococcus aureus	131[b],179
M-CSF	*Candida albicans*	24

Immunomodulator	Micro-organism	References
	Histoplasma capsulatum	149
	Listeria monocytogenes	102
	Mycobacterium avium	184[a]
GM-CSF	*Candida albicans*	133
	Histoplasma capsulatum	149
	Mycobacterium lepraemurium	43[b]
	Pseudomonas aeruginosa	133
	Salmonella typhimurium	81
	Staphylococcus aureus	133
	Streptococcus pneumoniae	216
tumor necrosis factor	*Candida albicans*	16,100
	Klebsiella pneumoniae	88
	Legionella pneumophila	14
	Listeria monocytogenes	44[b]
	Mycobacterium lepraemurium	43[b]
	Plasmodium falciparum	116
	Streptococcus pneumoniae	168
	Toxoplasma gondii	45
	Trypanosoma cruzi	45,141[b]
	cytomegalovirus	208
interferon-α	herpes simplex virus	175
	Toxoplasma gondii	5[ab]
interferon-ß	simian varicella-like virus	55
	Toxoplasma gondii	5[ab]
interferon-γ	various micro-organisms	Table 1.4

[a] no antimicrobial resistance enhancing effects are found
[b] in vitro studies
[c] immunocompromised host

of biological activities, such as antiviral activity and action on B-lymphocytes, natural killer cells and macrophages (95). IFN-γ stimulates macrophages to increase the production of its enzymes and monokines, as well as enhancing their tumoricidal activity in vitro as well as in vivo (13,95). In addition, it has been shown that in animals treated with IFN-γ, a resistance against microbial infections was found (87,132). Also after in vitro exposure to IFN-γ, macrophages show increased microbicidal activity. A summary of the studies of the antimicrobial resistance enhancing effects of IFN-γ in vivo, in the immunocompetent and immunocompromised host, as well as in vitro is given in Table 1.4. Most studies were in vitro studies, performed in monolayers or suspensions of macrophages.

To stimulate the MPS by administration of immunomodulators in immunocompromised patients who are prone to infections during a prolonged period of time, repeated

administration of these agents is anticipated. This may result in toxic side effects (18,55,117,188). To reduce the toxicity of these immunomodulating agents a less toxic formulation of these agents, such as encapsulation in liposomes, may be of importance. It is clearly demonstrated that conventional liposomes are taken up preferentially by the cells of the MPS. These liposomes can therefore be used in targeting immunomodulators to stimulate the MPS (162).

Liposomes as carriers of immunomodulators

Why liposomes?

Liposomes are microscopic vesicles consisting of one or more lipid bilayers surrounding an internal aqueous compartment (Figure 1.1). A variety of agents can be entrapped in liposomes, hydrophobic agents in the lipid bilayers and hydrophilic agents in the inner aqueous space. Depending on the method of production, liposomes can vary widely in size and in number of lamellae (unilamellar or multilamellar vesicles) and in their physicochemical characteristics. A vast body of literature is available describing the different physicochemical and biopharmaceutical aspects of these vesicular structures (63,99,123,200,215). The pharmacokinetics of liposomes in animals has gained extensive attention. Liposomes are quite similar to natural membranes and, as a result, they can be safely administered and are biodegradable. Data in humans are now rapidly becoming available. Central to any therapeutic use is the pattern of biodistribution of the liposomes after intravenous administration, and the ability to manipulate this biodistribution in order to target the liposomes selectively. The disposition of liposomes after parenteral administration strongly

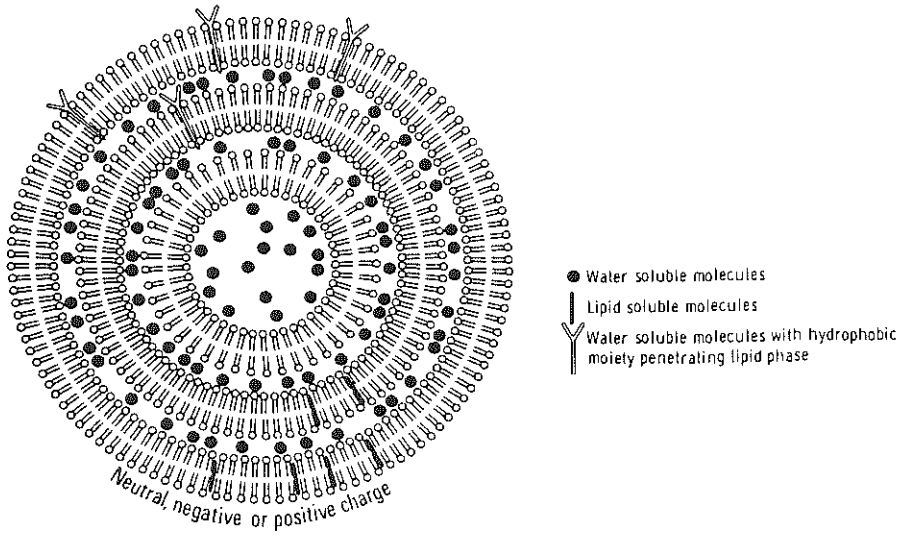

FIGURE 1.1 A liposome (multilamellar vesicle) (82)

TABLE 1.3 Micro-organisms studied with respect to the antimicrobial resistance enhancing effects of muramyl peptide derivatives

Micro-organism	Muramyl peptide derivative	Immunocompromising factor	References
in vivo studies			
Candida albicans	MDP, abuMDP, MTPPE	-	51,56
Escherichia coli	MDP, L18-MDP,MDP-Lys(L18)	-	130,154,158
	L18-MDP	irradiation or cyclophosphamide	155
Klebsiella pneumoniae	MDP, L18-MDP,	-	29,130,154[a],176,178
	murabutide, MDP-Lys(L18)	-	3,26,158,165,177
	MDP	starvation	73,74
	MDP, murabutide, MDP-Lys(L18)	neonatal	163,167
	MDP	cobra venom factor	33
	MDP	shock due to hypertonic glucose	4
	MDP	cyclophosphamide or hydrocortison	75
Listeria monocytogenes	MDP, norMDP, abuMDP	-	2,27,63[a],67,154[a]
Mycobacterium leprae	MDP	-	114[a]
Naegleria fowleri	MDP	-	121
Pseudomonas aeruginosa	MDP, norMDP, abuMDP,	-	27,130,154,165
	MDP-Lys(L18), L18MDP	cortison acetate	153,157
Salmonella enteritidis	MDP, MDP-Lys(L18)	-	151,158
	MDP, MDP-Lys(L18)	myeloperoxidase deficiency	152[a]
Stapylococcus aureus	L18MDP	-	154
Trypanozoma cruzi	MDP	-	109,121
Toxoplasma gondii	MDP	-	121
Friend leukemia virus	MDP, MTPPE	-	51
herpes simplex virus	MDP, MTPPE	-	51,112
influenza A,B	MDP, MTPPE, murabutide	-	32,46,51
murine hepatitis virus	MDP, murametide	-	128
parainfluenza	MDP, MTPPE	-	51
Rift Valley fever virus	MDP, MTPPE	-	51
in vitro studies			
Listeria monocytogenes	MDP	-	84
human immunodeficiency virus	MDP	-	129

[a] no antimicrobial resistance enhancing effects are found

depends on their physicochemical characteristics, particularly the size, the bilayer, rigidity and the charge (63,94).

Conventional liposomes, when administered intravenously, rapidly accumulate in cells of the MPS, primarily those residing in the liver and spleen. These liposomes, therefore, tend to have a short half-life in blood (124,200). This natural behaviour of liposomes can be exploited in the treatment of infectious diseases in two ways. Firstly, liposomes can be used as carriers of antibiotics in treatment of infections involving the MPS. Secondly, since cells of the MPS play a major role in the nonspecific host defense against infections in general,

TABLE 1.4 Microorganisms studied with respect to the antimicrobial resistance enhancing effects of interferon-γ

Micro-organism	Immunocompromising factor	references
in vivo studies		
Aspergillus fumigatus	chronic granulomatous disease	11
Candida albicans	-	104[a]
Klebsiella pneumoniae	-	86,87
Listeria monocytogenes	-	108,117,146, 203[a],204[a]
	neonatal	31
Mycobacterium tuberculosis	-	106
Salmonella enteritidis	-	77,132
Salmonella typhimurium	-	77,101,204[a]
Staphylococcus aureus	-	189
Trypanosoma cruzi	-	180
herpes simplex virus	-	175
in vitro studies		
Blastomyces dermatitidis	-	138,142
Candida albicans	-	16,21
Chlamydia psittaci	-	143
Coccidioides immitis	-	9,10
Legionella pneumophila	-	12,98,110,148
Leishmania donovani	-	48,90,143
Listeria monocytogenes	-	104,107,170,179
Mycobacterium avium	-	15[a]
Mycobacterium bovis	-	64
Mycobacterium microti	-	107
Mycobacterium tuberculosis	-	48[a]
Pseudomonas aeruginosa	-	104
Staphylococcus aureus	-	77
Toxoplasma gondii	-	5
Trypanosoma cruzi	-	141,142

[a] no antimicrobial resistance enhancing effects are found

liposomes can be used for targeting of immunomodulators, giving an enhancement of nonspecific resistance in infections caused by a variety of organisms. Prolongation of the circulation time and partial avoidance of liposome uptake by the MPS is important for a wider application of liposomes used as carriers of agents required outside the MPS. This can be accomplished by manipulation of the liposome size and composition and thereby influencing the biodistribution of the liposomes after intravenous administration.

Liposomes, therefore, seem to be useful as carriers of drugs because of their versatility. Through variation in size, number of bilayers, lipid composition, charge and surface characteristics, the cell specificity and the rate of intracellular degradation of liposomes can be manipulated and the therapeutic availability of the encapsulated agent can be increased. (8,62,99,123,200,215)

Liposomal encapsulation of immunomodulators

The most important goal of liposomal encapsulation of immunomodulators is reduction of toxicity and the targeting of these agents to the cells of the MPS in the liver and spleen. It has been shown that liposomal encapsulation of MTPPE and IFN-γ results in decreased

TABLE 1.5 Liposome-encapsulated immunomodulators with antimicrobial resistance enhancing properties

Immunomodulator	Micro-organism	References
LE[a]-MDP	*Candida albicans*	54
	Listeria monocytogenes	7[b]
	Rift Valley fever virus	111
LE-abuMDP	*Candida albicans*	54
	Pseudomonas aeruginosa	54
LE-MDP-GDP	*Listeria monocytogenes*	171
	Salmonella spp.	171
	Streptococcus pneumoniae	97,171
LE-MTPPE	Friend leukemia virus	51
	influenza virus	76[b]
	herpes simplex virus 2	51,112
	Leishmania donovani	89
	Rift Valley fever virus	105
LE-IFN-ß	simian varicella-like virus	55
LE-IFN-γ	*Leishmania donovani*	89
	Toxoplasma gondii	137

[a] LE=liposome-encapsulated
[b] in vitro studies

toxicity (58,90,188). Targeting of MTPPE and IFN-γ to cells of the MPS may be accomplished by encapsulation in large negatively charged liposomes consisting of multiple bilayers of phosphatidylcholine and phosphatidylserine (162,199). Until now studies regarding the stimulation of the nonspecific resistance against bacterial infections by liposome-encapsulated immunomodulators are limited (Table 1.5).

Aim of the study and introduction to experimental work

From the foregoing section it can be deduced that stimulation of the cells of the MPS by MTPPE or IFN-γ might be promising in the treatment of infections in the immunocompromised host. **The aim of this thesis was to obtain insight in the possibilities and restrictions of treatment with MTPPE and IFN-γ in the free form or liposome-encapsulated form.** To this end, different models of infection in mice were used.

In one experimental model infections were induced with *Listeria monocytogenes*, a bacterium that is rapidly taken up by cells of the MPS, but that can resist intracellular killing by escape from the phagosome into the cytoplasm. It was investigated whether the cells of the MPS, when stimulated by MTPPE or IFN-γ are able to effectively kill *L. monocytogenes*. *L. monocyto-genes* infection was induced by intravenous (i.v.) inoculation, resulting in uptake of all bacteria from the blood, particularly by liver and spleen. Thereafter bacterial numbers increased rapidly in both organs, while the blood remained sterile. This increase was followed by a decrease at five days after inoculation. Eventually all mice survived.

In other experimental models infections were induced with *Klebsiella pneumoniae*, a bacterium that, when it is not opsonized by specific antibodies, is poorly taken up by cells of the MPS. It is investigated whether in the non-immune host, the cells of the MPS, when stimulated by MTPPE, are able to effectively phagocytose *K. pneumoniae*. When *K. pneumoniae* infection was induced by i.v. inoculation about 80% of the bacteria were cleared from the blood by liver and spleen, resulting in death of all mice due to septicemia ('artificially-induced septicemia'). In a more clinically relevant model of infection *K. pneumoniae* was inoculated intraperitoneally (i.p.). In this model multiplication of bacteria i.p. resulted in appearance of bacteria in the blood at regular intervals. Eventually all mice died due to septicemia ('naturally-induced septicemia').

It is known that genetic factors contribute to the susceptibility of man to infections. In this thesis it was investigated **whether the difference in susceptibility is a restrictive factor in the usefulness of MTPPE and IFN-γ to induce resistance against *L. monocytogenes* infection.** Mouse strains differing in their innate resistance against *L. monocytogenes* were used. In these mice the different susceptibility is manifested by a different influx of leukocytes to the site of infection. The effects of MTPPE and IFN-γ on bacterial numbers were investigated in relation to the recruitment of leukocytes (Chapters 2 and 3).

Since in the immunocompromised host protection against infections is needed for a prolonged period of time, it was investigated **what the effects were of administration of immunomodulators in the less toxic liposome-encapsulated form.** In the model of artificially-induced *K. pneumoniae* septicemia the efficacy of a single dose of MTPPE and LE-MTPPE was studied in relation to the appearance of bacteria in the blood (Chapter 4).

In the model of naturally-induced *K. pneumoniae* septicemia single dose treatment as well as repeated administration of MTPPE and LE-MTPPE were investigated (Chapter 6).

Particularly in immunocompromised patients, enhancement of nonspecific resistance against infections by administration of immunomodulators may be of importance. These patients are often leukopenic. It was investigated **what the relative roles were of the recruitment of leukocytes, on the one hand, and the activation of tissue macrophages, on the other hand, both of which are induced by LE-MTPPE** (Chapter 5).

To obtain more insight in **which tissues and cell types are mainly involved in the by LE-MTPPE induced effects**, the tissue and cellular distribution of liposomes encapsulating MTPPE were investigated (Chapter 7).

When macrophages in vitro are exposed to both MTPPE and IFN-γ a synergistic effect in antibacterial resistance is found. The value of this synergistic effect for treatment of infections is questionable since exposure of macrophages to both immunomodulators simultaneously after i.v. administration of MTPPE and IFN-γ is expected to be minimal. However, this may be realized by administration of the immunomodulators co-encapsulated in the same liposome. **It was investigated what the therapeutic effects were of MTPPE and IFN-γ co-encapsulated in liposomes** (Chapter 8). This was studied in the model of *L. monocytogenes* infection. In this model **the importance of the immunomodulator to lipid ratio on the antibacterial effects of LE-MTPPE and LE-IFN-γ was also investigated.**

CHAPTER 2

EFFECT OF MURAMYL TRIPEPTIDE PHOSPHATIDYL ETHANOLAMIDE ON *LISTERIA MONOCYTOGENES* INFECTION IN GENETICALLY RESISTANT OR SUSCEPTIBLE MICE

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Abstract

The effect of the immunomodulator muramyltripeptide phosphatidylethanolamide (MTPPE) on the resistance against *Listeria monocytogenes* infection was investigated in mice which differed in their genetically determined innate resistance against this infection. It was shown that administration of MTPPE led to increased antibacterial resistance in terms of decreased numbers of bacteria in liver and spleen in so called resistant C57Bl mice but not in susceptible CBA mice. The effectiveness of MTPPE appeared to correlate with the MTPPE-induced capacity to recruit leukocytes from the bone marrow, since numbers of peripheral leukocytes were significantly increased in resistant mice but not in susceptible mice. Exposure of macrophages of resistant mice *in vitro* to MTPPE had no effect on the uptake and intracellular growth of *L. monocytogenes*, suggesting that besides the macrophage also other cell types play a role in the MTPPE-increased antibacterial resistance.

Introduction

From clinical experience it is well known that antibiotic treatment of severe infections especially in immunocompromised patients is not always successful. One of the factors which contribute to the lack of success of antibiotic treatment is the failure of the host defense to provide adequate support to antibiotic therapy. Administration of agents that stimulate the nonspecific antimicrobial resistance might prove beneficial in treatment of infections in the immunocompromised host (50,150,169). To this aim immunomodulatory agents such as muramyl peptides may be useful.

Muramyl dipeptide (MDP) is the minimal essential structure of the bacterial cell wall of Mycobacteria that is required for adjuvant activity of Freund's complete adjuvans (53). Muramylpeptides (MDP or analogs) have many effects on macrophages in terms of morphological changes (37,120,161), biochemical changes (50,217), enzyme production (96,120,134), monokine production (30,28,166) and macrophage-mediated tumoricidal activity (41,172,174,

202). In addition, muramyl peptides are able to stimulate macrophages to an increased production of colony stimulating factor resulting in an increase in the number of leukocytes in the blood (41,158). It is also demonstrated that muramyl peptides administered by various routes to animals resulted in enhancement of the nonspecific resistance to infections caused by a variety of organisms (158,20,167). However the role of muramyl peptides in resistance to infections caused by *Listeria monocytogenes* is still controversial (27,67,92,154,158,165).

Resistance to infection with *L. monocytogenes* in mice is genetically determined (127,185,192,198). Some strains of mice for example C57Bl mice are so called resistant to *L. monocytogenes*, whereas other strains among which CBA mice are susceptible. In susceptible mice the influx of leukocytes to the site of infection is delayed and less as compared to the influx in resistant mice (113,192,197,198). In the present study it was investigated whether treatment with the lipophilic derivative muramyltripeptide phosphatidylethanolamide (MTPPE) could increase the resistance against *L. monocytogenes* infection and whether the effect was related to the genetically determined resistance of the host against this infection. Because of the importance of peripheral leukocytes in the innate resistance against *L. monocytogenes* infection, also the change in the numbers of peripheral leukocytes with time due to treatment with MTPPE was studied.

Materials and methods

Animals. Specific pathogen free, 11 to 13 weeks old female C57Bl/Ka and male CBA/Rij mice were used (ITRI-TNO, Rijswijk, The Netherlands). The experiments with animals are approved by the local ethical committee according to the rules regarding the use of laboratory animals on the Erasmus University Rotterdam (1991, protocol no. 117.91.01).

Reagents. N-acetyl-muramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-[1,2-dipalmitoyl-sn-glycero-3-(hydroxy-phosphoryloxy)]ethanolamide (MTPPE) was a generous gift of Ciba Geigy (Basel, Switzerland). Dipalmitoyl phosphatidylethanolamine (DPPE) was obtained from Sigma (St Louis, Missouri, USA). All reagents were dissolved in phosphate-buffered saline (PBS).

Bacteria. A strain of *Listeria monocytogenes* type 1/2 was used. Bacteria were grown for 16 h at 37°C in Todd-Hewitt broth (Oxoid Ltd, Basingstoke, England) and preserved on ice. Directly before the inoculation of mice, the bacteria were washed twice in PBS. The i.v. 50% lethal dose (LD_{50}) was $2.6x10^4$ CFU in C57Bl/Ka mice and $5.9x10^3$ CFU in CBA/Rij mice.

Experimental infection caused by L. monocytogenes. Infections were induced by intravenous inoculation of $2x10^4$ CFU ($0.75xLD_{50}$) *L. monocytogenes* into C57Bl mice and $2x10^4$ CFU ($3.4xLD_{50}$) or $4.5x10^3$ CFU ($0.75xLD_{50}$) *L. monocytogenes* into CBA mice. At different intervals after inoculation blood samples were taken from the retroorbital plexus and twofold diluted in 3.8% sodium citrate (BDH Chemicals Ltd, Poole, England). Serial 10-fold dilutions in PBS were prepared and volumes of 0.2 ml of each dilution were spread on tryptone soya agar (TSA) plates (Oxoid Ltd, Basingstoke, England). Mice were then sacrificed and spleen and liver were removed and homogenized in 20 ml of PBS for 30 s at 10,000 rpm in a VirTis homogenizer (The VirTis Co. Inc., Gardiner, New York). Serial 10-fold dilutions of homogenate in PBS were prepared. Volumes of 0.2 ml of each dilution and

2 ml volumes of the undiluted homogenate were spread on TSA plates. The remainder of the homogenate together with an equal volume of double concentrated TSA was poured in plastic plates. All plates were incubated for 48 h at 37°C. The number of viable bacteria recovered from liver, spleen and blood was used as a parameter of therapeutic efficacy.

Quantitation of blood leukocytes. Bloodsamples (0.8 ml) were taken from the retroorbital plexus and collected in polypropylene tubes containing 1 mg 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 numbers of leukocytes were determined in duplicate in a Bürkers hemocytometer. The numbers of lymphocytes, granulocytes and monocytes were calculated from the total number of leukocytes and differential counts of 500 leukocytes in cytocentrifuge preparations of buffycoats, obtained by centrifugation of blood samples for 30 min at 1500 x g in hematocrit tubes (Tamson B.V., Zoetermeer, The Netherlands).

Monolayers of peritoneal macrophages infected with L. monocytogenes. Monolayers of $3.5x10^5$ macrophages from C57Bl mice were cultured at 37°C in chamber-slides (Miles laboratories Inc., Naperville, Illinois) in a humidified atmosphere of 7.5% CO_2 in air in culture medium containing Dulbecco modified Eagle medium (D-MEM, Flow Laboratories, Irvine, Scotland) with 1% glutamine and 15% fetal bovine serum (FBS, HyClone Laboratories Inc., Logan, Utah, USA) for 48 h before incubation with bacteria. After the first 2 h of incubation fresh culture medium was added to the monolayer. The cells were exposed 24 h before incubation with bacteria to various non-toxic concentrations of MTPPE or to PBS. At zero time bacteria were added in a ratio of 16 bacteria per macrophage. After an uptake period of 30 min the noningested bacteria were removed by washing the monolayer three times with culture medium. The macrophages were reincubated for 6 h. At different time intervals during the period of incubation monolayers were washed with icy-cold culture medium and the macrophages were disrupted by quickly freezing and thawing and vigorously mixing of the cell suspension. This procedure had no effect on the viability of *L. monocytogenes*. The intracellular numbers of viable bacteria were determined by preparing tenfold serial dilutions. Volumes of 0.2 ml of each dilution were spread on TSA plates. All plates were incubated for 48 h at 37°C.

Statistical analysis. Statistical evaluation of differences in numbers of bacteria between MTPPE-treated and PBS-treated groups of animals was performed by using the Mann-Whitney test. The Student-T test was used for evaluation of differences in numbers of leukocytes between MTPPE-treated and PBS-treated groups.

Results

Effect of dose and administration time of MTPPE on the course of L. monocytogenes infection in resistant C57Bl mice. Figure 2.1 shows that after i.v. inoculation of C57Bl mice with $2x10^4$ CFU *L. monocytogenes* (0.75x LD_{50}) within 1 h most bacteria were trapped in the liver and spleen. The number of bacteria in the liver and spleen increased until 72 h after bacterial inoculation followed by a stability in the liver and a decrease in the spleen. All mice survived (Table 1). Administration of 100 μg MTPPE per mouse (5.2 mg/kg) at 24 h before

before bacterial inoculation appeared to be the minimal dose from a 2-fold dose range that resulted in a significant decrease in the number of bacteria in liver and spleen at 120 h after inocula-tion. At 1, 24, or 72 h after inoculation the number of bacteria was the same in MTPPE-treated mice as in control mice. The blood was always sterile. A dose of 50 μg MTPPE per mouse (2.6 mg/kg) resulted in decreased numbers of bacteria in the spleen at 120 h, but not in the liver. A dose of 25 μg MTPPE per mouse (1.3 mg/kg) had no effect anymore on the numbers of bacteria in both the liver and spleen at 120 h.

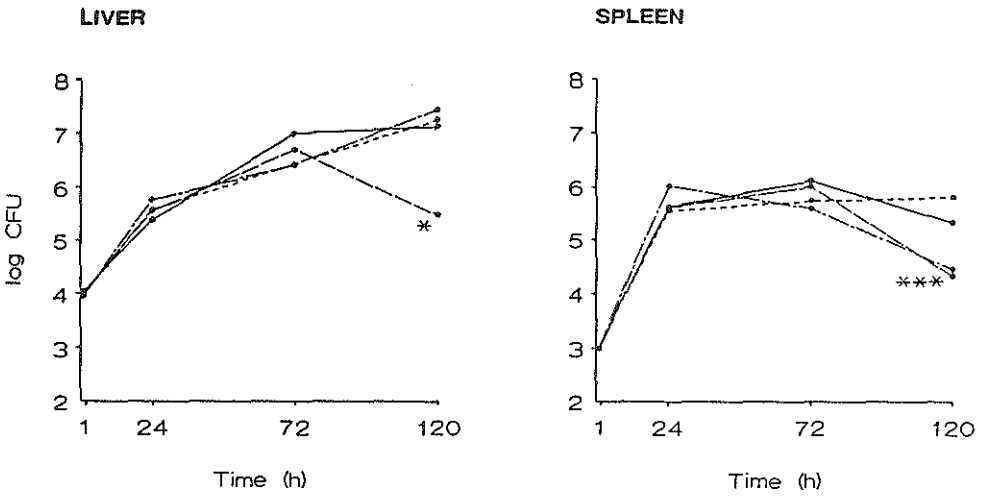

FIGURE 2.1 Effect of MTPPE on the numbers of *L. monocytogenes* in liver and spleen of C57Bl mice. Mice infected with $2x10^4$ CFU *L. monocytogenes* at zero time were treated i.v. at 24 h before bacterial inoculation with 100 μg MTPPE per mouse (5.2 mg/kg) (—— ——), 50 μg MTPPE (2.6 mg/kg) (— - —), 25 μg MTPPE (1.3 mg/kg) (- - - - -) or PBS (———). Each point represents the geometric mean for six mice. Significance: *$p \leq 0.05$ ***$p \leq 0.001$ versus PBS-treated mice.

Effect of MTPPE on the number of leukocytes in the blood of resistant C57Bl mice. Figure 2.2 shows that in uninfected mice the numbers of total leukocytes fluctuated around 3700 per mm^3 blood. Numbers of monocytes, granulocytes and lymphocytes fluctuated around respectively 70, 200, and 3400 per mm^3 blood. Administration of 100 μg MTPPE per mouse (5.2 mg/kg), the lowest dose from the 2-fold dose range which was effective in *L. monocytogenes* infection, to uninfected mice at 24 h before zero time resulted in a 3-fold increase of monocytes and an almost 2-fold increase of granulocytes as well as lymphocytes.

In mice infected with *L. monocytogenes* at zero time the numbers of monocytes, granulocytes and lymphocytes were significantly decreased at 48 h after bacterial inoculation to 35% of the numbers at 24 h after inoculation, followed by an increase. Pretreatment of infected mice with 100 μg MTPPE per mouse (5.2 mg/kg) at 24 h before bacterial inoculation resulted in 3-fold increased numbers of monocytes at 24 h after inoculation, whereas the

numbers of granulocytes and lymphocytes remained stable until 24 h after inoculation. At 48 h after bacterial inoculation numbers of monocytes, granulocytes and lymphocytes were significantly decreased to 25%, 50% or 45% respectively of the numbers at 24 h after inoculation. Hereafter numbers of monocytes, granulocytes and lymphocytes increased again.

Effect of MTPPE on the uptake and intracellular survival of L.monocytogenes in macrophages of C57Bl mice in monolayer. In Table 2.2 is shown that exposure of macrophages in monolayer to 4-fold increasing concentrations of MTPPE ranging from 0.4 up to 100 μg/ml for 24 h before incubation with *L. monocytogenes* had no effect on the uptake and intracellular survival of the bacteria, except that exposure to 100 μg MTPPE/ml or 25 μg MTPPE/ml resulted in slightly but significantly increased numbers of intracellular surviving bacteria at 4 h after bacterial uptake.

TABLE 2.1 Effect of MTPPE treatment on survival of C57Bl mice and CBA mice from *L. monocytogenes* infection

	Percentage survival[b]		
Treatment[a] (mg MTPPE/kg)	C57Bl infected with $2.0x10^4$ CFU	CBA infected with $2.0x10^4$ CFU	CBA infected with $4.5x10^3$ CFU
5.20	100	0	0
2.60	100	ND	ND
1.30	100	ND	50
0.64	ND	ND	100
0.32	ND	0	100
0.16	ND	0	ND
0	100	0	100

[a] Mice i.v. infected with $2x10^4$ or $4.5x10^3$ CFU *L. monocytogenes* were i.v. treated with a single dose MTPPE from a twofold increasing dose range at 24 h before inoculation.

[b] Survival was determined until 6 days after bacterial inoculation.

Effect of dose and administration time of MTPPE on the course of L. monocytogenes infection in susceptible CBA mice. Figure 2.3 shows that after i.v. inoculation of CBA mice with $2x10^4$ CFU *L. monocytogenes* within 1 h most bacteria were trapped in the liver and spleen. This inoculum was the same inoculum as used in the experiments with resistant C57Bl mice ($0.75xLD_{50}$), however in susceptible CBA mice this inoculum is equivalent to $3.4xLD_{50}$. Numbers of bacteria increased until the mice died at 96 h after bacterial inoculation (Table 2.1). Administration of a dose of 130 μg MTPPE per mouse (5.2 mg/kg) at 24 h before bacterial inoculation resulted in a significant increase in numbers of bacteria

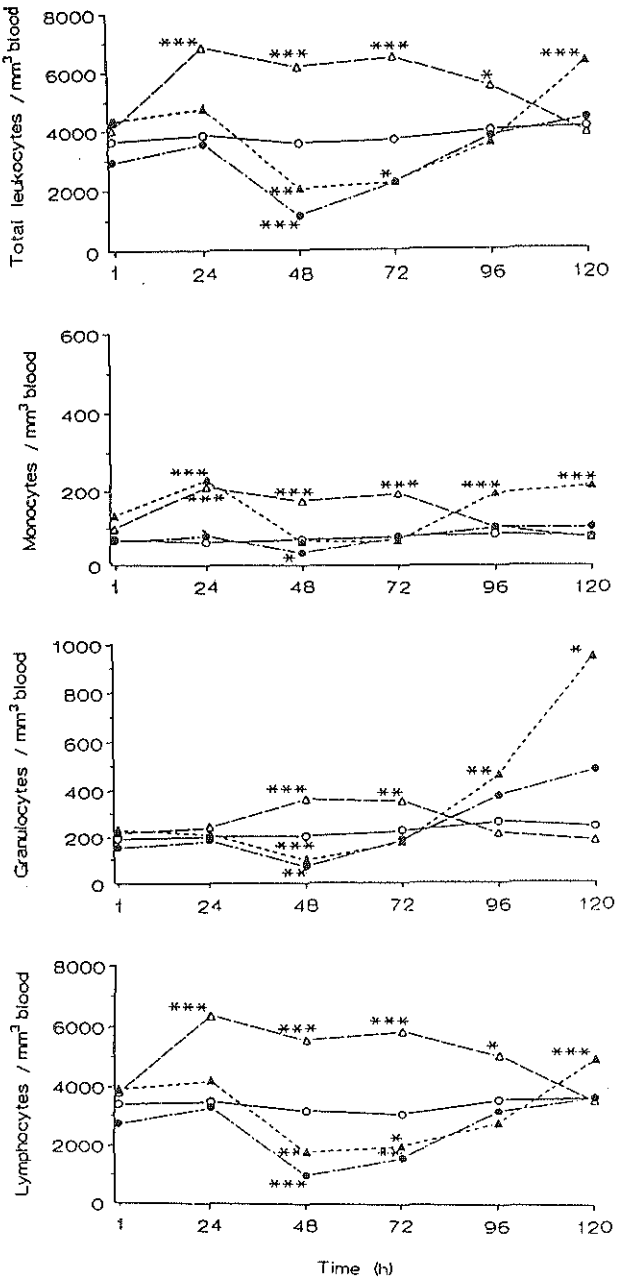

FIGURE 2.2 Effect of MTPPE on the numbers of total leukocytes, monocytes, granulocytes and lymphocytes in blood of C57Bl mice. Mice infected with $2x10^4$ CFU *L. monocytogenes* at zero time were treated i.v. at 24 h before bacterial inoculation with 100 μg MTPPE per mouse (5.2 mg/kg) (▲- - -▲) or PBS (●- - -●). Uninfected mice were treated i.v. at 24 h before zero time with 100 μg MTPPE per mouse (5.2 mg/kg) (△— —△) or PBS (○——○). Each point represents the mean for six mice. Significance: *$p \leq 0.05$ **$p \leq 0.01$ ***$p \leq 0.001$ versus uninfected PBS-treated mice.

TABLE 2.2 Effect of MTPPE on uptake and intracellular survival of *L. monocytogenes* in peritoneal macrophages of C57Bl mice in vitro[a]

Concentration MTPPE (μg/ml)	Log CFU *L. monocytogenes* per 10^6 macrophages[b] at times (h) after uptake of bacteria			
	0	1	4	6
100	5.57±0.03	5.76±0.08	6.18±0.09*	6.52±0.06
25	5.66±0.04	5.72±0.05	6.28±0.07**	6.56±0.04
6.2	5.57±0.05	5.56±0.08	5.86±0.10	6.29±0.04
1.6	5.55±0.06	5.62±0.12	6.09±0.16	6.58±0.04
0.4	5.50±0.06	5.58±0.12	6.07±0.07	6.41±0.08
0	5.67±0.08	5.84±0.11	5.96±0.03	6.52±0.11

[a] Before incubation with bacteria, macrophages in monolayer culture were exposed 24h to various concentrations of MTPPE.

[b] Data represent the geometric mean ± SEM for six observations (control) or four observations (MTPPE). Significance: *$p \leq 0.05$ **$p \leq 0.01$ versus control.

in the liver and spleen at 24 h after bacterial inoculation and in death of all mice at 48 h. Only in dead mice *L. monocytogenes* was recovered from the blood. From the 2-fold dose range of MTPPE 8.1 μg MTPPE per mouse (0.32 mg/kg) was the lowest dose that resulted in significant increased numbers of bacteria in the liver and spleen at 72 h after bacterial inoculation. A dose of 4 μg MTPPE per mouse (0.16 mg/kg) had no effect on the bacterial numbers compared to untreated control mice. Administration of MTPPE to susceptible BALB/c mice at 24 h before bacterial inoculation also resulted in significant increased bacterial numbers in liver and spleen, compared to numbers in untreated mice (data not shown).

Figure 2.4 shows that after i.v. inoculation of CBA mice with 4.5×10^3 CFU *L. monocytogenes* ($0.75 \times LD_{50}$) within 1 h most bacteria were trapped in the liver and spleen. Bacterial numbers in the liver and spleen increased till 72 h, followed by a decrease. All mice survived (Table 2.1). Administration of a dose of 130 μg MTPPE per mouse (5.2 mg/kg) at 24 h before bacterial inoculation resulted in a significant increase in numbers of bacteria in the liver and spleen at 24 h and 72 h and all mice died at 96 h. Only in dead mice bacteria were recovered from the blood. From the 2-fold dose range of MTPPE, 32.5 μg MTPPE per mouse (1.3 mg/kg) was the lowest dose that resulted in significant increased numbers of bacteria in liver and spleen at 24 h and at 72 h. A dose of 16.2 μg MTPPE per mouse (0.64 mg/kg) resulted in increased numbers of bacteria at 24 h and 120 h in the liver, but not in the spleen. A dose of 8.1 μg MTPPE per mouse (0.32 mg/kg) had no effect anymore on the numbers of bacteria both in the liver and spleen at 120 h.

Effect of DPPE on the course of L.monocytogenes infection in CBA mice. Figure 2.5 shows that administration of 22 μg DPPE per mouse (0.9 mg/kg, which is the equimolar amount of 1.3 mg MTPPE/kg) at 24 h before inoculation with 4.5×10^3 CFU *L. monocytoges*

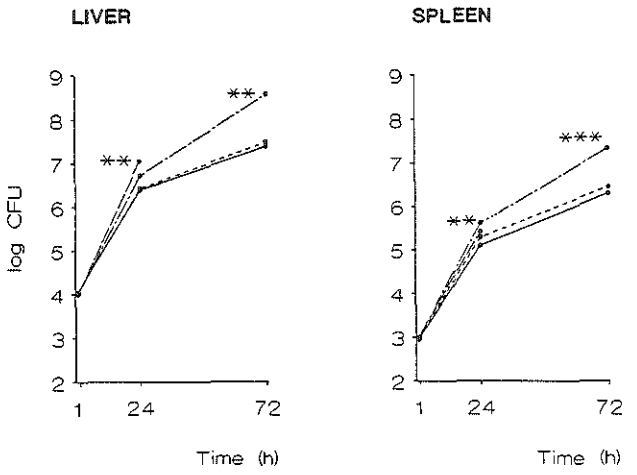

FIGURE 2.3 Effect of MTPPE on the numbers of *L. monocytogenes* in liver and spleen of CBA mice. Mice infected with $2x10^4$ CFU *L. monocytogenes* at zero time were treated i.v. at 24 h before bacterial inoculation with 130 μg MTPPE per mouse (5.2 mg/kg) (— —), 8 μg MTPPE per mouse (0.32 mg/kg) (— - —), 4 μg MTPPE per mouse (0.16 mg/kg)(- - - - - -) or PBS (———). Each point represents the geometric mean for six mice. If mice died a geometric mean was not calculated. Significance: $^{**}p \leq 0.01$ $^{***}p \leq 0.001$ versus PBS-treated mice.

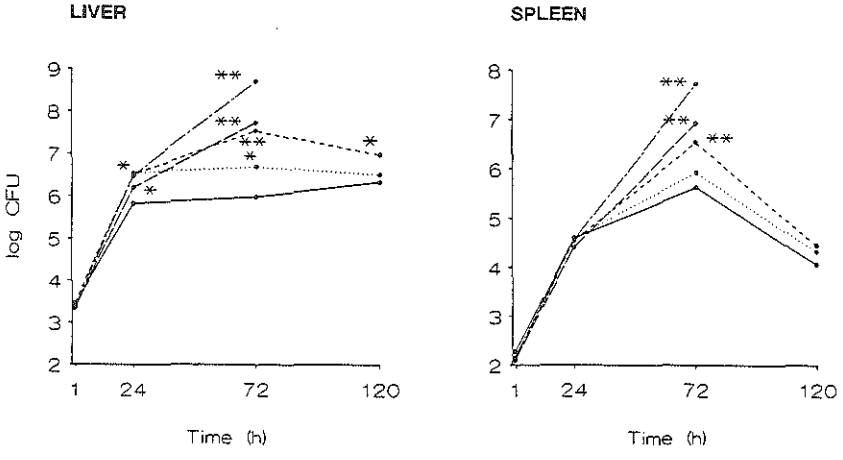

FIGURE 2.4 Effect of MTPPE on the numbers of *L. monocytogenes* in liver and spleen of CBA mice. Mice infected with $4.5x10^3$ CFU *L. monocytogenes* at zero time were treated i.v. at 24 h before bacterial inoculation with 130 μg MTPPE per mouse (5.2 mg/kg) (— - —), 32.5 μg MTPPE per mouse (1.3 mg/kg) (— —), 16 μg MTPPE per mouse (0.64 mg/kg)(- - - - -), 8 μg MTPPE per mouse (0.32 mg/kg) (·····) or PBS (———). Each point represents the geometric mean for six mice. If mice died a geometric mean was not calculated. $^{*}p \leq 0.05$ $^{**}p \leq 0.01$ versus PBS-treated mice.

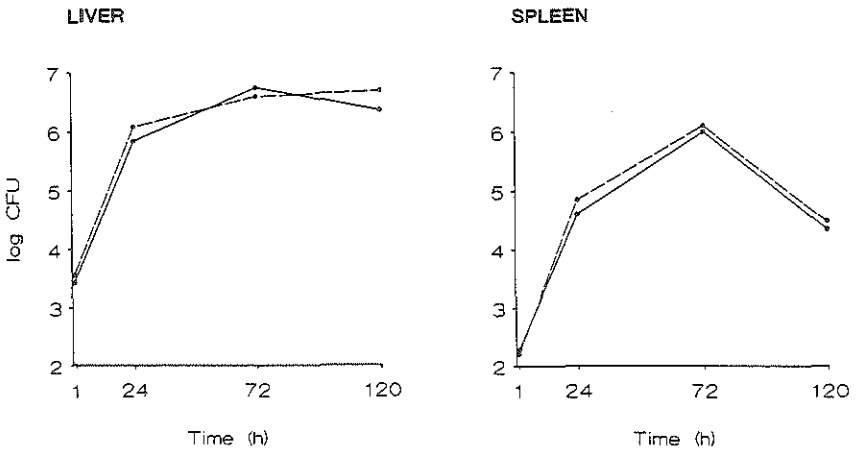

FIGURE 2.5 Effect of DPPE on the numbers of *L. monocytogenes* in liver and spleen of CBA mice. Mice infected with $4.5x10^3$ CFU *L. monocytogenes* at zero time were treated i.v. at 24 h before bacterial inoculation with 22 μg DPPE per mouse (0.9 mg/kg) (— —) or PBS (———). Each point represents the geometric mean for six mice.

($0.75xLD_{50}$) had no effect on the course of infection in the liver and spleen.

Effect of MTPPE on the number of leukocytes in the blood of susceptible CBA mice. Figure 2.6 shows that in uninfected mice the numbers of total leukocytes fluctuated around 5400 per mm^3 blood. The numbers of monocytes, granulocytes and lymphocytes fluctuated around 200, 400 and 4800 per mm^3 blood, respectively. Administration of 32.5 μg MTPPE per mouse (1.3 mg/kg), the lowest dose from the 2-fold dose range which was effective in *L. monocytogenes* infection, to uninfected mice at 24 h before zero time had no effect on the number of leukocytes except from a significant lower number at 48 h after administration.

In mice infected with $4.5x10^3$ CFU *L. monocytogenes* ($0.75xLD_{50}$) at zero time the numbers of monocytes, granulocytes and lymphocytes were significantly decreased at 48 h after bacterial inoculation to 50% of the numbers at 24 h after inoculation, followed by an increase. Pretreatment with 32.5 μg MTPPE per mouse (1.3 mg/kg) at 24 h before bacterial inoculation resulted in a significant decrease of monocytes, granulocytes and lymphocytes until 50% of the mice died.

Discussion

In this study it is shown that administration of MTPPE led to an increased resistance against *L. monocytogenes* infection in C57Bl mice, which are genetically determined resistant for *L. monocytogenes*. This protective effect appeared to be dependent on the dose of administration of MTPPE. An increased resistance against experimental infection by *L. monocytogenes* due to administration of muramyl peptides was also observed by other

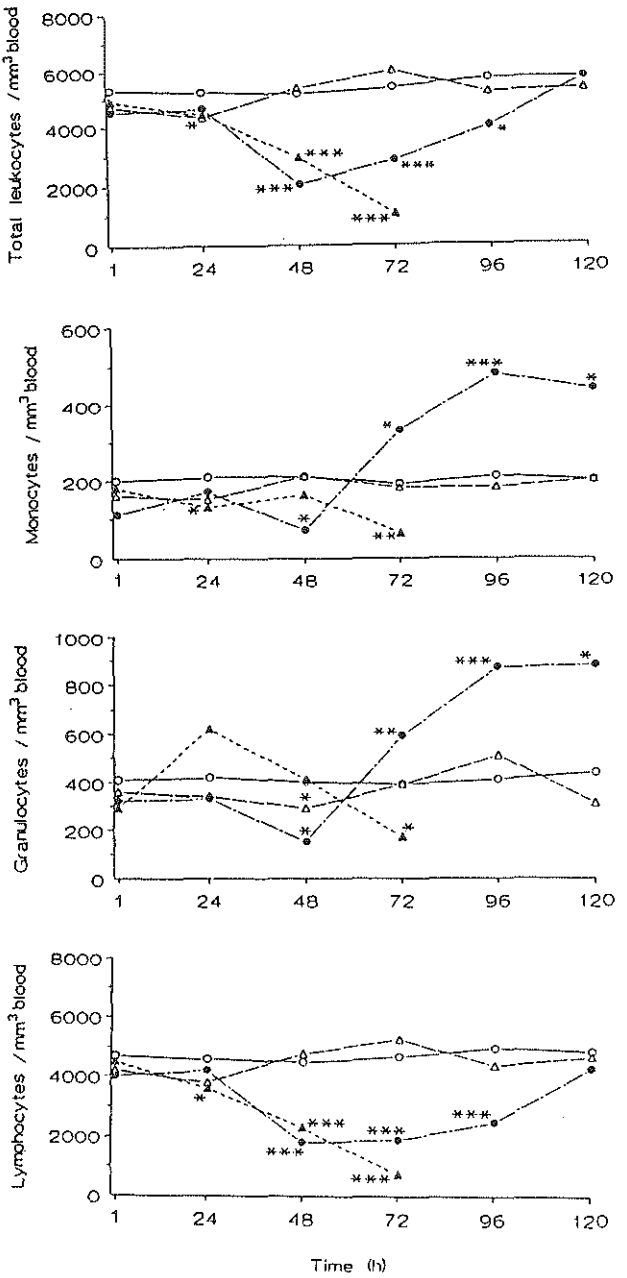

FIGURE 2.6 Effect of MTPPE on the numbers of total leukocytes, monocytes, granulocytes and lymphocytes in blood of CBA mice. Mice infected with 4.5×10^3 CFU *L. monocytogenes* at zero time were treated i.v. at 24 h before bacterial inoculation with 32.5 μg MTPPE per mouse (1.3 mg/kg) (▲----▲) or PBS (●— --●). Uninfected mice were treated i.v. at 24 h before zero time with 32.5 μg MTPPE per mouse (1.3 mg/kg) (△— —△) or PBS (○——○). Each point represents the mean for six mice. If mice died a mean was not calculated. Significance: *$p \leq 0.05$ **$p \leq 0.01$ ***$p \leq 0.001$ versus uninfected PBS-treated mice.

investigators (27,67,158,165). However some studies show that muramyl peptides had no effect on the course of *L. monocytogenes* infection (63,92,154,164). Exposure of macrophages to MTPPE *in vitro* had no effect on the uptake and intracellular survival of *L. monocytogenes*. This suggests that other cell types as well as macrophages are involved in the MTPPE-induced increased resistance against *L. monocytogenes*. With respect to this it was observed in uninfected C57Bl mice that administration of MTPPE led to a threefold increase in numbers of monocytes and a twofold increase in numbers of granulocytes and lymphocytes in the blood. In infected C57Bl mice MTPPE pretreatment resulted in a more than threefold increased efflux of monocytes from the blood between 24 h and 48 h after bacterial inoculation compared to untreated infected mice which indicates that the MTPPE-induced increased numbers of monocytes were also responsive. Yamaguchi et al. (218) also found that administration of a lipophilic muramyl peptide (MDP-Lys(L18)) to resistant mice resulted in increased numbers of leukocytes in the blood which was due to increased levels of CSF, a lymphokine which enhances the proliferation and differentiation of cells in the bone marrow (34).

It has been shown that in genetically determined resistant mice, such as C57Bl mice resistance to *L. monocytogenes* infection is caused by the Lr^r gene whose phenotype is manifested by a fast and great influx of monocytes to the site of infection (192,197,202) within 48 h after bacterial inoculation. Mice which are genetically determined susceptible such as CBA mice possess the Lr^S gene and the influx of monocytes to the site of infection is delayed and less. In this study it is observed that administration of MTPPE to susceptible CBA mice infected with the same inoculum of *L. monocytogenes* as was used in resistant C57Bl mice did not result in an increased resistance against *L. monocytogenes* infection, however even resulted in a decreased resistance. Lowering the bacterial inoculum for susceptible CBA mice to an inoculum at the same ratio to LD_{50} ($0.75xLD_{50}$) as used in resistant C57Bl mice did not change the effects of MTPPE in susceptible CBA mice. Regarding the observations on the effect of muramyl peptides in experimental *L. monocytogenes* infection in various mouse strains there is considerable discrepancy. Humphres et al. (92) demonstrated the ineffectiveness of MDP on *L. monocytogenes* infection in susceptible CBA mice but he also found that administration of MDP had no effect on *L. monocytogenes* infection in several other mouse strains among which resistant C57Bl mice. Fraser-Smith et al. (67) showed an increased survival from *L. monocytogenes* infection after treatment with several hydrophilic muramyl peptides in susceptible CBA mice but not in ICR-SPF mice which are also susceptible for *L. monocytogenes*. The MTPPE-induced decreased resistance in susceptible CBA mice observed in our study was probably caused by MTP since PE alone (DPPE) had no effect on the course of *L. monocytogenes* infection in these mice. In uninfected susceptible CBA mice, administration of MTPPE had no effect on the number of monocytes, granulocytes and lymphocytes except for a slight but significant lower number of monocytes and lymphocytes at 48 h after administration. The observations in resistant C57Bl mice and susceptible CBA mice on the effects of MTPPE in terms of resistance against *L. monocytogenes* infection and the numbers of leukocytes in the blood suggests that the effectiveness of MTPPE correlates with the capacity to recruit leukocytes from the bone marrow. The observations in resistant C57Bl mice and susceptible CBA mice on the effects of MTPPE in terms of resistance against *L. monocytogenes* infection and the numbers of

leukocytes in the blood suggests that the effectiveness of MTPPE correlates with the capacity to recruit leukocytes from the bone marrow. Since the number of leukocytes in the blood of MTPPE-treated infected mice is a resultant of an increase due to MTPPE treatment and a decrease due to migration from the blood to the site of infection, in MTPPE-treated infected C57Bl mice more leukocytes have migrated to the infected organs than in PBS-treated infected mice. The behaviour of leukocyte numbers in the infected organs is now under investigation.

The observation described in this study that the effect of treatment with the immunomodulator MTPPE was related to the genetically determined resistance of the host against this infection, may be of importance with respect to the treatment of human patients. In man the genetically determined resistance against bacterial infections (e.g. *Mycobacterium tuberculosis)* also may vary individually (22,193). This effect may depend on which immunomodulator is used. Therefore, in future studies the importance of genetically determined resistance of the host in treatment with other immunomodulators, besides MTPPE, will be investigated.

In summary, the protective effect in *L. monocytogenes* infection by MTPPE is dependent on the genetically determined resistance to *L. monocytogenes*. In resistant C57Bl mice, administration of MTPPE results in increased resistance, in susceptible CBA mice it leads to a decreased resistance. The effectiveness of MTPPE appears to be correlated with the MTPPE-induced capacity of mice to recruit leukocytes from the bone marrow. MTPPE can not stimulate macrophages *in vitro* to inhibition of intracellular growth of *L. monocytogenes.*

CHAPTER 3

EFFECT OF INTERFERON-GAMMA ON *LISTERIA MONOCYTOGENES* INFECTION IN GENETICALLY RESISTANT OR SUSCEPTIBLE MICE

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This study is submitted for publication.

Abstract

The effect of the immunomodulator interferon-γ (IFN-γ) on the resistance against *Listeria monocytogenes* infection was investigated in mice which differed in their genetically determined innate resistance against this infection. It was shown that administration of IFN-γ led to increased antibacterial resistance in resistant as well as susceptible mice. This seemed not to be correlated with the capacity to recruit leukocytes from the bone marrow, since numbers of leukocytes were increased in resistant mice but not in susceptible mice. Exposure of macrophages of so called resistant C57Bl or susceptible CBA mice *in vitro* to IFN-γ resulted in inhibition of intracellular growth of *Listeria monocytogenes* whereas exposure of macrophages of these mouse strains to MTPPE had no effect at all.

Introduction

It is well known from clinical experience that antibiotic treatment of severe infections especially in immunocompromised patients is not always successful. Several factors may contribute to the lack of success of treatment. One factor is the failure of the host defense to provide adequate support to antibiotic therapy. Administration of agents that stimulate the nonspecific antimicrobial resistance might prove benificial in treatment of infections in the immunocompromised host (50,150,169). To this aim immunomodulatory agents such as interferon-γ may be useful.

Interferon-gamma (IFN-γ) is a lymphokine produced by activated T lymphocytes which exerts a broad spectrum of biological activities (95) such as antiviral activity and action on B lymphocytes, T lymphocytes, NK cells and macrophages (95). It is demonstrated that administration of IFN-γ to animals resulted in enhancement of the nonspecific resistance to various microbial infections (142) including *L. monocytogenes* infection (31,108,117,146).

Resistance to infection with *L. monocytogenes* in mice is genetically determined (127,185,192,198). Some strains of mice for example C57Bl mice are so called resistant to *L. monocytogenes*, whereas other strains among which CBA mice are susceptible. In susceptible mice the influx of leukocytes to the site of infection is delayed and less as compared to

the influx in resistant mice (113,192,197,198). In a previous study we have shown that the efficacy of the immunomodulator muramyl tripeptide phosphatidylethanolamide (MTPPE) against *Listeria monocytogenes* depended on the genetically determined innate resistance of the host against this *L. monocytogenes*. To investigate whether this is a general effect of immunomodulation, or only restricted to treatment with MTPPE, we have studied the effect of IFN-γ, a cytokine very important in the innate resistance against *L. monocytogenes* (52,147).

Materials and methods

Animals. Specific pathogen free, 11 to 13 weeks old female C57Bl/Ka and male CBA/Rij mice were used (ITRI-TNO, Rijswijk, The Netherlands).

Reagents. Recombinant rat IFN-γ with a specific activity of 4.4×10^6 U/mg protein was kindly provided by Dr. P. van der Meide (ITRI-TNO, Rijswijk, The Netherlands) based on collaboration between the two laboratories. IFN-γ was dissolved in phosphate-buffered saline (PBS).

Bacteria. A strain of *Listeria monocytogenes* type 1/2 was used. Bacteria were grown for 16 h at 37°C in Todd-Hewitt broth (Oxoid Ltd, Basingstoke, England) and preserved on ice. Directly before the inoculation of mice, the bacteria were washed twice in PBS. The intravenous 50% lethal dose (LD_{50}) was 2.6×10^4 CFU in C57Bl/Ka mice and 5.9×10^3 CFU in CBA/Rij mice.

Experimental infection caused by L. monocytogenes. Infections were induced by intravenous inoculation of 2×10^4 CFU ($0.75 \times LD_{50}$) *L. monocytogenes* into C57Bl mice and 2×10^4 CFU ($3.4 \times LD_{50}$) *L. monocytogenes* into CBA mice. At different intervals after inoculation bloodsamples were taken from the retroorbital plexus and twofold diluted in 3.8% sodium citrate (BDH Chemicals Ltd, Poole, England). Serial 10-fold dilutions in PBS were prepared and volumes of 0.2 ml of each dilution were spread on tryptone soya agar (TSA) plates (Oxoid Ltd, Basingstoke, England). Mice were then sacrificed and spleen and liver were removed and each was homogenized in 20 ml of PBS for 30 s at 10,000 rpm in a VirTis homogenizer (The VirTis Co. Inc., Gardiner, New York). Serial 10-fold dilutions of homogenate in PBS were prepared. Volumes of 0.2 ml of each dilution and 2 ml volumes of the undiluted homogenate were spread on TSA plates. The remainder of the homogenate together with an equal volume of double concentrated TSA was poured in plastic plates. All plates were incubated for 48 h at 37°C. The number of viable bacteria recovered from liver, spleen and blood was used as a parameter of therapeutic efficacy.

Quantitation of blood leukocytes. Bloodsamples (0.8 ml) were taken from the retroorbital plexus and collected in polypropylene tubes containing 1 mg 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 numbers of leukocytes were determined in duplicate in a Bürkers hemocytometer. The numbers of lymphocytes, granulocytes and monocytes were calculated from the total number of leukocytes and differential counts of 500 leukocytes in cytocentrifuge preparations of buffycoats obtained by centrifugation of blood samples for 30 min at 1500 x g in hematocrit tubes (Tamson B.V., Zoetermeer, The Nether-

lands).

Monolayers of peritoneal macrophages infected with L. monocytogenes. Monolayers of peritoneal macrophages from C57Bl mice were cultured at 37°C in chamber-slides (Miles laboratories Inc., Naperville, Illinois) in an humidified atmosphere of 7.5% CO_2 in air in culture medium containing Dulbecco modified Eagle medium (D-MEM, Flow Laboratories, Irvine, Scotland) with 1% glutamine and 15% fetal bovine serum (FBS, HyClone Laboratories Inc., Logan, Utah, USA) for 48 h before incubation with bacteria. After the first 2 h of incubation fresh culture medium was added to the monolayer. The cells were exposed 24 h before incubation with bacteria to various concentrations of IFN-γ, or to PBS. At zero time bacteria were added in a ratio of 16 bacteria per macrophage. After an uptake period of 30 min the noningested bacteria were removed by washing the monolayer three times with culture medium. The macrophages were reincubated for 6 h. At different time intervals during the period of incubation monolayers were washed with icy-cold culture medium and the macrophages were disrupted by quickly freezing and thawing and vigorously mixing of the cell suspension. The intracellular numbers of viable bacteria were determined by preparing tenfold serial dilutions. Volumes of 0.2 ml of each dilution were spread on TSA plates. All plates were incubated for 48 h at 37°C.

Statistical analysis. Statistical evaluation of differences in numbers of bacteria between IFN-γ-treated and PBS-treated groups of animals was performed by using the Mann-Whitney test. The Student-T test was used for evaluation of differences in numbers of leukocytes between IFN-γ-treated and PBS-treated groups.

Results

Effect of IFN-γ on the course of L. monocytogenes infection in resistant C57Bl mice. In Figure 3.1 it is shown that administration of $1.25x10^4$ U IFN-γ per mouse ($6.4x10^5$ U/kg) at 24 h before inoculation with $2x10^4$ CFU *L. monocytogenes* ($0.75xLD_{50}$) was the minimal dose from a 2-fold dose range that resulted in a significant decrease in the number of bacteria in liver and spleen at 120 h after bacterial inoculation. All mice survived (Table 3.1). At 1, 24 or 72 h after inoculation the number of bacteria was the same in IFN-γ-treated mice as in control mice. The blood was always sterile. A dose of $6.2x10^3$ U IFN-γ per mouse ($3.2x10^5$ U/kg) had no effect on the number of bacteria in the liver and spleen at 120 h after bacterial inoculation.

Effect of IFN-γ on the number of leukocytes in the blood of resistant C57Bl mice. Figure 3.2 shows that in uninfected mice the numbers of the total leukocytes fluctuated around 3700 per mm^3 blood. numbers of monocytes, granulocytes and lymphocytes fluctuated sround respectively 70, 200 and 3400 per mm^3 blood. Administration of $1.25x10^4$ U IFN-γ per mouse ($6.4x10^5$ U/kg), the lowest dose from the 2-fold dose range which was effective in *L. monocytogenes* infection, to uninfected mice at 24 h before zero time resulted in a 1.5-fold increased number of lymphocytes from 24 h after zero time. Numbers of monocytes and granulocytes remained stable with time.

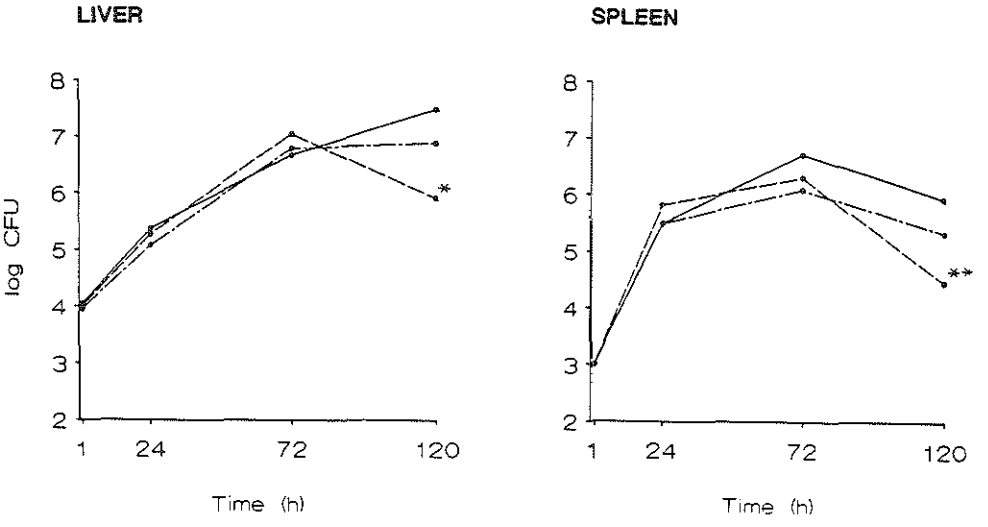

FIGURE 3.1 Effect of IFN-γ on the numbers of *L. monocytogenes* in liver and spleen of C57Bl mice. Mice infected with $2x10^4$ CFU *L. monocytogenes* at zero time were treated i.v. at 24 h before bacterial inoculation with $1.2x10^4$ U IFN-γ per mouse ($6.4x10^5$ U/kg) (— —), $3.2x10^3$ U IFN-γ ($1.2x10^5$ U/kg)(— - —), or PBS (———). Each point represents the geometric mean for six mice. If mice died a geometric mean was not calculated. Significance: $*p \leq 0.05$ $**p \leq 0.01$ versus PBS-treated mice.

In mice infected with *L. monocytogenes* at zero time the numbers of monocytes, granulocytes and lymphocytes were significantly decreased at 48 h after bacterial inoculation to 35% of the numbers at 24 h after inoculation followed by an increase. Pretreatment of infected mice with $1.25x10^4$ U IFN-γ per mouse ($6.4x10^5$ U/kg) at 24 h before bacterial inoculation resulted in 2-fold increased numbers of monocytes at 24 h after inoculation. Numbers of granulocytes and lymphocytes were not increased. At 48 h after bacterial inoculation numbers of monocytes, granulocytes and lymphocytes were significantly decreased to 60%, 75% and 60%, respectively, of the numbers at 24 h after inoculation. From 72 h, numbers of monocytes, granulocytes and lymphocytes increased again.

Effect of IFN-γ on the uptake and intracellular survival of L. monocytogenes in macrophages of C57Bl mice in monolayer. Table 3.2 shows that exposure of macrophages in monolayer to 1000 or 250 U IFN-γ/ml for 24 h before incubation with *L. monocytogenes* resulted in significant lower numbers of intracellular surviving bacteria at 4 h and 6 h after bacterial uptake. Compared to bacterial numbers directly after phagocytosis, intracellular growth was inhibited by IFN-γ. A concentration of 62 U IFN-γ/ml had no effect anymore on the number of bacteria at 6 h after bacterial uptake.

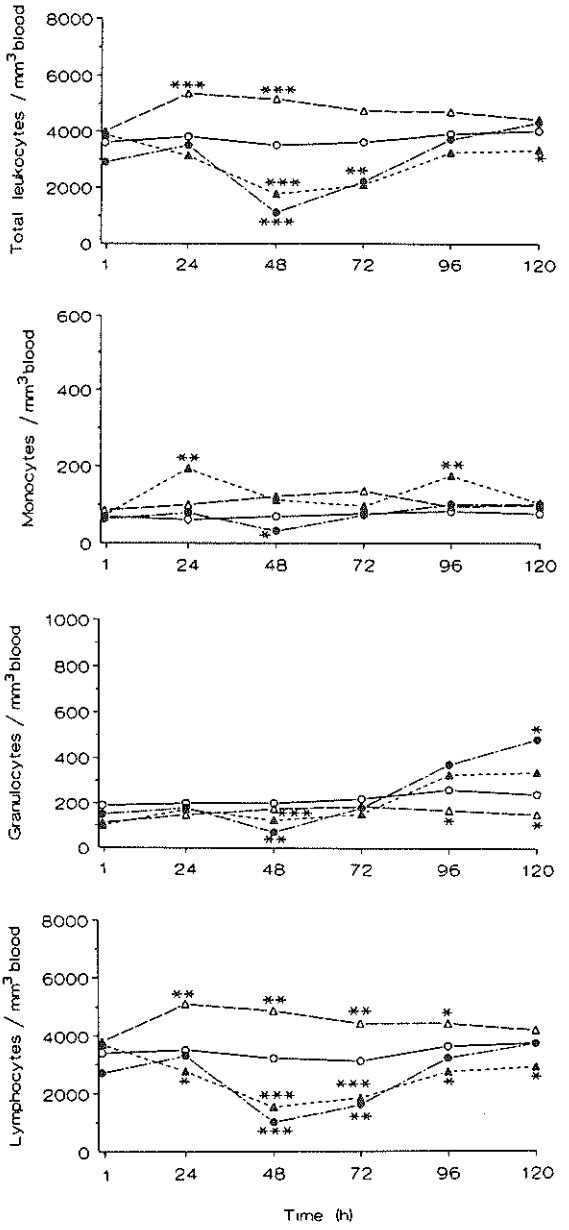

FIGURE 3.2 Effect of IFN-γ on the numbers of total leukocytes, monocytes, granulocytes and lymphocytes in blood of C57Bl mice. Mice infected with $2x10^4$ CFU *L. monocytogenes* at zero time were treated i.v. at 24 h before bacterial inoculation with $1.25x10^4$ U IFN-γ per mouse ($6.4x10^5$ U/kg) (▲----▲) or PBS (●——●). Uninfected mice were treated i.v. at 24 h before zero time with $1.25x10^4$ U IFN-γ per mouse ($6.4x10^5$ U/kg) (△— —△) or PBS (○——○). Each point represents the mean for six mice. Significance: *$p \leq 0.05$ **$p \leq 0.01$ ***$p \leq 0.001$ versus uninfected PBS-treated mice.

TABLE 3.1 Effect of IFN-γ treatment on survival of C57Bl mice and CBA mice from *L. monocytogenes* infection

Treatment[a] (U IFN-γ/kg)	Percentage survival[b]	
	C57Bl infected with $2x10^4$ CFU	CBA infected with $2x10^4$ CFU
$6.4x10^5$	100	100
$3.2x10^5$	100	ND
$2.0x10^4$	ND	100
$1.0x10^4$	ND	0
0	100	0

[a] Mice i.v. infected with $2x10^4$ or $4.2x10^3$ CFU *L. monocytogenes* were treated with a single dose MTPPE from a twofold increasing dose range at 24 h before inoculation.

[b] Survival was determined until 6 days after inoculation.

TABLE 3.2 Effect of IFN-γ on uptake and intracellular survival of *L. monocytogenes* in peritoneal macrophages of C57Bl mice in vitro[a]

Concentration of IFN-γ (U/ml)	log CFU *L. monocytogenes* per 10^6 macrophages[b] at times (h) after uptake of bacteria			
	0	1	4	6
1000	5.58±0.04	5.52±0.04	5.51±0.09**	6.03±0.03**
250	5.61±0.04	5.60±0.09	5.44±0.19**	6.13±0.05**
62	5.60±0.06	5.74±0.04	5.74±0.05**	6.21±0.16
15	5.55±0.03	5.85±0.04	5.91±0.06	6.26±0.18
0	5.67±0.08	5.84±0.11	5.96±0.03	6.52±0.11

[a] Before incubation with bacteria, macrophages in monolayer culture were exposed 24 h to various concentrations of IFN-γ.

[b] Data represent the geometric mean ± SEM for six observations (control) or four observations (IFN-γ). Significance: **$p \leq 0.01$ versus control.

Effect of IFN-γ on the course of L. monocytogenes infection in susceptible CBA mice. As shown in Figure 3.3 administration of $1.6x10^4$ U IFN-γ per mouse ($6.4x10^5$ U/kg) at 24 h before inoculation with $2x10^4$ CFU *L. monocytogenes* ($3.4xLD_{50}$) resulted in survival of all mice, whereas all control mice died at 96 h (Table 3.1). Only in dead mice bacteria were recovered from the blood. From a 2-fold dose range $5.0x10^2$ U per mouse ($2.0x10^4$ U/kg)

was the lowest dose that resulted in survival of all mice and in significant decreased numbers of bacteria in liver and spleen at 72 h after bacterial inoculation. A dose of $2.5x10^2$ U per mouse ($1.0x10^4$ U/kg) had no effect anymore on bacterial numbers in the liver and spleen compared to untreated control mice.

Effect of IFN-γ on the number of leukocytes in the blood of CBA mice. Figure 3.4 shows that in uninfected mice the numbers of total leukocytes fluctuated around 5400 per mm^3 blood. The numbers of monocytes, granulocytes and lymphocytes fluctuated around 200, 400 and 4800 per mm^3 blood, respectively. Administration of $5.0x10^2$ U IFN-γ per mouse ($2.0x10^4$ U/kg), the lowest dose from the 2-fold dose range which was effective in *L. monocytogenes* infection, to uninfected mice at 24 h before zero time resulted in 1.3 fold-decreased numbers of granulocytes and lymphocytes at 24 h after zero time. From 48 h after zero time numbers of monocytes, granulocytes and lymphocytes remained stable with time, except from an significantly higher number of lymphocytes at 72 h after zero time.

In mice infected with *L. monocytogenes* at zero time the numbers of granulocytes were 3.5-fold increased at 24 h 24 h after inoculation. Numbers of monocytes and lymphocytes were 2-fold and 3-fold decreased, respectively. From 48 h numbers of monocytes, granulo-

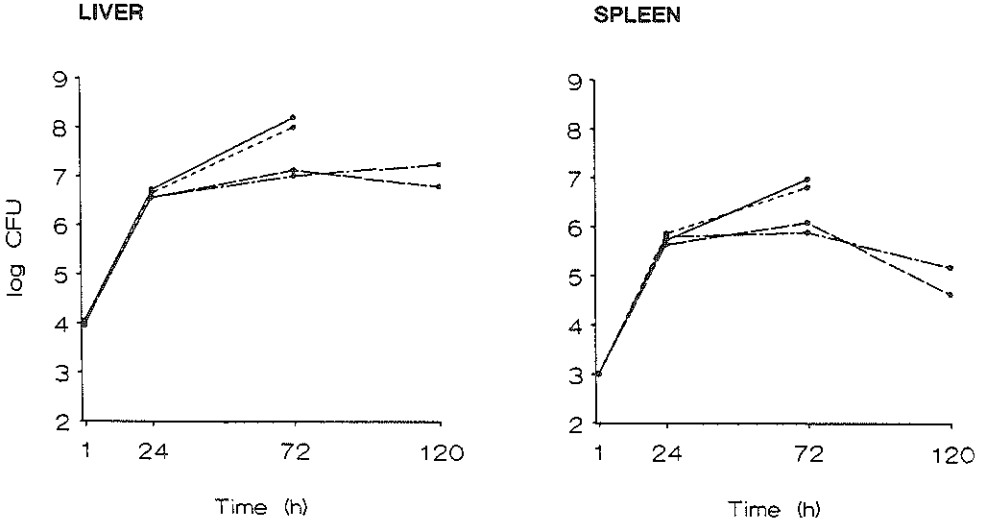

FIGURE 3.3 Effect of IFN-γ on the numbers of *L. monocytogenes* in liver and spleen of CBA mice. Mice infected with $2x10^4$ CFU *L. monocytogenes* at zero time were treated i.v. at 24 h before bacterial inoculation with $1.6x10^4$ U IFN-γ per mouse ($6.4x10^5$ U/kg) (— —), $5.0x10^2$ U IFN-γ per mouse ($2.0x10^4$ U/kg) (— - —), $2.5x10^2$ U IFN-γ per mouse ($1.0x10^4$ U/kg) (- - - -) or PBS (———). Each point represents the geometric mean for six mice. If mice died a geometric mean was not calculated. Significance: $*p \leq 0.05$ $**p \leq 0.01$ $***p \leq 0.001$ versus PBS-treated mice.

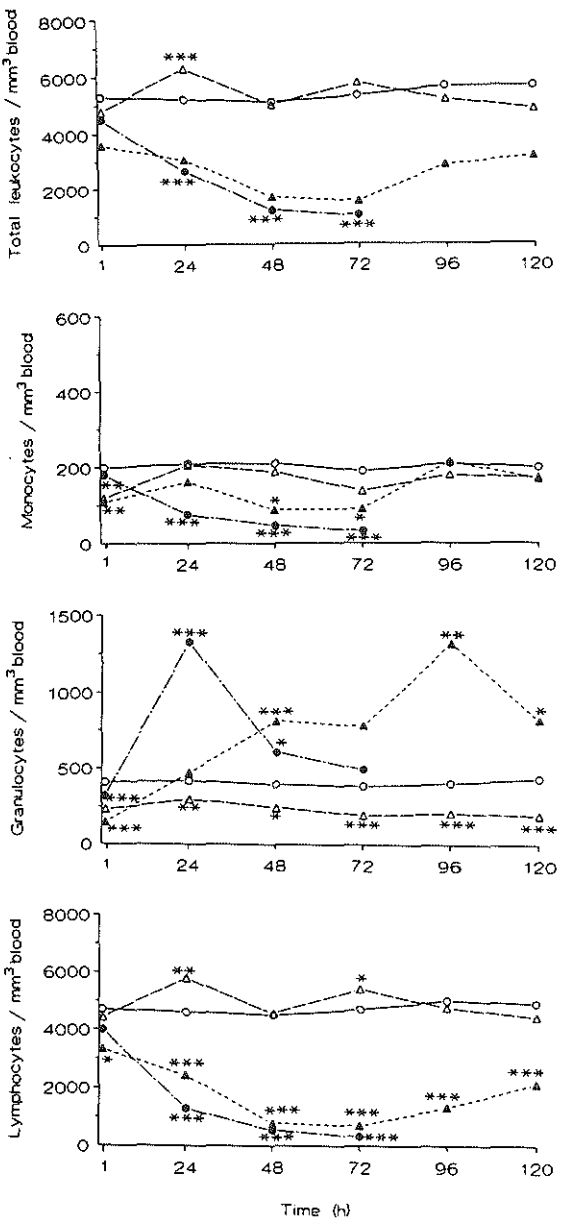

FIGURE 3.4 Effect of IFN-γ on the numbers of total leukocytes, monocytes, granulocytes and lymphocytes in blood of CBA mice. Mice infected with $2.0x10^4$ CFU *L. monocytogenes* at zero time were treated i.v. at 24 h before bacterial inoculation with $5.0x10^2$ U IFN-γ per mouse ($2.0x10^4$ U/kg) (▲---▲) or PBS (●---●). Uninfected mice were treated i.v. at 24 h before zero time with $5.0x10^2$ U IFN-γ per mouse ($2.0x10^4$ U /kg) (△— —△) or PBS (○——○). Each point represents the mean for six mice. If mice died a mean was not calculated. Significance: $*p \leq 0.05$ $**p \leq 0.01$ $***p \leq 0.001$ versus uninfected PBS-treated mice.

cytes and lymphocytes decreased further until all mice died. Pretreatment with $5.0x10^2$ U IFN-γ per mouse ($2.0x10^4$ U/kg) at 24 h before inoculation resulted in a 3-fold decrease in numbers of lymphocytes at 24 h after inoculation. Numbers of monocytes and granulocytes were not decreased. At 48 h after bacterial inoculation numbers of monocytes and lymphocytes were significantly decreased to 60% and 30%, respectively of the numbers at 24 h after inoculation. From 72 h numbers of monocytes granulocytes and lymphocytes increased.

Effect of IFN-γ on the uptake and intracellular survival of L. monocytogenes in macrophages of CBA mice in monolayer. Table 3.3 shows that exposure of macrophages in monolayer to 4-fold increasing concentrations of IFN-γ ranging from 1.5 up to 1000 U/ml for 24 h before incubation with *L. monocytogenes* resulted in significant lower numbers of intracellular surviving bacteria at 4 h and 6 h after bacterial uptake.

TABLE 3.3 Effect of IFN-γ on uptake and intracellular survival of *L. monocytogenes* in peritoneal macrophages of CBA mice in vitro[a]

Concentration of IFN-γ (U/ml)	log CFU *L. monocytogenes* per 10^6 macrophages[b] at times (h) after uptake of bacteria			
	0	1	4	6
1000	5.95±0.11	5.63±0.06	5.39±0.06*	5.73±0.09*
250	5.79±0.19	5.60±0.08	5.60±0.03*	5.84±0.04*
62	5.89±0.16	5.57±0.04	5.39±0.07*	5.88±0.02*
15	5.80±0.12	5.57±0.04	5.53±0.08	5.85±0.11*
0	5.73±0.07	5.80±0.09	6.02±0.03	6.51±0.06

[a] Before incubation with bacteria, macrophages in monolayer culture were exposed 24 h to various concentrations of IFN-γ.

[b] Data represent the geometric mean ± SEM for four observations (IFN-γ). Significance: *$p \leq 0.05$ versus control.

Discussion

In this study it was shown that administration of IFN-γ led to an increased resistance against *L. monocytogenes* infection in C57Bl mice as well as in CBA mice, mice which are genetically determined resistant or susceptible for *L. monocytogenes*, respectively. The protective effect appeared to be dependent on the dose of IFN-γ administered. These findings are in contrast to the effect of administration of the immunomodulator MTPPE which resulted in increased resistance in C57Bl mice and a decreased resistance in CBA mice (Chapter 2). These observations on IFN-γ are in agreement with those of other investigators who have also demonstrated an IFN-γ-induced increased resistance against *L. monocytogenes* in various resistant mouse strains (31,108,117). However, Van Dissel and others (203) did not find an

antibacterial effect of IFN-γ on *L. monocytogenes* infection in resistant C57Bl mice or susceptible CBA mice. Actually their results are not in discrepancy with our data since in their study the effect of administration of IFN-γ on numbers of bacteria in liver and spleen was only measured at 48 h after bacterial inoculation. In our study IFN-γ treatment also did not result in decreased numbers of bacteria in liver and spleen at 48 h after bacterial inoculation, however the effects were observed from 120 h after inoculation. Exposure of macrophages to IFN-γ *in vitro* resulted in stimulation of macrophages of both mouse strains to inhibit the intracellular growth of *L. monocytogenes* whereas MTPPE had no effect at all. Administration of IFN-γ to uninfected C57Bl mice only led to an increase in the number of lymphocytes, not in the number of monocytes and granulocytes. Administration of IFN-γ to uninfected CBA mice had no effect on the number of monocytes, granulocytes and lymphocytes except for a slight but significant lower number of granulocytes and a slight but significant higher number of lymphocytes at 48 h after administration. The efficacy of IFN-γ in terms of increasing the resistance against *L. monocytogenes* infection seems not to be correlated with the capacity to recruit leukocytes from the bone marrow. *In vitro* we observed a growth inhibition of *L. monocytogenes* in macrophages exposed to IFN-γ. These *in vivo* and *in vitro* data suggest that other cell types besides the macrophage are of minor importance for the IFN-γ-induced resistance against *L.monocytogenes* infection. This is in contrast to the MTPPE-induced resistance for which other cell types were shown to be of major importance.

Susceptible CBA mice seem to be more sensitive to IFN-γ treatment than resistant C57Bl mice *in vivo* as well as *in vitro*. *In vivo* in experimental infection in susceptible CBA mice the protective effect of IFN-γ was obtained at a 32-fold lower dose compared to resistant C57Bl mice. In *in vitro* infected macrophages of susceptible CBA mice the effect of IFN-γ was obtained at a 4-fold lower dose compared to resistant C57Bl mice. A possible explanation for this may be that macrophages of susceptible CBA mice are already in a primed state and therefore are able to respond more adequately on IFN-γ stimulation.

In summary, the protective effect in *L. monocytogenes* infection by IFN-γ is not dependent on the genetically determined resistance to *L. monocytogenes*. The effectiveness of IFN-γ is not correlated with the IFN-γ-induced capacity of mice to recruit leukocytes from the bone marrow. In addition IFN-γ is able to stimulate macrophages *in vitro* to inhibition of intracellular growth of *L. monocytogenes*.

CHAPTER 4

FREE VERSUS LIPOSOME-ENCAPSULATED MURAMYL TRIPEPTIDE PHOSPHATIDYLETHANOLAMIDE IN TREATMENT OF EXPERIMENTAL INFECTION DUE TO *KLEBSIELLA PNEUMONIAE*

Pernella M.B. Melissen, Wim van Vianen, Yvonne Rijsbergen, and Irma A.J.M. Bakker-Woudenberg.

Infection and Immunity 1992;60:95-101

Abstract

The effect of free and liposome-encapsulated muramyl tripeptide phosphatidylethanolamide (MTPPE) on the resistance to *Klebsiella pneumoniae* infection was investigated in mice. It was shown that administration of MTPPE, at 24 h before bacterial inoculation, led to a dose-dependent antibacterial resistance in terms of increased clearance of bacteria from the blood and bacterial killling in various organs. The lowest effective dose of MTPPE was 50 μg per mouse. Administration of liposome-encapsulated MTPPE was also effective at a dose of 25 μg per mouse. The time of administration of both free and liposome-encapsulated MTPPE, with respect to the appearance of bacteria in the blood, was very important and indicated that repeated administration is necessary to obtain protection for a prolonged period. In view of the toxicity of MTPPE it was an important observation that repeated administration of MTPPE in the liposome-encapsulated form also produced antibacterial resistance. Administration of free and liposome-encapsulated MTPPE resulted in increased numbers of granulocytes, monocytes and lymphocytes in the blood of uninfected mice and prevented leukopenia in infected mice.

Introduction

It is well-known from clinical experience, that antibiotic treatment of severe infections, particularly in immunocompromised patients, is not always successful. Several factors may contribute to the lack of success of antimicrobial treatment. One factor is the failure of the host defense to provide adequate support to antibiotic therapy. Administration of agents that stimulate the nonspecific antimicrobial resistance might prove beneficial in treatment of infections in the immunocompromised host (50,150,169). To this aim the immunomodulating agent muramyl tripeptide phosphatidyl-ethanolamide (MTPPE) may be useful.

MTPPE is a lipophilic derivative of muramyl dipeptide (MDP) which is the minimal active structure that can substitute for Mycobacteria in Freund's complete adjuvant (53). Muramyl dipeptide derivatives have many effects on macrophages *in vitro* such as morpholo-

gical and biochemical changes, production of monokines and enzymes and macrophage-mediated tumoricidal activity (2,29,120). It has also been demonstrated in animals, that muramyl dipeptide derivatives administered by various routes resulted in enhancement of the non-specific resistance to infections caused by a variety of organisms (20,158,166).

Since immunocompromised patients are prone to infections during a prolonged period of time, it is expected that repeated administration of immunomodulating agents is needed. However, repeated administration of MTPPE has been shown to result in toxic side effects (18,58,188). Encapsulation of MTPPE in liposomes may therefore be important since, in this form, a reduction in MTPPE toxicity has been found (58,188). The cells of the mononuclear phagocyte system (MPS) play a major role in the host defense system against infections, and targeting of macrophage modulators such as MTPPE by liposomal encapsulation can be effectively accomplished (35,124).

The aim of this study was to investigate the effect of MTPPE in the free form and liposome-encapsulated form on the clearance of *Klebsiella pneumoniae* from the blood after intravenous inoculation. In addition, the killing of bacteria in liver and spleen, organs rich in cells of the MPS, as well as bacterial killing in lung and kidney, organs containing fewer of the MPS, was investigated.

Materials and methods

Animals. Specific pathogen free, 11 to 13 weeks old female C57Bl/Ka mice were used (ITRI-TNO, Rijswijk, The Netherlands).

Reagents. N-acetyl-muramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-[1,2-dipalmitoyl-sn-glycero-3-(hydroxy-phosphoryloxy)]ethanolamide (MTPPE) was kindly provided by Ciba-Geigy (Basel,Switzerland). Liposomes containing MTPPE and placebo liposomes were a generous gift of Ciba-Geigy Ltd. (Basel, Switzerland). Liposomes were prepared from a dry lyophilisate composed of 250 mg phosphatidylcholine and phosphatidylserine in a molar ratio of 7:3 (with or without 1 mg MTPPE) and shaken with 2.5 ml of phosphate buffered saline (PBS) (CGP 19835A lipid). The average diameter of the constituted liposomes was between 2.0 and 3.5 μm and at least 80% of the liposomes were larger than 1.5 μm (205).

Bacteria. A strain of *K. pneumoniae* capsular serotype 2 (ATCC 43816) was used. Bacteria were grown for 16 h at 37°C in Todd-Hewitt broth (Oxoid Ltd, Basingstoke, England) and preserved on ice. Directly before the inoculation of mice the bacteria were washed three times in PBS.

Experimental infection caused by K. pneumoniae. Infections were induced by intravenous inoculation of 10^3 CFU *K. pneumoniae* into C57Bl/Ka mice. At different intervals after inoculation blood samples were taken from the retroorbital plexus and twofold diluted in 3.8% sodium citrate (BDH Chemicals Ltd, Poole, England). Serial tenfold dilutions were prepared and volumes of 0.2 ml of each dilution were spread on tryptone soya agar (TSA) plates (Oxoid Ltd, Basingstoke, England). Mice were then sacrificed and spleen, lungs, kidneys and liver were removed and homogenized in 20 ml of PBS for 30 s at 10,000 rpm in a VirTis homogenizer (The VirTis Co. Inc. Gardiner, New York). Serial 10-fold dilutions of homogenate in PBS were prepared. Volumes of 0.2 ml of each dilution and 2 ml volumes

of the undiluted homogenate, were spread on TSA plates. The remainder of the homogenate together with an equal volume of double concentrated TSA, was poured in plastic plates. All plates were incubated overnight at 26°C. The number of viable bacteria recovered from liver, spleen, lungs, kidneys and blood was used as a parameter for therapeutic efficacy.

Quantitation of blood leukocytes. Blood samples (0.8 ml) were taken from the retroorbital plexus and collected in polypropylene tubes containing 1 mg 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 numbers of lymphocytes, granulocytes and monocytes were calculated from the total number of leukocytes and the differential counts of 500 leukocytes in cytocentrifuge preparations of buffycoats obtained by centrifugation of blood samples for 30 min at 1500 x g in hematocrit tubes (Tamson B.V., Zoetermeer, The Netherlands).

Statistical analysis. Statistical evaluation of the differences in numbers of bacteria between MTPPE, liposome-encapsulated MTPPE-treated and PBS-treated groups of animals was performed by using the Mann-Whitney test. The Student-T test was used for the evaluation of differences in numbers of leukocytes between test groups (MTPPE-treated or liposome-encapsulated MTPPE-treated) and control (PBS-treated) groups in uninfected and infected animals. Differences in percentage survival were evaluated by using the Fisher-exact test.

Results

Effect of MTPPE on the course of K. pneumoniae infection. After i.v. inoculation of mice with 10^3 CFU *K. pneumoniae* most bacteria were trapped in the liver and spleen within 1 h. Thereafter, the number of bacteria in liver and blood increased until the mice died at 72 h after bacterial inoculation (Figure 4.1 and Table 4.1). Similar data were obtained in spleen, lung and kidney (data not shown). Administration of 50 μg MTPPE per mouse at 24 h before bacterial inoculation appeared to be the minimal dose, from a twofold dose range, that resulted in significantly decreased numbers of bacteria in the organs from 24 h until 96 h after bacterial inoculation and also the survival of all mice. In these mice, the blood was sterile from 48 h after bacterial inoculation. A twofold lower dose of 25 μg MTPPE per mouse also resulted in decreased numbers of bacteria in the organs at 24 h and at 48 h after bacterial inoculation however, only 50% of the mice survived.

Effect of time of administration of MTPPE on the course of K. pneumoniae infection. Administration of 50 μg MTPPE per mouse at 48 h before inoculation resulted in significantly decreased numbers of bacteria in liver and blood at 24 h and 48 h after bacterial inoculation (Figure 4.2). Thereafter, numbers of bacteria increased and all mice died (Table 4.1). Similar data were obtained in spleen, lung and kidney (data not shown). Administration of 50 μg MTPPE per mouse at 1 h after bacterial inoculation also resulted in a significant lower number of bacteria in the organs and blood at 48 h after inoculation and with 50% of the mice surviving. Administration of 50 μg MTPPE per mouse at 6 h after bacterial inoculation was not effective, the numbers of bacteria in the organs and blood being similar to those found in the untreated controls and all of the mice died.

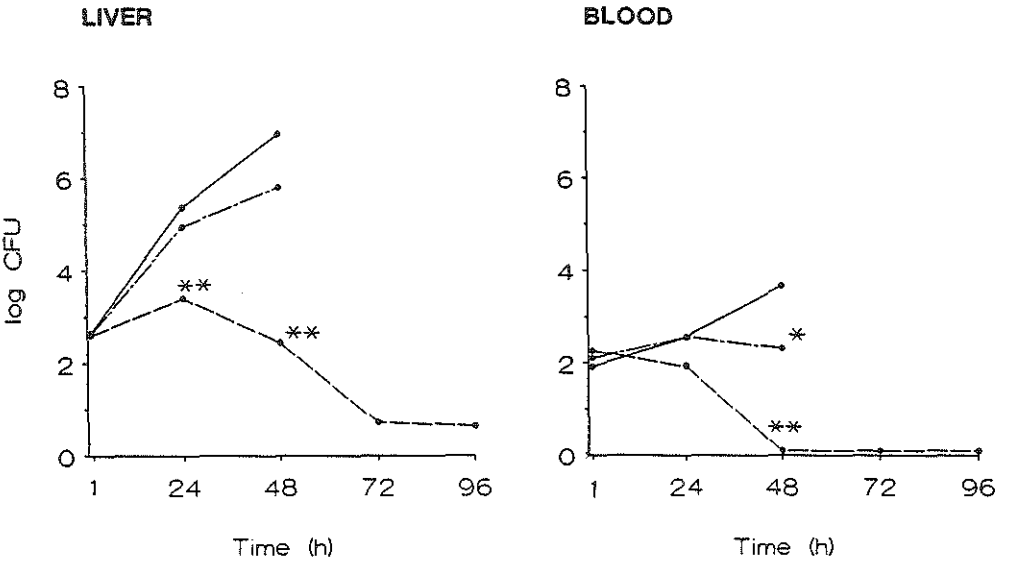

FIGURE 4.1 Effect of MTPPE on the numbers of *K. pneumoniae* in liver and blood of mice. Mice i.v. infected with 10^3 CFU *K. pneumoniae* at zero time were treated i.v. at 24 h before bacterial inoculation with 50 μg MTPPE per mouse (—· —), 25 μg MTPPE (— - —) or PBS (———). Each point represents the geometric mean for six mice. If mice died a geometric mean was not calculated. Significance:*$p \leq 0.05$ **$p \leq 0.01$ versus PBS-treated mice.

TABLE 4.1 Effect of time of administration of 50 μg MTPPE on survival of mice from *K. pneumoniae* infection[a]

	No. of surviving mice / no. of mice treated	
Time of administration	day 7	day 14
-48 h	0/6	0/6
-24 h	6/6	6/6
+1 h	3/6	3/6
+6 h	0/6	0/6
control	0/6	0/6

[a] Mice i.v. infected with 10^3 CFU *K. pneumoniae* at zero time were treated i.v. with 50 μg MTPPE at several times before (-) or after (+) inoculation.

Effect of liposome-encapsulated MTPPE on the course of K. pneumoniae infection. Figure 4.3 shows that administration of 25 μg liposome-encapsulated MTPPE per mouse, at 24 h before bacterial inoculation, appeared to be the minimal dose, from a twofold dose range, that resulted in significantly decreased numbers of bacteria in liver from 24 h until 96 h after bacterial inoculation. Similar data were obtained for spleen, kidney and lung (data not shown). Eventually all mice survived (Table 4.2). The blood was sterile from 72 h after bacterial inoculation. A twofold lower dose of 12.5 μg liposome-encapsulated MTPPE per mouse also resulted in decreased numbers of bacteria in the organs at 24 h and 48 h after bacterial inoculation however, only 17% of the mice survived. A dose of 6.25 mg placebo liposomes (which is equivalent to the amount of lipid in which 25 μg of MTPPE is encapsulated) had no effect on the course of the *K. pneumoniae* infection.

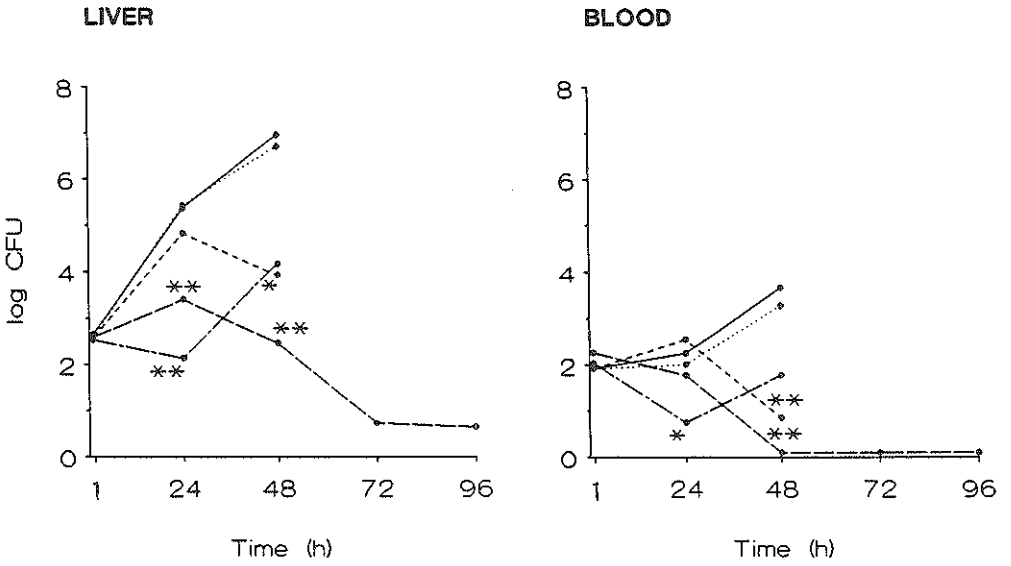

FIGURE 4.2 Effect of time of administration of MTPPE on the numbers of *K. pneumoniae* in liver and blood of mice. Mice i.v. infected with 10^3 CFU *K. pneumoniae* at zero time were treated i.v. with 50 μg MTPPE per mouse at 48 h before (— - —) or at 24 h before bacterial inoculation (— —), or at 1 h after (- - - - -) or at 6 h after inoculation (· · · · · ·), or with PBS (———) at 24 h before inoculation. Each point represents the geometric mean for six mice. If mice died a geometric mean was not calculated. Significance: *$p \leq 0.05$ **$p \leq 0.01$ versus PBS-treated mice.

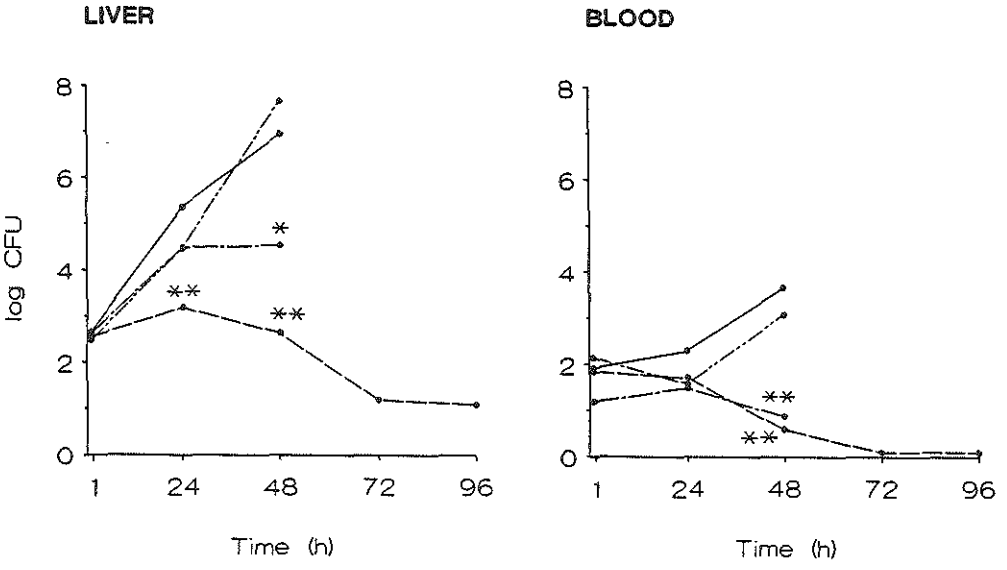

FIGURE 4.3 Effect of liposome-encapsulated MTPPE on the numbers of *K. pneumoniae* in liver and blood of mice. Mice i.v. infected with 10^3 CFU *K. pneumoniae* at zero time were treated i.v. at 24 h before bacterial inoculation with 25 μg liposome-encapsulated MTPPE per mouse (— —), 12.5 μg liposome-encapsulated MTPPE (— · —), placebo liposomes (— -- —) or PBS (———). Each point represents the geometric mean for six mice. If mice died a geometric mean was not calculated. Significance: *$p \leq 0.05$ **$p \leq 0.01$ versus PBS-treated mice.

TABLE 4.2 Effect of time of administration of 25 μg liposome-encapsulated MTPPE on survival of mice from *K. pneumoniae* infection[a]

	No. of surviving mice / no. of mice treated	
Time of administration	day 7	day 14
-48 h	4/6	2/6
-24 h	6/6	6/6
+1 h	0/6	0/6
+6 h	0/6	0/6
control	0/6	0/6

[a] Mice i.v. infected with 10^3 CFU *K. pneumoniae* at zero time were treated i.v. with 25 μg liposome-encapsulated MTPPE at several times before (-) or after (+) inoculation.

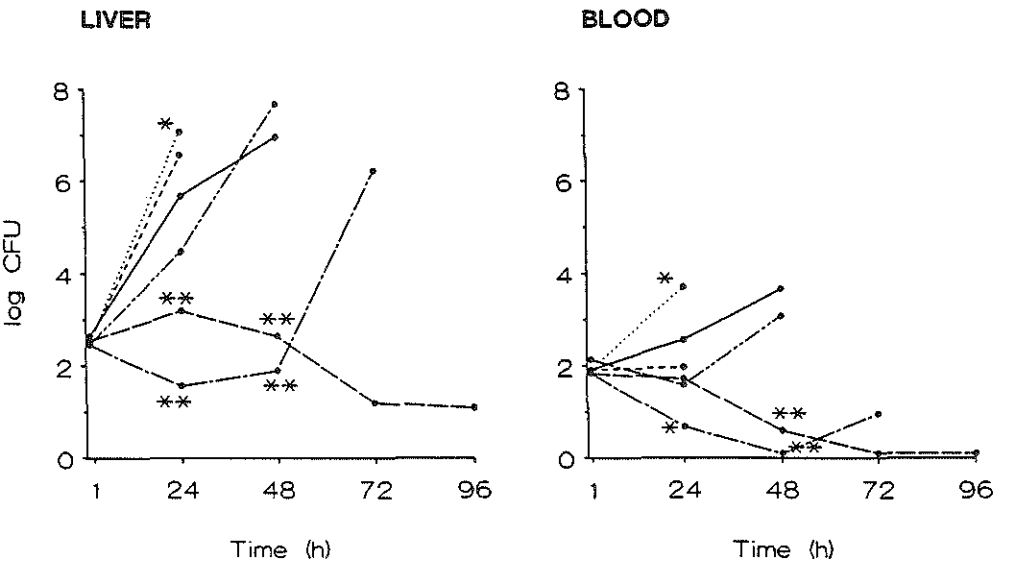

FIGURE 4.4 Effect of time of administration of liposome-encapsulated MTPPE on the numbers of *K. pneumoniae* in liver and blood of mice. Mice i.v. infected with 10^3 CFU *K. pneumoniae* at zero time were treated i.v. with 25 μg liposome-encapsulated MTPPE per mouse at 48 h before (— - —) or at 24 h before bacterial inoculation (—— ——), or at 1 h after (- - - - -) or at 6 h after inoculation (·····), or with placebo liposomes (—— - - —) or with PBS (———) at 24 h before inoculation. Each point represents the geometric mean for six mice. If mice died a geometric mean was not calculated. Significance: *$p \leq 0.05$ **$p \leq 0.01$ versus PBS-treated mice.

Effect of time of administration of liposome-encapsulated MTPPE on the course of K. pneumoniae infection. When 25 μg liposome-encapsulated MTPPE per mouse was administered at 48 h before inoculation, complete elimination of the bacteria from the organs was not found, and only 33% of the mice survived (Table 4.2 and Fig. 4.4). Administration of 25 μg liposome-encapsulated MTPPE per mouse at 1 h or at 6 h after bacterial inoculation was not effective. The numbers of bacteria in the organs and blood were similar to or higher than those found in the untreated controls and all of the mice died.

Effect of repeated administration of liposome-encapsulated MTPPE. Table 4.3 shows that all mice survived when a single dose of liposome-encapsulated MTPPE was administered at 24 h before bacterial inoculation. However, administration at 48 h before inoculation resulted in the survival of only 40% of the mice, but was increased to 80% when a second dose of liposome-encapsulated MTPPE was given at 1 h or 24 h after inoculation (ie. 48 h or 72 h after the first dose). When the second dose was given at 48 h after inoculation (ie.

96 h after the first dose) the survival rate was not increased. A single dose of 25 μg liposome-encapsulated MTPPE administered at 1 h or at 24 h after inoculation had no significant therapeutic effect.

Effect of MTPPE and liposome-encapsulated MTPPE on the number of leukocytes in the blood. Figure 4.5 shows that, in uninfected mice, the number of granulocytes, monocytes and lymphocytes was around 200, 70, and 3400 per mm^3 blood, respectively. Administration of 50 μg MTPPE or 25 μg liposome-encapsulated MTPPE to uninfected mice at 24 h before zero time resulted in a 2.0-fold, 2.3-fold and a 1.5-fold increase in the number of granulocytes, monocytes and lymphocytes, respectively, compared to the numbers in uninfected PBS-treated mice. The rise in granulocytes and lymphocytes due to liposome-encapsulated MTPPE was 24 h later compared to that due to MTPPE in the free form. Administration of placebo liposomes had no effect on the number of leukocytes.

Figure 4.6 shows that, in mice infected with *K. pneumoniae* at zero time, the numbers of granulocytes, monocytes and lymphocytes in the blood were decreased to 40% of those in uninfected mice at 48 h after zero time. In infected mice treated with 50 μg MTPPE or 25 μg liposome-encapsulated MTPPE at 24 h before bacterial inoculation the number of leukocytes in the blood did not change with time. Only the numbers of monocytes increased 10-fold compared to those in infected PBS-treated mice. The numbers of granulocytes and lymphocytes remained stable throughout, except for a significant decrease at 1 h after bacterial inoculation. Pretreatment with placebo liposomes had no effect on the numbers of monocytes, granulocytes and lymphocytes compared to the numbers in infected PBS-treated mice.

TABLE 4.3 Effect of repeated administration of 25 μg liposome-encapsulated MTPPE on survival of mice from *K. pneumoniae* infection[a]

	No. of surviving mice / no. of mice treated	
Time of administration	day 7	day 14
-24 h	10/10	10/10 ***
-48 h	6/10	4/10 *
-48 h, +1 h	8/10	6/10 **
-48 h, +24 h	8/10	8/10 ***
-48 h, +48 h	4/10	4/10 *
+1 h	0/10	0/10
+24 h	4/10	2/10
control	0/10	0/10

[a] Mice i.v. infected with 10^3 CFU *K. pneumoniae* at zero time were treated i.v. with 25 μg liposome-encapsulated MTPPE at several times before (-) and after (+) inoculation. Significance: *$p \leq 0.05$ **$p \leq 0.01$ ***$p \leq 0.001$ versus control mice.

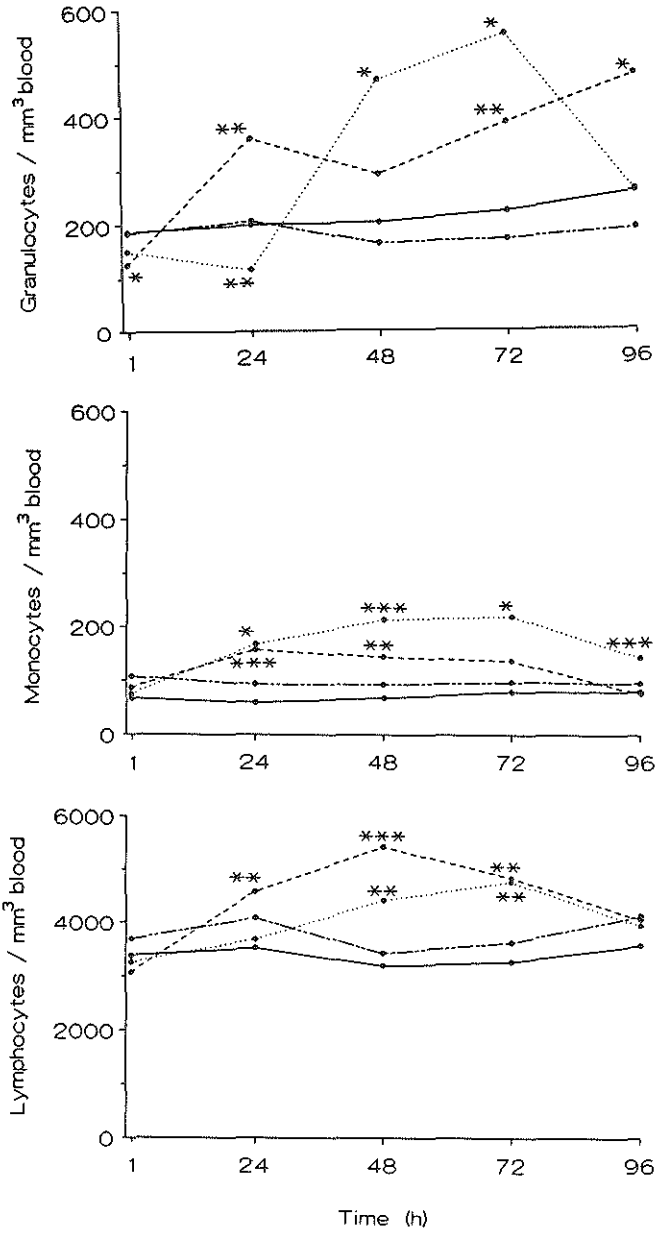

FIGURE 4.5 Effect of MTPPE or liposome-encapsulated MTPPE on the numbers of leukocytes in blood of uninfected mice. Mice were treated i.v. at 24 h before zero time with 50 μg MTPPE per mouse (- - - - -), 25 μg liposome-encapsulated MTPPE (· · · · ·), placebo liposomes (— - - —) or PBS (———). Each point represents the mean for six mice. Significance: *$p \leq 0.05$ **$p \leq 0.01$ ***$p \leq 0.001$ versus uninfected PBS-treated mice.

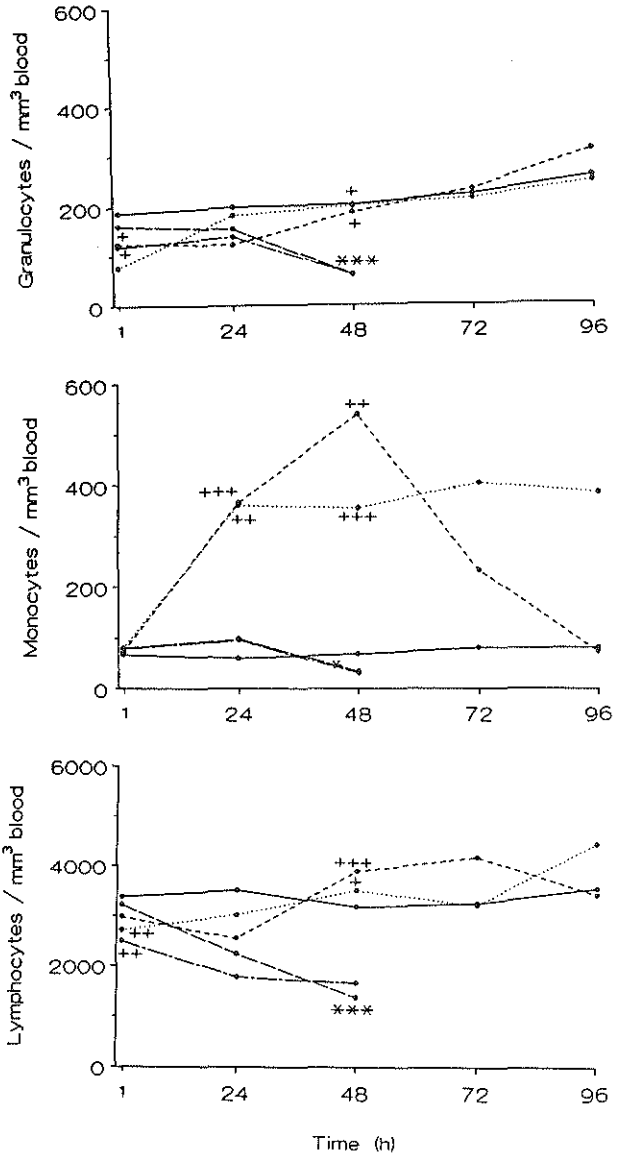

FIGURE 4.6 Effect of MTPPE or liposome-encapsulated MTPPE on the numbers of leukocytes in blood of infected mice. Mice i.v. infected with 10^3 CFU *K. pneumoniae* at zero time were treated i.v. at 24 h before bacterial inoculation with 50 μg MTPPE per mouse (- - - - -), 25 μg liposome-encapsulated MTPPE (·····), placebo liposomes (— - —) or PBS (— —). Uninfected mice were treated i.v. at 24 h before zero time with PBS (————). Each point represents the mean for six mice. If mice died a mean was not calculated. Significance: *$p \leq 0.05$ **$p \leq 0.01$ ***$p \leq 0.001$ versus uninfected PBS-treated mice or +$p \leq 0.05$, ++$p \leq 0.01$, +++$p \leq 0.001$ versus infected PBS-treated mice.

Discussion

In this study, it was shown that administration of MTPPE led to an increased resistance against *K. pneumoniae* infection in mice in terms of clearance of bacteria from the blood and bacterial killing in various organs. This protective effect appeared to be dependent on the dose of MTPPE. An increased resistance against experimental infection with *K. pneumoniae* in mice due to administration of MDP or -derivatives was also observed by other investigators using either systemic infection or wound infection models with the survival of animals (29,75,165,177), or the decrease in bacterial numbers in the infected organs (3,75,178), used as parameters for therapeutic efficacy. In addition, our study indicated that the protective effect of MTPPE was dependent on the time of administration of MTPPE with regards to the appearance of bacteria in the blood. Since the immunocompromised host is prone to infections during a prolonged period of time, repeated administration of immunomodulating agents is needed. It has been shown that repeated administration of MTPPE has toxic side effects (18,58,188), the most prominent of which being vascular lesions due to cellular accumulation. These lesions often result in necrotic foci in several organs of rabbits, dogs and guinea pigs (18,58,188). Mice, however, could tolerate high doses of MTPPE (58). Schumann et al. (188) showed that the highest non-toxic dose of liposome-encapsulated MTPPE was 10-fold higher as the non-toxic dose of MTPPE in the free form. In addition Fidler et al. (58) found that, due to liposomal encapsulation, the same dose of MTPPE was not toxic anymore.

This study shows that administration of MTPPE in the liposome-encapsulated form also led to antibacterial resistance, even at a 2-fold lower dose than MTPPE in the free form. This 2-fold increased therapeutic efficacy is probably not related to an increased level of liposome-encapsulated MTPPE in the the main clearing organ, the liver, because Fogler et al. (66) showed that liposomal encapsulation did not result in increased distribution of MTPPE in the liver of mice. However, liposomal encapsulation did result in increased levels of MTPPE in spleen and lungs (68). An improved therapeutic efficacy due to liposomal encapsulation of MTPPE was also observed in experimental infections in mice caused by herpes simplex virus (112), influenza virus (76), Rift Valley fever virus and Friend leukemia virus (51). Izbicki et al. (97) also found a protective effect of liposome-encapsulated MTPPE against *Streptococcus pneumoniae* infection in pigs, but the effect of free MTPPE was not investigated. For several hydrophilic MDP-derivatives, it was demonstrated that liposomal encapsulation led to a 20- to 1000-fold increase in efficacy in experimental infections or in monolayers of infected macrophages (7,54,68) as well as in *in vivo* and *in vitro* models of tumorcytotoxicity (41,172).

The protective effect of liposome-encapsulated MTPPE observed in our study was also dependent on the time of administration with regards to the appearance of bacteria in the blood. Administration of liposome-encapsulated MTPPE, as well as MTPPE in the free form, at 24 h before bacterial inoculation appeared to be most effective. This is in agreement with the findings of Ausobsky et al. (3) who demonstrated that, administration of muramyl dipeptide (MDP) at 24 h before inoculation with *K. pneumoniae* was more effective than administration at 2 h, at 4 h or at 6 h after inoculation. Otani et al. (158) also found that the highest efficacy of MDP, as well as the lipophilic MDP-derivatives L18-MDP and MDP-Lys(L18), was achieved when administered at 24 h before bacterial inoculation.

Administration of these agents at the time of bacterial inoculation had no effect anymore. In our study, administration of MTPPE at 1 h after bacterial inoculation was effective for only part of the animals: 50% of the mice survived. Administration of liposome-encapsulated MTPPE at 1 h after bacterial inoculation was not effective. The dose of free MTPPE that was effective when administered at 24 h before inoculation, was not effective when given at 48 h before bacterial inoculation. However, administration of liposome-encapsulated MTPPE at 48 h before inoculation was still effective for part of the animals, 33% of the mice surviving. Probably MTPPE is slowly released from the liposomes and some time is needed to appropriately stimulate macrophages. Fogler and others (66) showed that when, encapsulated in the same liposomes as were used in our study and i.v. administered to mice, MTPPE did not leak out of the liposomes before 4 h after administration and at 24 h after injection only part of MTPPE was released from the liposomes.

Repeated administration of liposome-encapsulated MTPPE in our *K. pneumoniae* infection model seems to be only beneficial when the second dose is administered not later than 72 h after the first dose (given 48 h before bacterial inoculation). When the second dose of liposome-encapsulated MTPPE was administered 96 h after the first dose there was no increase in the survival rate. This was not due to high numbers of bacteria in the organs and blood at the time of administration of the second dose but was probably due to the time needed for MTPPE to be released from the liposomes and stimulate macrophages properly.

Our study shows that administration of MTPPE in the free, or liposome-encapsulated form, led to significantly increased numbers of granulocytes, monocytes and lymphocytes in the blood. Only at 1 h and 24 h after zero time were the numbers of granulocytes significantly decreased. Wachsmuth (213) also found that administration of free MTPPE to rabbits resulted in an initial decrease followed by an increase in the numbers of granulocytes and lymphocytes in the blood. Yamaguchi et al. (218) also showed this effect in the numbers of granulocytes, monocytes and lymphocytes at 24 h after administration of MDP-Lys(L18) to mice. The increased numbers of granuluocytes, monocytes and lymphocytes are probably due to increased levels of colony stimulating factor (CSF), a lymphokine which enhances the proliferation and differentiation of cells in the bone marrow (34). This correlation was confirmed by Yamaguchi et al. (218) who found that administration of the lipophilic MDP-derivative MDP-Lys(L18) to mice resulted in increased levels of CSF and increased numbers of leukocytes in the blood. The rise in numbers of leukocytes due to administration of liposome-encapsulated MTPPE in our study was later than that observed with MTPPE in the free form. This also indicates that it takes time for MTPPE to be released from the liposomes.

In PBS-treated infected mice a decrease of granulocytes, monocytes and lymphocytes in the blood was observed, which was probably due to migration of these cells to the infected organs. In infected mice treated with liposome-encapsulated MTPPE or MTPPE in the free form, the numbers of granulocytes and lymphocytes were not changed, which probably resulted from the increase in numbers due to treatment with MTPPE counteracting the decrease in numbers due to migration from the blood to the infected organs. The reason for the 10-fold increase in numbers of monocytes found in infected mice treated with free MTPPE remains obscure.

In summary, antibacterial resistance against *K. pneumoniae* infection in terms of clearance of bacteria from the blood as well as bacterial killing in various organs is achieved

by administration of free MTPPE. The time of administration in relation to the time of appearance of bacteria in the blood is very important indicating that repeated administration is necessary to obtain protection for a prolonged period. In view of the reported toxicity of free MTPPE it is an important observation that repeated administration of lower doses of MTPPE in the liposome-encapsulated form also leads to antibacterial resistance. Administration of MTPPE in the free form or in the liposome-encapsulated form results in increased numbers of granulocytes, monocytes and lymphocytes in the blood. Future studies will elucidate the factors contributing to the MTPPE-induced antibacterial resistance, such as the increased numbers of leukocytes recruited from the bone marrow and the stimulation of resident macrophages in liver and spleen.

CHAPTER 5

ROLES OF PERIPHERAL LEUKOCYTES AND TISSUE MACROPHAGES IN ANTIBACTERIAL RESISTANCE INDUCED BY FREE OR LIPOSOME-ENCAPSULATED MURAMYL TRIPEPTIDE PHOSPHATIDYLETHANOLAMIDE

Pernella M.B. Melissen, Wim van Vianen, and Irma A.J.M. Bakker-Woudenberg.

Infection and Immunity 1992;60:4891-7.

Abstract

Administration of free muramyl tripeptide phosphatidylethanolamide (MTPPE) or liposome-encapsulated MTPPE (LE-MTPPE) in a twofold lower dose at 24 h before bacterial inoculation resulted in clearance of intravenously inoculated *Klebsiella pneumoniae* by tissue macrophages, whereas in control mice bacteria were not effectively cleared from the blood. In addition, MTPPE and LE-MTPPE led to increased numbers of leukocytes in the blood, which could compensate for the leukopenia in mice resulting from infection with *K. pneumoniae*. In an attempt to elucidate the relative contribution of the activation of tissue macrophages and the recruitment of leukocytes to the antibacterial resistance induced by MTPPE and LE-MTPPE, mice were infected intraperitoneally with *K.pneumoniae*. In these MTPPE- and LE-MTPPE treated mice, intraperitoneal influx of leukocytes and phagocytic capacity of leukocytes were not higher than in untreated control mice. However, MTPPE- and LE-MTPPE-treated mice survived much longer; eventually 33% of the LE-MTPPE-treated mice survived, whereas all untreated control mice died as a result of bacterial septicemia. This prevention of early death appeared to be the result of an increased clearance of bacteria from the blood by activated tissue macrophages. It was observed that depletion of these tissue macrophages in liver and spleen abrogates the effect of LE-MTPPE-treatment, indicating that tissue macrophages are of major importance in the by LE-MTPPE induced resistance against *K. pneumoniae* infection.

Introduction

Antibiotic therapy of infections, especially in the immunocompromised host, is not always effective. The failure of the host immune system to give adequate support to antibiotic treatment may contribute to this lack of success (150,169). In this respect it would be of great value to stimulate the non-specific antimicrobial defense system of the host (50,169). Stimulation of the non-specific antimicrobial defenses, in particular the cells of the mononuclear phagocyte system, can be achieved with various compounds including

interferons, colony stimulating factors, bacterial lipopolysaccharides and muramylpeptides. Muramyl peptides have been found to be highly active in vitro and in vivo, in terms of inducing tumoricidal (172,174,181,196), antiviral (46,97,128,129) and bactericidal (97,136) activities. Encapsulation of muramyl peptides in liposomes led to improved therapeutic efficacy in in vitro and in vivo models of tumorcytotoxicity (41,172,196) as well as in experimental microbial infections in mice (51,76,112,136). The precise mechanisms by which muramylpeptides exert their protective effect remain incompletely understood. It is shown that muramyl peptides have many effects on macrophages in terms of several morphologic and biochemical changes, enzyme production and monokine production (19,96,120,134). In addition, it is shown that administration of several muramylpeptides, among which muramyl tripeptide phosphatidylethanolamide {N-acetyl-muramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-[1,2-dipalmitoyl-*sn*-glycero-3-(hydroxy-phosphoryloxy)]ethanolamide (MTPPE)}, resulted in increased numbers of leukocytes in the blood of uninfected mice (136,213,218) which could compensate for the leukopenia in infected mice (97,136).

The aim of this study was to elucidate the relative contribution of the recruitment of blood leukocytes and the activation of tissue macrophages in the antibacterial resistance against *Klebsiella pneumoniae*, effects induced by free MTPPE and liposome-encapsulated MTPPE (LE-MTPPE). This question was investigated in mice which were intraperitoneally inoculated with *K. pneumoniae* after treatment with MTPPE or LE-MTPPE. In these infected mice, it was possible to discriminate between the roles of recruited blood leukocytes and activated tissue macrophages, as numbers and activity of recruited leukocytes now could be quantitated.

Materials and methods

Animals. Specific pathogen free, 11- to 13-weeks old female C57BL/Ka mice were used (ITRI-TNO, Rijswijk, The Netherlands).

Reagents. MTPPE was kindly provided by Ciba-Geigy Ltd. (Basel, Switzerland). Liposomes were prepared from a dry lyophilisate composed of 250 mg phosphatidylcholine and phosphatidylserine in a molar ratio of 7:3 with or without 1 mg MTPPE by shaking with 2.5 ml of phosphate buffered saline (PBS) (CGP 19835A lipid). All MTPPE was encapsulated in the liposomes (205). The average diameter of the constituted liposomes was between 2.0 and 3.5 μm and at least 80% of the liposomes were larger than 1.5 μm (205). Liposome-encapsulated dichloromethylene diphosphonate (LE-DMP) was a kind gift of Dr N. van Rooijen (Free University, Amsterdam, The Netherlands).

Bacteria. A strain of *K. pneumoniae* capsular serotype 2 (ATCC 43816) was used. Bacteria were grown for 16 h at 37°C in Todd-Hewitt broth (Oxoid Ltd, Basingstoke, England) and preserved on ice. Directly before the inoculation of mice the bacteria were washed three times with PBS.

Experimental infections caused by K. pneumoniae. Intravenous (i.v.) infections were induced by i.v. inoculation of 10^4 CFU *K. pneumoniae* into C57BL/Ka mice. At different intervals after inoculation, blood samples were taken from the retroorbital plexus and collected in polypropylene tubes containing 1 mg dried EDTA (BDH Chemical Ltd, Poole,

England). Serial tenfold dilutions were prepared and volumes of 0.2 ml of each dilution were spread on tryptone soya agar (TSA) plates (Oxoid Ltd, Basingstoke, England). Then groups of six mice per time point were sacrificed. Spleen, lung, kidney and liver were removed and each was homogenized in 20 ml of PBS for 30 s at 10,000 rpm in a VirTis homogenizer (The VirTis Co Inc Gardiner, New York). Serial 10-fold dilutions of homogenate in PBS were prepared. Volumes of 0.2 ml of each dilution and 2 ml volumes of the undiluted homogenate were spread on TSA plates. The remainder of the homogenate together with an equal volume of 2 times concentrated TSA was poured in plastic plates. All plates were incubated at 26°C. The number of viable bacteria recovered from liver, spleen, lung, kidney and blood was used as a parameter for therapeutic efficacy.

Intraperitoneal (i.p.) infections were induced by i.p. inoculation of 10^3 CFU *K. pneumoniae* into C57Bl/Ka mice. At different intervals after inoculation blood samples were taken from the retroorbital plexus and collected in polypropylene tubes containing 1 mg dried EDTA. Serial tenfold dilutions in PBS were prepared and volumes of 0.2 ml of each dilution were spread on TSA plates. Then groups of six mice per time point were sacrificed and 4 ml of peritoneal cell suspension was collected after i.p. injection of 5 ml PBS containing 10 U heparin (Organon Teknika B.V., Boxtel, The Netherlands) per ml. To determine the total numbers of peritoneal bacteria, cells were lysed by 10-fold dilution in distilled water, followed by 1 min vigorously mixing. Serial 10-fold dilutions of peritoneal cell suspension in PBS were prepared. Volumes of 0.2 ml of each dilution was spread on TSA plates. In order to quantitate only the intracellular bacteria, peritoneal cells were washed 3 times with PBS (10 min at 110 x g). Thereafter cells were lysed and 10-fold dilutions in PBS were spread on TSA plates, as described above.

Treatment of mice. Mice were treated i.v. with 50 μg MTPPE in the free form or 25 μg liposome-encapsulated MTPPE (LE-MTPPE) at 24 h before i.v. or i.p. bacterial inoculation. These doses appeared most effective in an earlier study in which different doses of free and LE-MTPPE were compared in the model of i.v. infection with *K. pneumoniae* (136).

Quantitation of leukocytes in blood and peritoneal cell suspension. For total leukocyte counts blood or peritoneal cell suspension from groups of six mice per time point was diluted 1:10 with Türk solution (0.1% crystal violet in 1% acetic acid) and numbers of leukocytes were determined in duplicate in a Bürkers hemocytometer. The numbers of lymphocytes, granulocytes and monocytes were calculated from the total number of leukocytes and differential counts of 500 leukocytes in cytocentrifuge preparations of buffycoats obtained by centrifugation of blood samples for 30 min at 1500 x g in hematocrit tubes (Tamson B.V., Zoetermeer, The Netherlands).

Elimination of spleen and liver macrophages. Intravenous injection of liposome-encapsulated dichloromethylene diphosphonate (LE-DMP) resulted in depletion of macrophages in spleen and liver of mice within 1 day after treatment as described by Van Rooijen and others (209) From 1 week after injection macrophages reappeared in the liver and spleen (210,211). This is confirmed for C57Bl/Ka mice used in this study. In brief, cryostat sections of liver and spleen were made at 1 day, 3 days, 5 days, 7 days, and 14 days after i.v. injection of LE-DMP. These sections were stained with monoclonal rat anti-mouse antibodies directed to different subsets of macrophages which were kindly provided by Dr P.L. Leenen

(Department of Cell Biology II, Erasmus University Rotterdam, The Netherlands).

Statistical analysis. Statistical evaluation of differences in numbers of bacteria as well as numbers of peritoneal leukocytes between MTPPE-treated, LE-MTPPE-treated, PBS-treated, and empty liposome-treated groups of animals was performed by using the Mann-Whitney test. Analysis of variance followed by Bonferroni t-test was used for evaluation of differences in the numbers of leukocytes between MTPPE-treated, LE-MTPPE-treated, PBS-treated and empty liposome-treated groups of uninfected and infected animals.

Results

Effects of MTPPE and LE-MTPPE on the course of K. pneumoniae infection. Within 30 min after i.v. inoculation of *K. pneumoniae*, 80% of the bacteria were trapped in liver and spleen; thereafter, bacterial numbers in liver, spleen, kidney and lung increased until mice died at 72 h after bacterial inoculation (Figure 5.1). Although initially 80% of the bacteria were cleared from the blood, they were not effectively cleared and bacterial numbers increased resulting in death of mice. From a twofold dose range, 50 μg of MTPPE or 25 μg of LE-MTPPE administered at 24 h before bacterial inoculation, was the minimal effective dose resulting in significantly lower bacterial numbers in the organs from 8 h after inoculation, compared with the numbers in control mice and all mice survived (135). Treatment of mice with MTPPE or LE-MTPPE also led to significant lower numbers of bacteria in the blood from 8 h after bacterial inoculation, compared with numbers in control mice. Empty liposomes had no effect on the course of *K. pneumoniae* infection in the organs and in the blood.

Effects of MTPPE and LE-MTPPE on the number of leukocytes in the blood. Table 5.1 shows that administration of 50 μg of MTPPE or 25 μg of LE-MTPPE at 24 h before bacterial inoculation resulted in 1.5-fold-increased numbers of leukocytes in the blood of uninfected mice from 24 h (MTPPE) or 48 h (LE-MTPPE) after zero time. In mice i.v. infected with *K. pneumoniae*, treatment with MTPPE or LE-MTPPE led to a slight increase in blood leukocyte numbers, whereas in PBS- or empty liposome-treated mice the numbers decreased to 40% of the numbers in uninfected mice at 48 h after bacterial inoculation.

Effects of MTPPE and LE-MTPPE on numbers of peritoneal leukocytes and bacteria after an i.p. challenge with K. pneumoniae. Figure 5.2 shows that after i.p. inoculation of *K. pneumoniae,* numbers of leukocytes in the peritoneal cavity sharply increased within 24 h. Between 24 and 48 h after bacterial inoculation, numbers of peritoneal leukocytes remained unchanged. Administration of MTPPE and LE-MTPPE at 24 h before bacterial inoculation had no effect on the number of peritoneal leukocytes compared with the numbers in mice treated with PBS or empty liposomes, except for a significant higher number of peritoneal leukocytes at 24 h after bacterial inoculation in mice treated with MTPPE. There were no differences in the relative numbers of peritoneal monocytes, granulocytes or lymphocytes between MTPPE- or LE-MTPPE-treated infected mice and infected mice treated with PBS or empty liposomes at 48 h after bacterial inoculation, except for a decreased number of granulocytes and an increased number of lymphocytes in LE-MTPPE-treated mice (Table 5.2).

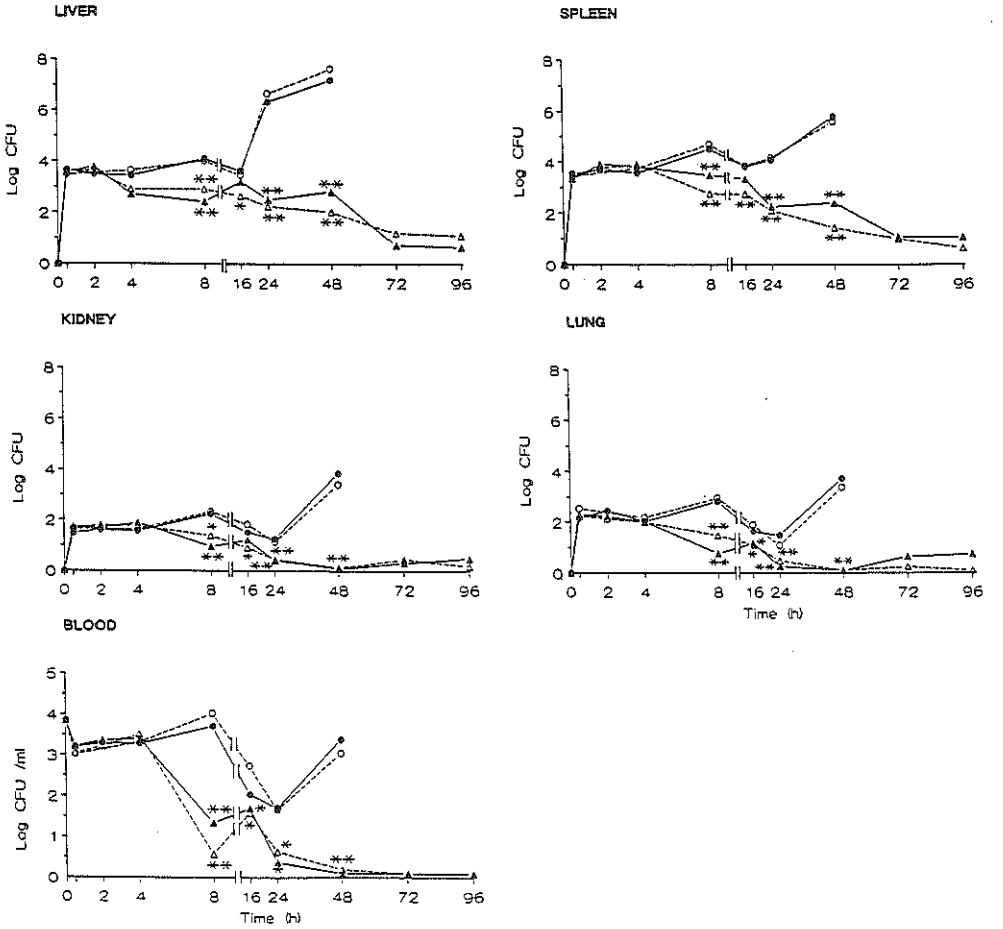

FIGURE 5.1 Effects of MTPPE and LE-MTPPE on the numbers of *K. pneumoniae* in liver, spleen, kidney, lung and blood of mice. Mice i.v. infected with 10^4 CFU *K. pneumoniae* at zero time were treated i.v. at 24 h before bacterial inoculation with 50 μg MTPPE (▲——▲), 25 μg LE-MTPPE (△----△), PBS (●——●) or empty liposomes (○---○). Each point represents the geometric mean for six mice. If mice died a geometric mean was not calculated. Significance: *$p \leq 0.05$ or **$p \leq 0.01$ versus PBS-treated mice.

TABLE 5.1 Effects of MTPPE and liposome-encapsulated MTPPE (LE-MTPPE) on the numbers of leukocytes in blood of uninfected and infected mice

Treatment[a]	Numbers of leukocytes at times after zero time[b]				
	1 h	24 h	48 h	72 h	96h
Uninfected mice					
MTPPE	3283±370	5100±233*	5866±274**	5366±369**	4616±282
LE-MTPPE	3482±242	3966±423	5100±194*	5533±145**	4350±111
PBS	3633±240	3783±164	3466±257	3566±362	3916±314
EL	3983±380	4383±674	3683±666	3900±176	4400±215
Infected mice					
MTPPE	3183±135	3048±177	4648±227**	4665±723	3811±234
LE-MTPPE	2873±101*	3566±341*	4150±790**	3850±337	5200±171
PBS	3466±98	2503±369	1471±314	-[c]	-[d]
EL	2698±183**	2020±103	1758±178	-[c]	-[d]

[a] Mice (six per group) were treated i.v. at 24 h before zero time with 50 μg MTPPE, 25 μg LE-MTPPE, PBS or empty liposomes (EL).

[b] At zero time, part of the mice were injected with PBS (uninfected mice) and part of the mice were inoculated with 10^4 CFU *K. pneumoniae* (infected mice). Significance: *$p \leq 0.05$, **$p \leq 0.01$ versus PBS-treated mice.

[c] Only part of the mice survived, and a mean was not calculated.

[d] All mice died.

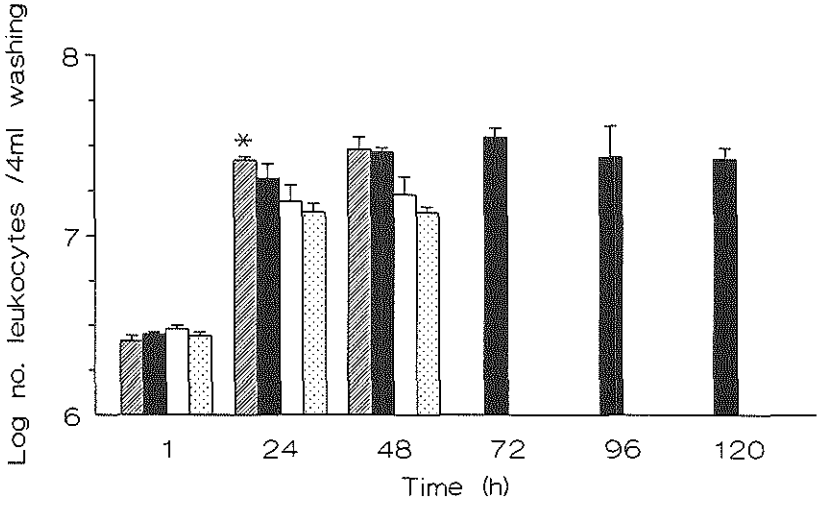

FIGURE 5.2 Effects of MTPPE and LE-MTPPE on numbers of leukocytes in the peritoneal cavity of mice. Mice i.p. infected with 10^3 CFU *K. pneumoniae* at zero time were treated i.v. at 24 h before bacterial inoculation with 50 μg MTPPE (▨), 25 μg LE-MTPPE (■), PBS (□) or empty liposomes (⁘). Each point represents the geometric mean for six mice ± SEM. If mice died a geometric mean was not calculated. Significance: *$p \leq 0.05$ versus PBS-treated mice.

Figure 5.3 shows that after i.p. inoculation of *K. pneumoniae*, bacterial numbers in the peritoneal cavity sharply increased, and between 48 h and 96 h, all PBS-treated mice died. Administration of MTPPE and LE-MTPPE at 24 h before bacterial inoculation had no effect on the total numbers of bacteria from 1 h until 48 h after inoculation. Thereafter, numbers of intraperitoneal bacteria in LE-MTPPE-treated mice were slightly decreased at 72 h and increased again until to about 10^8 CFU per 4 ml washing at 120 h. In the surviving MTPPE-treated mice (some mice died between 48 h and 72 h) numbers of intraperitoneal bacteria also increased until 10^8 CFU per 4 ml washing at 120 h. In addition, intracellular bacterial numbers were not affected (data not shown). However, MTPPE- and LE-MTPPE-treated mice survived much longer than did the PBS-treated mice. MTPPE-treated mice died between 48 and 264 h. After treatment with LE-MTPPE some mice died after 120 h; eventually, 33% of the mice survived.

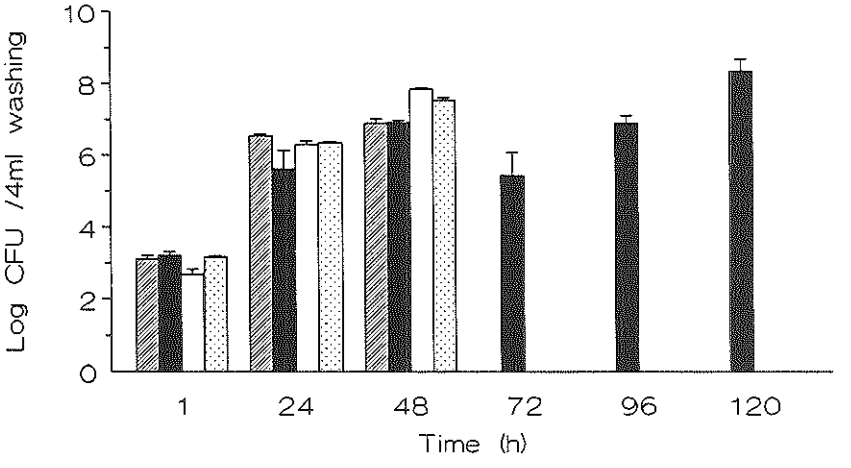

FIGURE 5.3 Effects of MTPPE and LE-MTPPE on bacterial numbers in the peritoneal cavity of mice. Mice i.p. infected with 10^3 CFU *K. pneumoniae* at zero time were treated i.v. at 24 h before bacterial inoculation with 50 μg MTPPE (▨), 25 μg LE-MTPPE (■), PBS (□) or empty liposomes (⁘). Each point represents the geometric mean for six mice ± SEM. If mice died a geometric mean was not calculated.

TABLE 5.2 Numbers of total leukocytes and the percentage of monocytes, granulocytes and lymphocytes in peritoneal washing at 48 h after bacterial inoculation

Treatment[a]	Determination (mean ± SEM)			
	Total leukocytes[b]	Monocytes(%)	Granulocytes(%)	Lymphocytes(%)
MTPPE	7.48±0.07	42.3±1.5	54.6±1.7	3.2±0.4
LE-MTPPE	7.47±0.02	45.7±2.3	48.5±2.4**	5.9±0.9**
PBS	7.23±0.08	39.4±2.5	57.9±2.9	2.7±0.4
EL	7.13±0.03	39.9±1.8	57.8±2.0	2.3±0.3

[a] Mice (six per group) were treated i.v. at 24 h before i.p. inoculation with 10^3 *K. pneumoniae* with 50 μg MTPPE, 25 μg LE-MTPPE, PBS or empty liposomes (EL). Significance: **$p \leq 0.01$ versus PBS-treated mice.

[b] Log number of leukocytes in 4 ml peritoneal wash fluid.

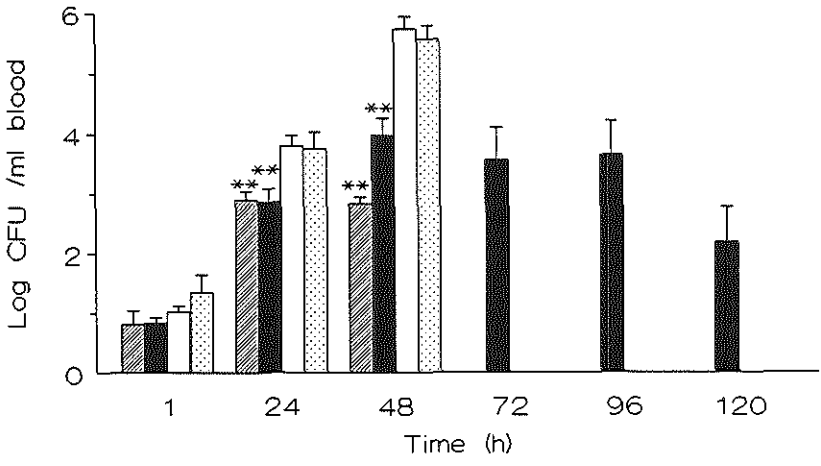

FIGURE 5.4 Effects of MTPPE and LE-MTPPE on numbers of *K. pneumoniae* in blood of mice. Mice i.p. infected with 10^3 CFU *K. pneumoniae* at zero time were treated i.v. at 24 h before bacterial inoculation with 50 μg MTPPE (▨), 25 μg LE-MTPPE (■), PBS (□) or empty liposomes (⁘). Each point represents the geometric mean for six mice ± SEM. If mice died a geometric mean was not calculated. Significance: **$p \leq 0.01$ versus PBS-treated mice.

Effects of MTPPE and LE-MTPPE on bacterial numbers in the blood after i.p. challenge with K. pneumoniae. Figure 5.4 shows that after i.p. inoculation of *K. pneumoniae*, bacterial numbers in the blood sharply increased until all mice died. Administration of MTPPE and LE-MTPPE resulted in a slower increase in bacterial numbers in the blood, and in LE-MTPPE-treated mice, bacterial numbers remained stable from 24 until 96 h after bacterial inoculation; thereafter, numbers decreased. In the surviving MTPPE-treated mice bacterial numbers in the blood further increased to $2x10^5$ bacteria per ml blood at 120 h.

Effect of LE-MTPPE on bacterial numbers in the blood after i.p. challenge with K. pneumoniae in mice depleted of tissue macrophages in liver and spleen by LE-DMP. Figure 5.5 shows that LE-DMP treatment at 48 h before i.p. inoculation with *K. pneumoniae* resulted in a much faster increase in bacterial numbers in the blood, compared with the increase in control mice, and mice died between 24 and 48 h after bacterial inoculation. When LE-MTPPE was given to LE-DMP-treated mice at 24 h before bacterial inoculation, numbers of bacteria in the blood sharply increased in the same way. The numbers of leukocytes in the blood of control mice decreased 1.5-fold between 1 and 24 h after bacterial inoculation. After administration of LE-DMP leukocyte numbers in the blood decreased in the same way as in control mice.

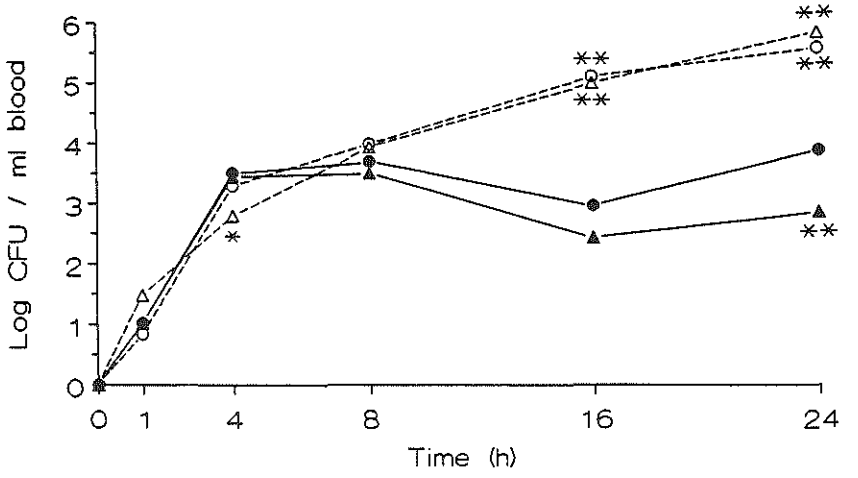

FIGURE 5.5 Effect of liposome-encapsulated MTPPE (LE-MTPPE) on numbers of *K. pneumoniae* in the blood of mice after elimination of tissue macrophages from liver and spleen. Mice were i.v. treated with liposome-encapsulated dichloromethylene diphosphonate (LE-DMP) or PBS at 48 h before i.p. inoculation with 10^3 CFU *K. pneumoniae*. To LE-DMP-treated mice at 24 h before bacterial inoculation 25 μg LE-MTPPE (▲- - -▲) or PBS (○- - - -○) was administered. To PBS-treated mice at 24 h before bacterial inoculation 25 μg LE-MTPPE (▲——▲) or PBS (●——●) was administered. Each point represents the geometric mean of six mice. Significance: *$p \leq 0.05$, **$p \leq 0.01$ versus PBS-treated mice.

Discussion

Increased resistance against experimental *K. pneumoniae* infection due to treatment with muramyl dipeptide (MDP) or derivatives was observed by several investigators (3,29,73,35,165,177,178). The precise mechanisms by which muramyl peptides exert their protective effects remain incompletely understood. In this study it was observed that in mice treated with free MTPPE or liposome-encapsulated MTPPE at a twofold lower dose, the clearance of i.v. inoculated *K. pneumoniae* from the blood by tissue macrophages was observed. In control mice, however, bacteria were not effectively cleared from the blood and bacterial numbers increased again, resulting in death of mice. This finding is in agreement with data of Izbicki et al. (97) who also found an increased clearance of bacteria from the blood of LE-MTPPE-treated minipigs during the first 2 h after i.v. inoculation with *Streptococcus pneumoniae*. In our study differences in clearance of bacteria between mice treated with free MTPPE or LE-MTPPE and control mice were at first seen at 8 h after bacterial inoculation and from that time also bacteria in the organs were killed. Bacterial numbers in blood and organs were positively correlated. This finding is in agreement with the study of Polk et al. (178), who also found this positive correlation between bacterial numbers in blood, liver, lungs and spleen in MDP-treated mice intramuscularly inoculated with *K. pneumoniae*.

Our study also shows that treatment of mice with free MTPPE or LE-MTPPE resulted in increased numbers of leukocytes in the blood. This result is in agreement with those of other investigators who also found increased numbers of leukocytes in the blood after treatment of uninfected rabbits or mice with MTPPE or MDP-Lys(L18), respectively (213,218). To elucidate the relative contributions of the recruitment of blood leukocytes and the activation of tissue macrophages to resistance against *K. pneumoniae* infection, induced by MTPPE and LE-MTPPE, we used mice which were i.p. inoculated with *K. pneumoniae*. In these mice, it was possible to discriminate between both effects of treatment with MTPPE or LE-MTPPE, since recruited leukocytes now could be quantitated. It was shown that after the i.p. bacterial challenge, numbers of peritoneal leukocytes in MTPPE and LE-MTPPE-treated mice were not significantly greater than numbers in control mice. These observations suggest that the leukocytes recruited by MTPPE and LE-MTPPE do not have increased potency to respond to a bacterial challenge. In addition, peritoneal leukocytes did not show an enhanced phagocytic capacity. These observations are in agreement with our in vitro data showing that exposure of peritoneal macrophages to various concentrations of MTPPE had no effect on the uptake or killing of *K. pneumoniae* (unpublished observations). Ozaki et al. (160) showed that i.v. administration of the lipophilic MDP derivative, MDP-Lys(L18), led to increased numbers of granulocytes but not of macrophages in bronchoalveolar lavage fluid (BAL) of rats, intratracheal inoculated with *Pseudomonas aeruginosa*. These granulocytes and macrophages had an increased phagocytic activity for *P. aeruginosa* in vitro. Lamont et al (119) also found increased numbers of granulocytes around an intramuscular suture, impregnated with *K. pneumoniae*, in mice subcutaneously pretreated with MDP. These granulocytes had an increased phagocytic capacity.

Although in our study, numbers of peritoneal leukocytes and their phagocytic capacities did not differ between MTPPE- or LE-MTPPE-treated mice and control mice, LE-MTPPE-

treated mice survived, whereas only some of the MTPPE-treated mice survived and all control mice died. The increased efficacy of LE-MTPPE compared to MTPPE in the free form found in the i.p. infection may be explained as follows. After i.p. infection, there is a constant influx of bacteria from the peritoneal cavity into the blood. Resident macrophages have to be activated for a prolonged period of time, which may be achieved by administration of MTPPE in the liposome-encapsulated form. In the i.v. infection, in which the doses of MTPPE and LE-MTPPE used appear to be equally effective, bacteria stay in the blood for only a short time period and in this case it is not necessary that macrophages be activated for a prolonged period of time. The prevention of early death from bacterial septicemia and the increased survival time in LE-MTPPE treated and i.p. infected mice appeared to be the result of an increased clearance of bacteria from the blood by tissue macrophages following treatment with LE-MTPPE. Polk et al. (178) also found lower numbers of bacteria in the blood of MDP-treated mice implanted with a *K. pneumoniae*-infected suture in an extremity than in the blood of control mice. When one compares the results of both studies, it must be borne in mind that because of regional anatomical differences between the peritoneal cavity and the extremities, recruitment of different defence mechanisms may be involved. In addition, Polk et al. (178) concluded that because of MDP-treatment, lymphatic filtration, but not the clearance of bacteria from the blood by tissue macrophages, was improved. To obtain more insight into the role of the tissue macophages, mice were treated with LE-DMP, an agent which caused elimination of macrophages in the liver and the red pulp and of marginal zone and metallophylic marophages in the spleen within 1 day (209). Numbers of peritoneal macrophages were not affected after i.v. administration of LE-DMP (210). The first macrophages reappeared at 1 week after treatment with LE-DMP. The effect of LE-DMP on numbers of granulocytes in liver and spleen was assumed to be very limited, since before injection of LE-DMP, there are only few numbers of granulocytes in liver and spleen. In addition, numbers of granulocytes in the blood are not changed after injection of LE-DMP and therefore it is very likely that an influx of granulocytes in reponse to infection in LE-DMP-treated mice is not drastically changed. In mice depleted of tissue macrophages in the liver and spleen, bacterial numbers in the blood increased rapidly and mice died between 24 h and 48 h after bacterial inoculation. Administration of LE-MTPPE to these mice had no effect anymore on the antibacterial resistance, numbers of bacteria in the blood increased and subsequently all mice died. This increased mortality was not due to decreased numbers of leukocytes in the blood. These observations clearly show that the tissue macrophages in liver and spleen are of major importance in LE-MTPPE induced resistance against *K. pneumoniae*. Whether other cell types are also involved in the induced activation of macrophages remains to be elucidated.

In summary, treatment of mice with MTPPE or LE-MTPPE results in efficient clearance and killing of *K. pneumoniae* in liver, spleen, kidney, and lung. The data suggest that increased numbers of leukocytes in the blood due to MTPPE or LE-MTPPE do not contribute significantly to this induced antibacterial resistance. However, tissue macrophages appear to be of major importance in this phenomenon, since depletion of tissue macrophages abrogates the effect of LE-MTPPE treatment.

CHAPTER 6

TREATMENT OF *KLEBSIELLA PNEUMONIAE* SEPTICEMIA IN NORMAL AND LEUKOPENIC MICE BY LIPOSOME-ENCAPSULATED MURAMYL TRIPEPTIDE PHOSPHATIDYLETHANOLAMIDE

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This study is submitted for publication.

Abstract

The effect of muramyl tripeptide phosphatidylethanolamide (MTPPE), in the free form or in the less toxic liposome-encapsulated form (LE-MTPPE), on *Klebsiella pneumoniae* septicemia resulting from intraperitoneal bacterial inoculation was investigated in mice. When administering a single dose at 24 h before bacterial inoculation, 50 μg MTPPE and 25 μg LE-MTPPE appeared to be most effective doses from a twofold dose range. The percentage survival was 55% and 40%, respectively, whereas all control mice died. The efficacy of repeated administration of LE-MTPPE at various intervals was investigated. After administration of additional therapeutic doses the percentage survival did not exceed 40%. An increased percentage survival was obtained when prophylactic treatment with LE-MTPPE was started 7 days before bacterial inoculation, percentage survival increased up to 85%. This prophylactic treatment schedule was not dependent on increased leukocyte numbers in the blood following administration of LE-MTPPE, since this treatment schedule was effective in cyclophosphamide-treated leukopenic mice.

Introduction

Administration of agents that activate the nonspecific antimicrobial host defense may be of great value in treatment of severe infections particularly in the immunocompromised host. From clinical experience it is well known that these 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 to antibiotic treatment. In this respect it is of great importance to stimulate the nonspecific host defense, in particular the mononuclear phagocyte system. This can be effectuated by the immunomodulating agent muramyl tripeptide phosphatidylethanolamide (MTPPE).

MTPPE is a lipophilic derivative of muramyl dipeptide. Muramyl peptides stimulate macrophages to morphological and biochemical changes, increased production of monokines and enzymes as well as enhanced tumoricidal activity (61,65,134,172,181). In addition,

muramyl peptides have been proven to be effective in stimulating the nonspecific host defense against several bacterial infections (158,167). In a previous study we observed that administration of a single dose of MTPPE at 24 h before intravenous (i.v.) inoculation with *Klebsiella pneumoniae* led to increased antibacterial resistance of mice against infection in terms of decreased numbers of bacteria in blood, liver, spleen, lung and kidney (136). *K. pneumoniae* is an important cause of infection and mortality of hospitalized patients and is a serious complication of patients with malignancies (36). Since mostly these immunocompromised patients are prone to infections during a prolonged period of time it is expected that repeated administration of immunomodulating agents is needed. However, repeated administration of MTPPE has been shown to result in toxic side effects (18,58,188). Encapsulation of MTPPE in liposomes may therefore be of importance, since in this form a reduction in MTPPE toxicity has been demonstrated (58,188). In addition, due to liposomal encapsulation MTPPE is targeted to the cells of the mononuclear phagocyte system (124) which have been shown to be of major importance in the antibacterial resistance. In the model of i.v. induced *K. pneumoniae* infection administration of a single dose of liposome-encapsulated MTPPE (LE-MTPPE) at 24 h before bacterial inoculation was also effective even in a two-fold lower dose as MTPPE in the free form (137). In the present study we used an infection model in which *K. pneumoniae* was inoculated intraperitoneally resulting in appearance of *K. pneumoniae* in the blood at regular intervals eventually leading to septicemia. This model has a clinical relevance as immunocompromised patients are also prone to develop septicemia from a local infection. The efficacy of several prophylactic and therapeutic treatment schedules with free MTPPE or LE-MTPPE was investigated.

Materials and methods

Animals. Specific pathogen free, 11 to 13 weeks old male C57Bl/Ka mice were used (ITRI-TNO, Rijswijk, The Netherlands).

Reagents. N-acetyl-muramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-[1,2-dipalmitoyl-sn-glycero-3-(hydroxy-phosphoryloxy)]ethanolamide was kindly provided by Ciba-Geigy (Basel,Switzerland). Liposomes containing MTPPE and placebo liposomes were a generous gift of Ciba-Geigy Ltd. (Basel, Switzerland). Liposomes were prepared from a dry lyophilisate composed of 250 mg phosphatidylcholine and phosphatidylserine in a molar ratio of 7:3 (with or without 1 mg MTPPE) and shaken with 2.5 ml of phosphate buffered saline (PBS) (CGP 19835A lipid). The average diameter of the constituted liposomes was between 2.0 and 3.5 μm and at least 80% of the liposomes were larger than 1.5 μm (205).

Bacteria. A strain of *K. pneumoniae* capsular serotype 2 (ATCC 43816) was used. Bacteria were grown for 16 h at 37°C in Todd-Hewitt broth (Oxoid Ltd, Basingstoke, England) and preserved on ice. Directly before the inoculation of mice the bacteria were washed three times in PBS.

Induction of leukopenia. Leukopenia was induced by i.p. injections with cyclophosphamide (Sigma Diagnostics, St Louis, Missouri, USA), a first dose of 120 mg/kg at eleven days before bacterial inoculation followed by doses of 80 mg/kg at every third day thereafter. At 7 days before bacterial inoculation i.v. treatment with LE-MTPPE started.

Quantitation of blood leukocytes. Blood samples (0.8 ml) were taken from the

retroorbital plexus and collected in polypropylene tubes containing 1 mg 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.

Experimental infection caused by K. pneumoniae. Infections were induced by i.p. inoculation of 10^3 CFU of *K. pneumoniae* into C57Bl/Ka mice. Then mice were housed in seperate 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.

Quantitation of bacterial numbers in the blood. At different intervals after i.v. inoculation with 10^4 *K. pneumoniae* blood samples (0.8 ml) were taken from the retroorbital plexus and collected in polypropylene tubes containing 1 mg dried EDTA (BDH). Serial tenfold dilutions were prepared and volumes of 0.2 ml of each dilution were spread on trypto-ne soya agar (TSA) plates (Oxoid Ltd, Basingstoke, England). All plates were incubated overnight at 26°C.

Statistical analysis. Statistical evaluation of the differences in the survival curves between MTPPE-treated, LE-MTPPE-treated and PBS-treated groups of animals was performed by using the log rank test. This test examines the decrease in survival with time as well as the eventual percentage of survival. The Mann-Whitney test was used for evaluation of the differences in the numbers of leukocytes and bacteria between LE-MTPPE-treated and PBS-treated groups of animals.

Results

Effect of a single (prophylactic) dose of free MTPPE or LE-MTPPE on the survival of mice from K. pneumoniae infection. 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 blood within 4 hours and 10^6 CFU per ml blood after 2 days, eventually leading to the death of mice (Figure 6.1). Figure 6.2 shows that all mice died within 6 days after bacterial inoculation. Most mice died between day 3 and day 4. Only *K. pneumoniae* was recovered from the blood of all dead mice. Administration of 50 μg MTPPE per mouse at 24 h before bacterial inoculation resulted in survival of 55% of the mice. When a twofold higher dose was given, 100 μg MTPPE per mouse, survival rate and percentage survival was not increased. Administration of 6.3 μg MTPPE per mouse at 24 h before bacterial inoculation appeared to be the lowest dose from a twofold dose range that resulted in a significantly increased survival rate compared to PBS-treated mice. A dose of 3.1 μg had no significant effect (data not shown).

Figure 6.3 shows that administration of 25 μg LE-MTPPE per mouse at 24 h before bacterial inoculation resulted in survival of 40% of the mice. A dose of 6.3 mg placebo liposomes, which is equivalent to the amount of lipid in which 25 μg MTPPE is encapsulated, had no effect. A dose of 25 μg LE-MTPPE was the maximum dose that could be administered, since at higher doses the equivalent amount of placebo liposomes had toxic effects. Administration of 3.1 μg of LE-MTPPE per mouse at 24 h before bacterial inoculation appeared to be the lowest dose from a twofold dose range that resulted in a

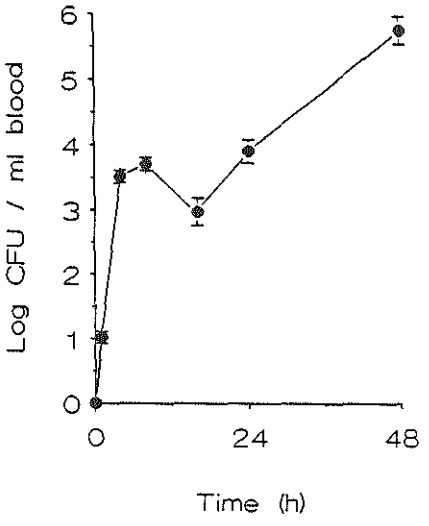

FIGURE 6.1 Numbers of *K. pneumoniae* in the blood of mice after i.p. inoculation. At 24 h before i.p. inoculation with 10^3 CFU *K. pneumoniae*, mice were i.v. treated with PBS. Each point represents the geometric mean for six mice ± SEM.

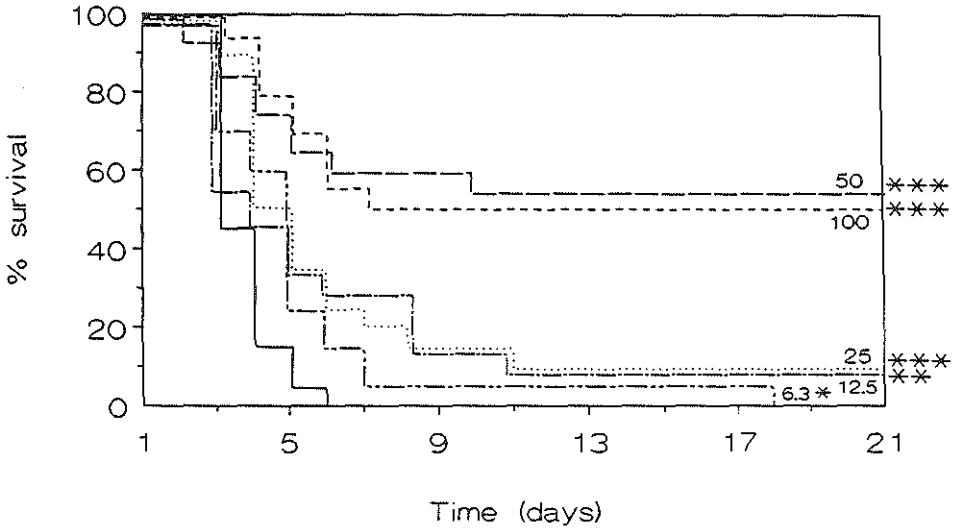

FIGURE 6.2 Effect of administration (prophylactic) of a single dose of free MTPPE on survival of mice from *K. pneumoniae* infection. At 24 h before i.p. inoculation with 10^3 CFU *K. pneumoniae*, mice were i.v. treated with a single dose of free MTPPE from a twofold dose range (100-6.3 μg per mouse) or with PBS. Groups of twenty mice were used. Significance: $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ versus PBS-treated mice.

significantly increased survival rate compared to PBS-treated control mice. A dose of 1.5 μg had no significant effect (data not shown).

Effect of repeated administration (prophylactic and therapeutic) of LE-MTPPE on the survival of mice from K. pneumoniae infection. Compared to administration of a single dose of 25 μg LE-MTPPE at 24 h before bacterial inoculation, which treatment resulted in 40% survival of the mice (Figure 6.3), administration of a second dose of 25 μg LE-MTPPE at 24 h after bacterial inoculation and a third dose at 72 h after bacterial inoculation did not further enhance the survival rate and percentage survival significantly (Figure 6.4). Repeated administration of 6.3 mg placebo liposomes had no effect.

Whereas administration of 3.1 μg LE-MTPPE at 24 h before bacterial inoculation resulted in a significantly increased survival compared to that in PBS-treated mice (percentage survival was 5%) (Figure 6.3), administration of a second dose at 24 h after bacterial inoculation led to a survival percentage of 30% (Figure 6.5). When a third dose was given at 72 h after bacterial inoculation survival was not significantly further enhanced. Changing the interval of administration of doses of 3.1 μg LE-MTPPE into 24 h (instead of 48 h) effected a further increase in survival rate, percentages being 5%, 45%, and 55%,

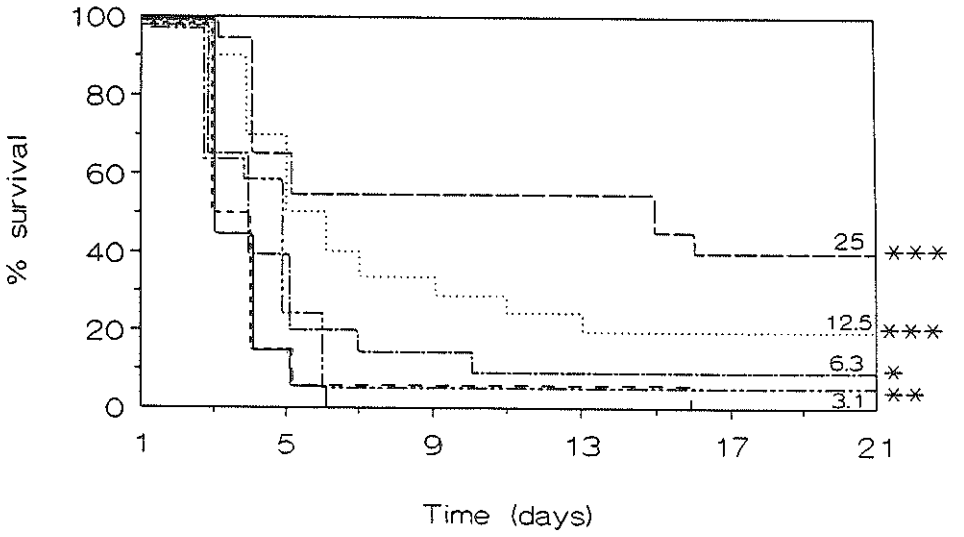

FIGURE 6.3 Effect of administration (prophylactic) of a single dose of liposome-encapsulated MTPPE (LE-MTPPE) on survival of mice from *K. pneumoniae* infection. At 24 h before i.p. inoculation with 10^3 CFU *K. pneumoniae*, mice were i.v. treated with a single dose of LE-MTPPE from a twofold dose range (25-3.1 μg per mouse), or with placebo liposomes (- - - - -) or with PBS (———). Groups of twenty mice were used. Significance: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ versus PBS-treated mice.

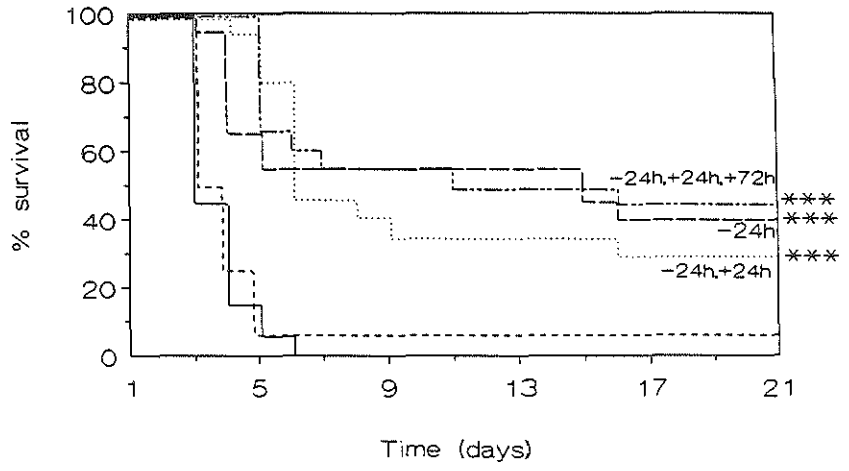

FIGURE 6.4 Effect of repeated administration (prophylactic and therapeutic) of 25 μg liposome-encapsulated MTPPE (LE-MTPPE) on survival of mice from *K. pneumoniae* infection. Mice were i.p. inoculated with 10^3 CFU *K. pneumoniae* at zero time. Mice were i.v. treated with 25 μg LE-MTPPE at 24 h before bacterial inoculation (— —), or at 24 h before and at 24 h after inoculation (. . . . .), or at 24 h before and at 24 h after and at 72 h after inoculation (— - —). Control mice were i.v. treated with placebo liposomes at 24 h before and at 24 h after and at 72 h after bacterial inoculation (- - - - -) or with PBS at 24 h before inoculation (———). Groups of twenty mice were used. Significance: ***$p \leq 0.001$ versus PBS-treated mice.

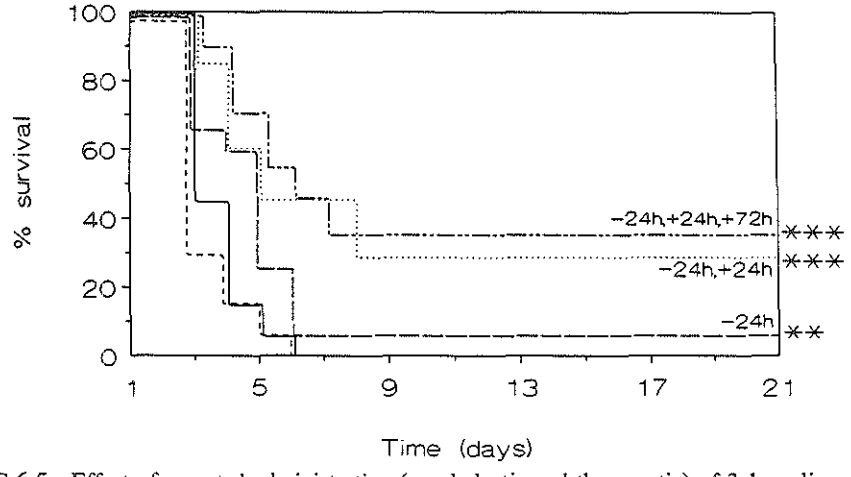

FIGURE 6.5 Effect of repeated administration (prophylactic and therapeutic) of 3.1 μg liposome-encapsulated MTPPE (LE-MTPPE) on survival of mice from *K. pneumoniae* infection. Mice were i.p. inoculated with 10^3 CFU *K. pneumoniae* at zero time. Mice were i.v. treated with 3.1 μg LE-MTPPE at 24 h before bacterial inoculation (— —), or at 24 h before and at 24 h after inoculation (. . . . .), or at 24 h before and at 24 h after and at 72 h after inoculation (— - - —). Control mice were i.v. treated with placebo liposomes at 24 h before and at 24 h after and at 72 h after bacterial inoculation (- - - - -) or with PBS at 24 h before inoculation (———). Groups of twenty mice were used. Significance: **$p \leq 0.01$, ***$p \leq 0.001$ versus PBS-treated mice.

a further increase in survival rate, the percentages being 5%, 45%, and 55%, respectively (Figure 6.6). Repeated administration of 0.8 mg placebo liposomes, equivalent to the amount of lipid in which 3.1 μg MTPPE was encapsulated, had no effect.

Effect of repeated administration (prophylactic) of LE-MTPPE on the survival of mice from K. pneumoniae infection. Doses of 3.1 μg LE-MTPPE were administered, the last dose being given at 24 h before bacterial inoculation. Administration at a 48 h interval, of two doses resulted in a survival of 40%, of three doses resulted in 70% survival and of four doses in 85% survival (Figure 6.7). Repeated administration of 0.8 mg placebo liposomes with an interval of 48 h had no effect. Changing the interval of administration of doses of 3.1 μg LE-MTPPE into 24 h (instead of 48 h) resulted in a survival of 40% when two doses were given, and in 75 % survival after three doses (Figure 6.8). When a fourth dose was given the survival rate and percentage survival did not change anymore. Repeated administration of 0.8 mg placebo liposomes with an interval of 24 h also resulted in a significantly increased survival compared to PBS-treated mice ($p=0.04$).

Effect of repeated administration (prophylactic) of LE-MTPPE on the survival of leukopenic mice from K. pneumoniae infection. Figure 6.9 shows that leukopenic mice i.p. infected with 10^3 CFU *K. pneumoniae* all died within 3 days after bacterial inoculation. Most mice died at day 2. Prophylactic administration of 4 doses of 3.1 μg LE-MTPPE to leuko-

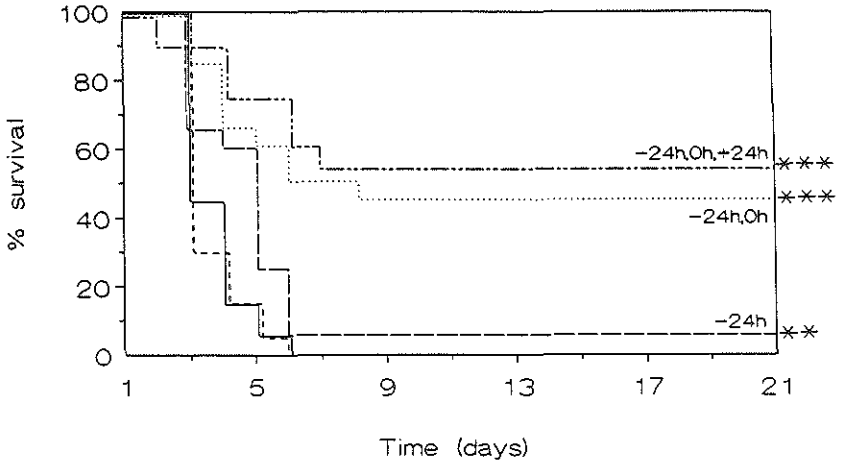

FIGURE 6.6 Effect of repeated administration (prophylactic and therapeutic) of 3.1 μg liposome-encapsulated MTPPE (LE-MTPPE) on survival of mice from *K. pneumoniae* infection. Mice were i.p. inoculated with 10^3 CFU *K. pneumoniae* at zero time. Mice were i.v. treated with 3.1 μg LE-MTPPE at 24 h before bacterial inoculation (—— ——), or at 24 h before and at zero time (·····), or at 24 h before and at zero time and at 24 h after inoculation (— · · —). Control mice were i.v. treated with placebo liposomes at 24 h before and at zero time and at 24 h after bacterial inoculation (- - - - - -) or with PBS at 24 h before inoculation (————). Groups of twenty mice were used. Significance: $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ versus PBS-treated mice.

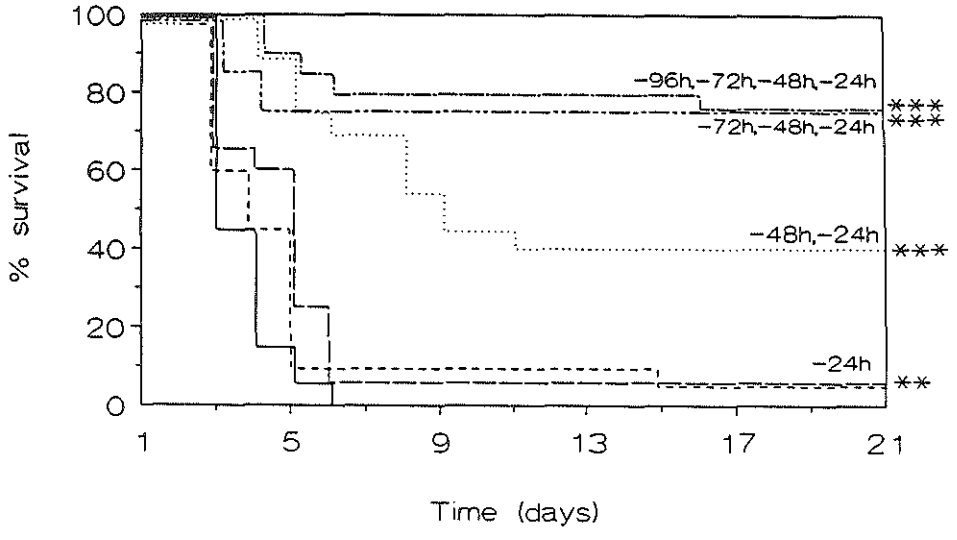

FIGURE 6.7 Effect of repeated administration (prophylactic) of 3.1 μg liposome-encapsulated MTPPE (LE-MTPPE) on survival of mice from *K. pneumoniae* infection. Mice were i.p. inoculated with 10^3 CFU *K. pneumoniae* at zero time. Mice were i.v. treated with 3.1 μg LE-MTPPE at 24 h before bacterial inoculation (— —), or at 24 h and 72 h before inoculation (· · · · · ·), or at 24 h and 72 h and 120 h before inoculation (— · · —), or at 24 h and 72 h and 120 h and 168 h before bacterial inoculation (— · —). Control mice were i.v. treated with placebo liposomes at 24 h and 72 h and 120 h and 168 h before bacterial inoculation (– – – – – –) or with PBS at 24 h before inoculation (—————). Groups of twenty mice were used. Significance: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ versus PBS-treated mice.

TABLE 6.1 Clearance of *K. pneumoniae* from the blood of normal and leukopenic mice[a]

Mice	Log numbers[b] of *K. pneumoniae* per ml blood at several times after i.v. bacterial inoculation			
	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 i.v. inoculated with 10^4 CFU *K. pneumoniae*. Leukopenia was induced by i.p. injections with cyclophosphamide, a first dose of 120 mg/kg at eleven days before bacterial inoculation followed by doses of 80 mg/kg at every third day thereafter.

[b] Geometric mean ± SEM for six mice.

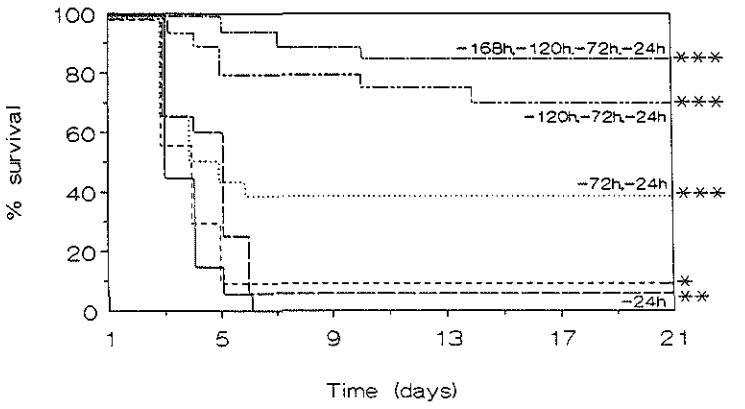

FIGURE 6.8 Effect of repeated administration (prophylactic) of 3.1 μg liposome-encapsulated MTPPE (LE-MTPPE) on survival of mice from *K. pneumoniae* infection. Mice were i.p. inoculated with 10^3 CFU *K. pneumoniae* at zero time. Mice were i.v. treated with 3.1 μg LE-MTPPE at 24 h before bacterial inoculation (—— ——), or at 24 h and 48 h before inoculation (- - - - - -), or at 24 h and 48 h and 72 h before inoculation (—— - - ——), or at 24 h and 48 h and 72 h and 96 h before bacterial inoculation (—— - ——). Control mice were i.v. treated with placebo liposomes at 24 h and 48 h and 72 h and 96 h before bacterial inoculation (- - - - - -) or with PBS at 24 h before inoculation (————). Groups of twenty mice were used. Significance: **$p \leq 0.01$, ***$p \leq 0.001$ versus PBS-treated mice.

penic mice with an interval of 48 h (the treatment schedule which was most effective in immunocompetent mice) led to an increased survival rate compared to that in PBS-treated leukopenic mice. Numbers of leukocytes in the blood of LE-MTPPE-treated leukopenic mice did not differ from numbers in PBS-treated leukopenic mice. They were 670 ± 25 and 687 ± 26 per μl blood (mean±SEM for six mice), respectively. Treatment of mice with cyclophosphamide did not lead to a decreased MPS capacity to clear *K. pneumoniae* from the blood compared to the clearance capacity of PBS-treated normal mice (Table 6.1).

Discussion

This study shows that administration of a single dose of MTPPE resulted in resistance against *K. pneumoniae* septicemia resulting from i.p. bacterial inoculation in terms of an increased percentage survival and an increased survival time. Administration of a single dose of LE-MTPPE was also effective but at a twofold lower dose. This is in agreement with our earlier observations in *K. pneumoniae* septicemia induced by i.v. inoculation where bacterial numbers in the organs and blood were used as parameter of therapeutic efficacy (136). However in the i.v. induced infection administration of a single dose of MTPPE or LE-MTPPE resulted in survival of all mice, whereas in the present model of i.p. induced infection only 55% or 40% of the infected mice survived after administration of a single dose

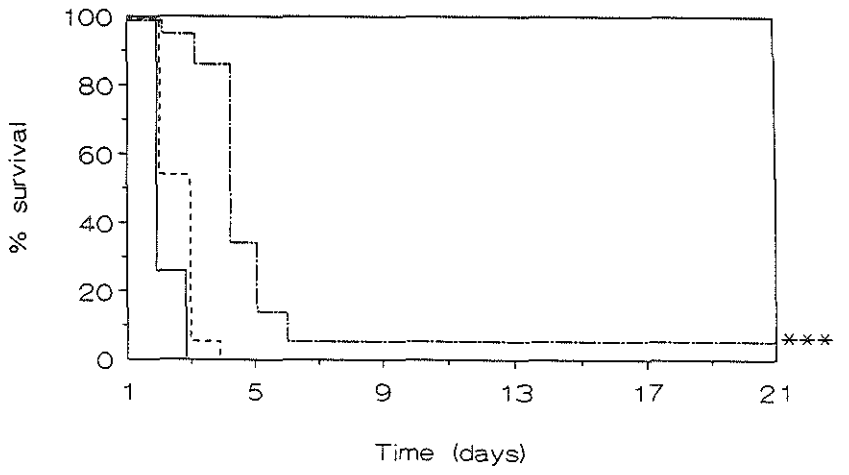

FIGURE 6.9 Effect of repeated administration (prophylactic) of 3.1 μg liposome-encapsulated MTPPE (LE-MTPPE) on survival of leukopenic mice from *K. pneumoniae* infection. Leukopenic mice were i.p. inoculated with 10^3 CFU *K. pneumoniae* infection at zero time. Mice were i.v. treated with 3.1 μg LE-MTPPE at 24 h and 72 h and 120 h and 168 h before bacterial inoculation (— - —). Control mice were i.v. treated with placebo liposomes at 24 h and 72 h and 120 h and 168 h before bacterial inoculation (- - - - -) or with PBS at 24 h before inoculation (———). Groups of twenty mice were used. Significance: ***$p \leq 0.001$ versus PBS-treated mice.

of MTPPE or LE-MTPPE, respectively. The increased efficacy of a single dose of MTPPE and LE-MTPPE in the i.v. induced infection may be explained as follows. After i.v. inoculation bacteria are present in the blood for only a short period of time and activation of macrophages is only needed for a rather short period of time, whereas after i.p. inoculation influx of bacteria from the peritoneal cavity into the blood occurs at regular intervals and macrophages have to be activated for a prolonged period of time by repeated doses LE-MTPPE. An increased resistance against experimental *K. pneumoniae* infection due to administration of muramyl dipeptide (MDP) or its derivatives was also observed by other investigators using either systemic infections or wound infection models with the survival of animals (30,75,169,178), or the decrease in bacterial numbers in the infected organs (3,178) used as parameters for therapeutic efficacy. Administration of a single dose of MTPPE, LE-MTPPE, MDP or other MDP-derivatives seemed to be most effective when given at 24 h before bacterial inoculation (3,136,158). The results obtained with repeated administration of MDP derivatives were in discrepancy (46,67,109,112,151,167). Various microorganisms were used (viruses, bacteria or parasites) and in most studies only few treatment schedules were tested. However, a detailed study related to the effects of prophylactic or therapeutic treatment schedules of MDP derivatives was not performed before.

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. It is known that repeated administration of MTPPE in the free

form results in toxic side effects (18,58,188). By encapsulation of MTPPE in liposomes this toxicity can be reduced, in addition, targeting of MTPPE to the cells of the mononuclear phagocyte system can be achieved (124,188). The present study shows that the survival of mice treated at 24 h before bacterial inoculation with the maximum tolerated dose of LE-MTPPE (25 μg per mouse) could not be further increased by additional doses of LE-MTPPE at a 48-h interval. Since LE-MTPPE had to be given repeatedly, it was preferred to administer the lowest possible doses of LE-MTPPE to exclude any toxic side effects. A single dose of 3.1 μg LE-MTPPE was the lowest dose from the two-fold dose range that significantly improved survival of mice. Additional doses of 3.1 μg LE-MTPPE could enhance the percentage survival up to 55%. Administration of three doses of 3.1 μg LE-MTPPE led to a better survival than administation of three doses of 25 μg LE-MTPPE. These results suggest that for the degree of macrophage activation by LE-MTPPE the total administered dose is of less importance. The highest therapeutic effect was obtained when all doses of LE-MTPPE were given prophylactically with an interval of 48 h, the percentage survival of infected mice was increased to 85%. The number of doses as well as the time of administration of LE-MTPPE are of major importance. It can be concluded 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. Studies about efficacy of treatment schedules using MDP derivatives are rare. Parant et al. (169) found an increased efficacy of the lipophilic MDP derivative, murabutide, when it was i.p. administered once a day at 3, 2, and 1 day before i.v. inoculation of *K. pneumoniae*. However, they compared this effect only with the efficacy of administration of a single dose either at 3 or 1 day before inoculation and not with other treatment schedules.

It is known from clinical experience that particularly immunocompromised patients often suffer from severe infections which can not always be treated successfully with antibiotics. One factor which contributes to this lack of success is the failure of the host defense system to give adequate support to antibiotic treatment. Therefore, stimulation of nonspecific host defenses may be very important in these patients. In the present study we have investigated the effect of stimulating host defenses in leukopenic mice. These mice were immunocompromised due to treatment with cyclophosphamide resulting in a 85% decrease in numbers of peripheral leukocytes. The effects of repeated prophylactic administration of LE-MTPPE we found in immunocompetent mice: increased survival rate and increased percentage survival we also observed in leukopenic mice. By others only the effect of single doses of MDP derivative, administered at 24 h before bacterial inoculation, was assessed (73,74,167,178). Galland et al. (73,74) found an increased survival time of starved, cyclophosphamide-treated or hydrocortison-treated mice infected with a *K. pneumoniae* loaden suture after a single s.c. dose of MDP. Parant (167) observed an increased survival of 7 day old mice from an intragastric *K. pneumoniae* infection after a single intragastric dose of murabutide. Polk et al. (178) demonstrated an increased survival and decreased bacterial numbers in the blood of starved mice infected with an *K. pneumoniae* loaden suture after administration of a single dose of MDP.

Whereas in immunocompetent mice we found increased numbers of leukocytes in the

blood due to LE-MTPPE treatment (136), the present study demonstrated that in leukopenic mice after repeated administration of LE-MTPPE, numbers of leukocytes in the blood were not increased. This was due to the cyclophosphamide induced blockade of the recruitment of leukocytes. Therefore it may be concluded that activation of tissue macrophages by LE-MTPPE is of major importance in the induced antibacterial resistance in leukopenic mice. In immunocompetent mice we have also found that activation of tissue macrophages is of great importance in the increased resistance against *K. pneumoniae* infection (135). Our results are in agreement with findings of Nakajima et al. (145) that leukocyte numbers in the blood of cyclophosphamide-treated mice were not increased after treatment with the lipophilic MDP-Lys(L18).

CHAPTER 7

TISSUE DISTRIBUTION AND CELLULAR DISTRIBUTION OF LIPOSOMES ENCAPSULATING MURAMYLTRIPEPTIDE PHOSPHATIDYL ETHANOLAMIDE

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This study is submitted for publication.

Abstract

In a previous study it was shown that administration of liposome-encapsulated MTPPE (LE-MTPPE) led to resistance against *Klebsiella pneumoniae* infection. To get more insight into the cell types that are involved in this induced antibacterial resistance by LE-MTPPE, the tissue distribution of liposomes encapsulating MTPPE and the cellular distribution in the main target organs were investigated. After intravenous injection of the liposomes in mice a substantial amount was recovered from liver and spleen and a smaller amount from the lung. In the liver 83% of the liposomes was taken up by the Kupffer cells. In the spleen also most liposomes were taken up by macrophages of the red and white pulp as well as by dendrocytes. The liver and spleen were also the organs in which, after intravenous inoculation, *K. pneumoniae* was trapped. It was observed that a great part of the cells containing LE-MTPPE had not taken up bacteria. Most bacteria, about 73%, were found in cells not containing liposomes. The capacity of the liposome-containing cells to take up bacteria did not change with time. This suggests that the by LE-MTPPE immunostimulating effect is due to the production of cytokines by the cells that take up LE-MTPPE. These cytokines might stimulate other cells to the killing of bacteria.

Introduction

Stimulation of the nonspecific host defense, in particular the mononuclear phagocyte system can be effected by immunomodulators either from bacterial origin, such as LPS or muramyl peptides or by cytokines such as interleukin-1, colony stimulating factor or interferon-γ. In previous studies we showed that administration of the lipophilic muramyl peptide derivative, muramyl tripeptide phosphatidylethanolamide (MTPPE) to mice led to resistance against *Klebsiella pneumoniae* infection (135,136). From these studies it appeared that for effective treatment the nonspecific host defense has to be stimulated for a prolonged period of time and repeated administration of MTPPE is needed. It has been shown that repeated administration of MTPPE has toxic side effects (58,188). Encapsulation of MTPPE in liposomes (LE-MTPPE) has been proven to reduce this toxicity (58,188). The liposomes

encapsulating MTPPE are rather large (diameter of 2.0-3.5μm, 205) and are consisting of phosphatidylcholine and phosphatidylserine rendering them negatively charged. It is expected that these liposomes are preferentially taken up by cells of the mononuclear phagocyte system (MPS) in the liver and spleen (124,200).

In a previous study we observed that administration of LE-MTPPE resulted in an increased antimicrobial resistance against *K. pneumoniae* infection compared to administration of MTPPE in the free form (135,136). An improved efficacy of MTPPE after liposomal encapsulation was also found in studies investigating the stimulation of the nonspecific defenses against several viruses (51,76,112). To get more insight into the cell types that are involved in the by LE-MTPPE induced enhancement of the nonspecific resistance, in the present study the tissue distribution of LE-MTPPE and the distribution of LE-MTPPE over the cells in the main target organs was investigated. In addition, the capacity of cells containing LE-MTPPE to take up *K. pneumoniae* was examined.

Materials and methods

Animals. Specific pathogen free, 11 to 13 weeks old female C57Bl/Ka mice were used (ITRI-TNO, Rijswijk, The Netherlands).

Reagents. Liposomes containing MTPPE were a generous gift of Ciba-Geigy Ltd. (Basel, Switzerland). Liposomes were prepared from a dry lyophilisate composed of 250 mg phosphatidylcholine and phosphatidylserine in a molar ratio of 7:3 (with or without 1 mg MTPPE) and shaken with 2.5 ml of phosphate buffered saline (PBS) (CGP 19835A lipid). The average diameter of the constituted liposomes was between 2.0 and 3.5 μm and at least 80% of the liposomes were larger than 1.5 μm (205). Rhodamine phosphatidylethanolamine (Rho-PE) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). Tetramethyl-rhodamine isothiocyanate (Tritc) and fluorescein isothiocyanate (Fitc) were obtained from Sigma (St. Louis, Missouri, USA). ^{67}Gallium-citrate and ^{111}Indium-chloride were from Frosst (Quebec, Canada). Deferoxamine mesylate (DF) was from Ciba-Geigy Ltd (Basel, Switzerland).

Radiolabeling of liposomes. Liposomes were radiolabeled with ^{67}Gallium-deferoxamine (^{67}Ga-DF) as described by Gabizon et al. (71). The labeling resulted in formation of a ^{67}Ga-DF complex in the aqueous interior of liposomes. Non-entrapped ^{67}Ga-oxine was removed by two times centrifugation for 1 h at 48,000xg at 4°C.

Fluorescent labeling of liposomes. Liposomes were passively labeled with 0.03 mol percent of Rho-PE, a marker of the lipid bilayers, or with 1 mg Tritc per ml PBS, a marker of the aquous phase. Non-encapsulated Tritc was removed by two times centrifugation for 1 h at 48,000xg at 4°C.

Biodistribution of liposomes. A dose of 6.25 mg of liposomes encapsulating 25 μg MTPPE and labeled with ^{67}Ga-DF was intravenously injected. At several times after administration samples of 0.2 ml blood were obtained by retroorbital bleeding under CO_2 anesthesia. Then mice were sacrificed (six mice per time point) and liver, spleen, kidney and lung were removed. Radioactivity was quantitated in a γ-counter (Minaxy 5530, Packard Instruments, Downers Grove, USA).

Immunohistochemical staining of sections from liver and spleen. At 1 h after injection of liposomes, passively labeled with Rho-PE in the lipid bilayers or with Tritc in the aqueous phase, mice were sacrificed. Spleen, lung and liver were removed and embedded in Tissue-Tek 11 (Miles laboratories Inc, Kankakee, USA) on a specimen stub and frozen immediately. Five μm sections were cut on a cryostat (model 1720, Leitz, Germany) and collected on micoscope slides precoated with a solution containing 0.1% gelatin and 0.01% chromium potassium sulphate. Slides were air-dried and used immediately or stored at -20°C. Prior to use sections were fixed for 10 min in acetone and air-dried. After fixation sections were incubated for 1 h at 37°C with PBS supplemented with 0.05% bovine serum albumin (Sigma, St Louis, Missouri, USA) (PBS-BSA). Then sections were incubated for 30 min at room temperature with monoclonal antibodies directed to different subsets of macrophages. The monoclonal antibody BM8 was used to identify Kupffer cells in the liver and red pulp macrophages in the spleen. MOMA-1 binds to marginal metallophilic macrophages, ER-TR9 to marginal zone macrophages, NLDC-145 to dendritic cells, CD3 to T-cells, and RA3 6B2 to B-cells (122). After rinsing the sections with PBS-BSA, the sections were incubated for 30 min at room temperature with a 1:60 dilution of Fitc-conjugated rabbit-anti-(rat IgG) (Cappel, Organon Teknika Corp, Boxtel, The Netherlands) in PBS-BSA containing 2% normal mouse serum. Sections were rinsed with PBS-BSA and embedded in PBS with 10% glycerol (Merck, Darmstadt, Germany) and 0.1% phenylene diamine (BDH Chemicals Ltd, Poole, England).

Labeling of bacteria. An overnight culture of *K. pneumoniae* was washed three times with PBS. Then the pellet of bacteria was incubated with 100 μg Fitc/ml PBS for 1 h at 37°C. The nonbinding Fitc molecules were removed by washing three times with PBS containing 1% fetal bovine serum (HyClone Laboratories Inc., Logan, Utah, USA).

Results

Tissue distribution of liposomes encapsulating MTPPE. Mice were injected i.v. with 6.25 mg of ^{67}Ga-DF labeled liposomes encapsulating 25 μg MTPPE being the lowest effective dose from a twofold dose range that resulted in enhanced resistance against *K. pneumoniae* infection (136). In Table 7.1 it is shown that at 5 min after injection 96.9% of the initially injected liposomes was recovered from blood and several organs: 55.1% of the liposomes was recovered from the blood, the remainder was distributed over several organs, particularly in the liver. During the first 30 min the total recovery of the injected liposomes decreased substantially to 65.2% of the injected dose. From 30 min after injection the recovery of liposomes from spleen, lung and blood slowly decreased with time, whereas from the liver remained stable. Incubation of liposomes containing MTPPE in mouse serum for 24 h at 37°C in vitro did not affect the stability of the liposomes (data not shown).

Cellular distribution of liposomes encapsulating MTPPE in the liver, spleen and lung. It was investigated among which cell types in the liver, spleen and lung liposomes encapsulating MTPPE, labeled with Rho-PE, were distributed after i.v. injection (Figure 7.1). In addition, the cells of a certain cell type that had taken up liposomes encapsulating MTPPE were quantitated (Table 7.2). It was found that at 1 h after i.v. injection 83.2% of the

TABLE 7.1 Biodistribution of liposomes encapsulating MTPPE labeled with ^{67}Ga-DF in mice at various time intervals after injection [a]

	Tissue radioactivity (% of injected dose)[b]			
	5 min	30 min	60 min	120 min
liver	25.0±0.5	29.1±1.4	31.7±0.7	29.9±0.8
spleen	6.6±0.7	18.1±1.6	17.0±0.6	14.2±0.4
lung	8.4±0.3	5.6±0.2	4.8±0.1	4.0±0.3
kidney	1.2±0.3	1.6±0.1	1.3±0.1	1.2±0.1
blood[c]	55.1±1.5	10.8±0.6	7.4±0.6	5.4±0.4
recovery	96.9±1.5	65.2±1.3	62.2±1.3	54.6±1.0

[a] Mice were injected at zero time with 6.25 mg ^{67}Ga-DF labeled liposomes in which 25 μg MTPPE was encapsulated. At several times distribution of radiolabeled liposomes in blood and organs was shown as percentage radioactivity of the total injected amount of radioactivity. Values were corrected for the blood content in the tissues, which was determined after i.v. injection of ^{111}Indium labeled syngeneic erythrocytes in a seperate group of twelve mice. Labeling of erythrocytes was performed as described by Heaton et al. (85).

[b] Percentages are the mean ± SEM for six mice.

[c] Per total amount of blood.

TABLE 7.2 Percentage of cell types in liver and spleen containing liposomes encapsulating MTPPE[a]

organ	cell type	percentage positive cells[b]
liver	macrophages	11.2-13.0
spleen	RP macrophages	23.6-25.4
	MM macrophages	13.6-15.2
	MZ macrophages	10.2-12.4
	dendritic cells	3.8-6.0
	T-cells	1.0-1.4
	B-cells	0.8-1.1

[a] Mice were i.v. injected with 6.25 mg liposomes encapsulating 25 μg MTPPE and labeled with Rho-PE. At 1 h thereafter mice were sacrificed and spleen and liver were removed and slides were prepared as described in the materials and methods section. Each slide was stained with a different Fitc-conjugated monoclonal antibody directed against the cell type mentioned. RP macrophages, red pulp macrophages. MM macrophages, marginal metallophilic macrophages. MZ macrophages, marginal zone marcophages.

[b] The percentage of 500 monoclonal antibody positive cells that had taken up liposomes. Values of two mice are shown.

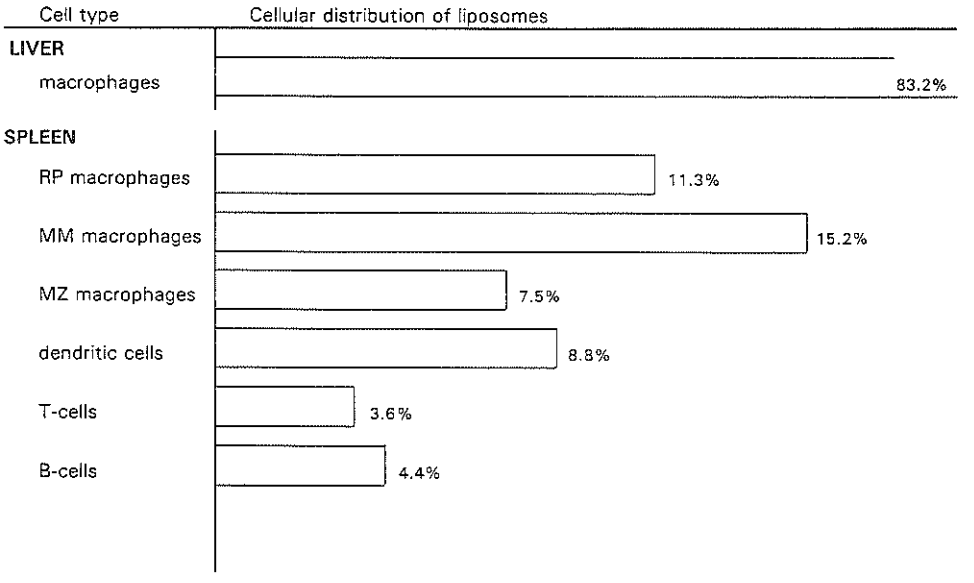

FIGURE 7.1 Cellular distribution of liposomes encapsulating MTPPE in liver and spleen. Mice were i.v. injected with 6.25 mg liposomes encapsulating 25 μg MTPPE and labeled with Rho-PE. After 1 h mice were sacrificed and liver and spleen were removed. Slides were prepared and each of them was stained with a different monoclonal antibody. On each slide the percentage of 500 liposome-containing cells that were positive for the monoclonal antibody (▭) was determined. The mean and range was given for the values of two mice. RP macrophages, red pulp macrophages. MM macrophages, marginal metallophilic macrophages. MZ macrophages, marginal zone macrophages.

liposomes that were recovered from the liver were taken up by macrophages. Of all Kupffer cells 12.1% contained liposomes. At 1 h after i.v. injection 79.5% of the liposomes were recovered from the white pulp in the spleen. Most liposomes were found in macrophages and in dendritic cells, percentages being 34.0% and 8.8% of the liposomes that were recovered from the spleen, respectively. T- and B-cells had taken up 8.0% of the liposomes. In addition, 41.0% of the injected liposomes were adhered to these T- and B-cells. From the cell types in the spleen the BM8 positive macrophages contained relatively the most liposomes: 24.5% of these cells had taken up liposomes. In slides of the lung only few liposomes were

observed. Liver and spleen contained a small number of granulocytes which had not taken up liposomes (data not shown).

Distribution of K. pneumoniae in the liver and spleen of mice treated with liposomes encapsulating MTPPE. At various times after i.v. injection of 6.25 mg Tritc-labeled liposomes containing 25 μg MTPPE, mice were i.v. inoculated with 10^7 Fitc-labeled *K. pneumoniae*. The cellular distribution of liposomes labeled with Tritc was the same as that of liposomes labeled with Rho-PE. Thirty minutes after inoculation when most bacteria were cleared from the blood (data not shown), distribution of *K. pneumoniae* was investigated in the spleen. In Table 7.3 it is shown that about 27% of the inoculated bacteria were taken up by cells that contained liposomes, whereas about 73% of the bacteria were found in the cells that had not ingested liposomes. With time no significant change was observed in this distribution. With respect to the liposome-containing cells: about 20% had taken up one or more bacteria (Table 7.4). This percentage also did not change significantly with time. The total number of cells that contained liposomes did not change until 24 h after injection (data not shown).

TABLE 7.3 Uptake of bacteria by cells in the spleen [a]

Number of bacteria per cell	Number of cells containing bacteria at various intervals after injection of liposomes[b]			
	1 h	4 h	8 h	24 h
cells containing liposomes				
1	108-143	121-158	116-161	101-133
2-5	7-14	4-6	2-3	8-9
>5	0	0	0	0
cells not containing liposomes				
1	343-370	336-366	333-379	349-384
2-5	7-8	2-7	2-5	6-10
>5	0	0	0	0

[a] At several times after i.v. injection of 6.25 mg of Tritc-labeled liposomes encapsulating 25 μg MTPPE, mice were i.v. injected with 10^7 Fitc-labeled *K. pneumoniae*. Thirty minutes after bacterial inoculation mice were sacrificed and spleen was removed. Slides were prepared as described in the materials and methods section.

[b] On each slide 500 cells which contained bacteria were microscopically screened for the uptake of liposomes. Values of two mice are shown.

TABLE 7.4 Uptake of bacteria by liposome containing cells in the spleen.[a]

Numbers of bacteria per cell	Number of cells containing bacteria at various intervals after injection of liposomes[b]			
	1 h	4 h	8 h	24 h
0	372-398	400-416	408-431	419-422
1	91-116	73-92	58-83	67-68
2-5	11-12	8-11	9-11	11-13
>5	0	0	0	0

[a] At several times after i.v. injection of 6.25 mg of Tritc-labeled liposomes encapsulating 25 μg MTPPE, mice were i.v. injected with 10^7 Fitc-labeled *K. pneumoniae*. Thirty minutes after bacterial inoculation mice were sacrificed and spleen was removed. Slides were prepared as described in the materials and methods section.

[b] On each slide 500 liposome-containing cells were microscopically screened for the numbers of intracellular bacteria. Values of two mice are shown.

Discussion

In a previous study we showed that administration of the liposome-encapsulated immunomodulator MTPPE resulted in increased resistance against *K. pneumoniae* in mice (135,136). MTPPE encapsulated in liposomes (LE-MTPPE) appeared to be effective in a twofold lower dose compared to MTPPE in the free form (135). Since it is expected that in patients with impaired host defense MTPPE has to be given at regular intervals, administration of the liposome-encapsulated form is preferred because of its reduced toxicity (58,188). The mechanism by which LE-MTPPE exerts its antibacterial resistance enhancing effects, particularly which cell types are involved, is not fully understood. To get more insight into this we studied the tissue distribution of LE-MTPPE and its distribution over the cells in those organs from which the most liposomes were recovered.

To study the tissue distribution of liposomes we used the radioactive marker ^{67}Ga-DF. This complex is located in the aqueous phase of the liposome. Gabizon et al. (71) showed that there is a minimal translocation of the radioactive label to plasma proteins as well as a rapid renal clearance rate when the label is released from the liposomes. Since a high amount of liposomes was still in the blood during the first 30 min, we corrected the percentage of liposomal recovery from a certain organ for the blood content in that organ.

It appeared that a substantial amount of the liposomes injected was recovered from liver and spleen. This distribution was as expected when injecting large (diameter between 2.0 μm and 3.5 μm) negatively charged liposomes consisting of phosphatidylcholine and phosphatidylserine (molar ratio 7:3) (124,200). In the lung, liposomes are probably trapped in the capillaries of the lung rather than taken up by macrophages, since in sections of lung only few liposomes were observed. Between 5 and 30 min after injection, recovery of the liposomes from the blood decreased substantially. Since we found in in vitro studies that the

aqueous marker was not released from the liposomes after 24 h incubation in mouse serum, it is not likely that the observed decrease in recovery of liposomes was the result of degradation of the liposomes in serum. Probably most of the liposomes are taken up from the blood by liver and spleen within 30 min after injection, followed by rapid degradation of liposomes and release of ^{67}Ga-DF.

The tissue distribution of LE-MTPPE has also been studied in rats by Schumann et al. (188) using ^{14}C-phosphatidylcholine (^{14}C-PC) as marker for LE-MTPPE. In these studies it was shown that at 30 min after injection of the liposomes almost 100% was recovered: 54% from the liver, 14% from the spleen, 19% from the lung and 12% from the blood. At 6 h after injection of the liposomes still 60% was recovered: a decrease was only observed in liver,lung and blood. This discrepancy between these data and our observations may be explained by the liposomal marker used: Partial degradation of the liposomes results in a rapid loss of a marker of the aqueous phase (^{67}Ga-DF), whereas a marker of the lipid bilayer (^{14}C-PC) will be only partly released. In addition, after degradation of liposomes the breakdown products of ^{14}C-PC are used by the cell for de novo synthesis of phospholipids, with the radioactive label remaining inside the cell (7).

In the liver almost all liposomes were taken up by the macrophages. Most liposomes in the spleen were also taken up by macrophages: either red pulp macrophages or white pulp macrophages such as marginal metallophilic macrophages and marginal zone macrophages. In addition, a substantial part of the liposomes was taken up in the dendrocytes in the white pulp. Most liposomes were found in or associated with cells of the white pulp, the areas of lymphoid tissue in the spleen. The cells of the red pulp contained a higher percentage liposomes compared to the percentage found in each cell type in the white pulp. This might be explained by the localization of the red pulp macrophages. When liposomes in the bloodstream enter the spleen, they first reach the cells of the red pulp and thereafter the cells of the white pulp.

Although the organs primarily involved in the uptake of liposomes, liver and spleen, were also the organs in which most of *K. pneumoniae* are trapped after i.v. inoculation (136), a great part of the cells in the spleen containing liposomes had not taken up *K. pneumoniae*. Most bacteria, about 73%, were found in cells not containing liposomes. In agreement with our observations, Phillips et al. (173) did not find a positive correlation between uptake of liposome-encapsulated MDP-GDP and tumoricidal activity of murine alveolar macrophages. More specifically, the macrophages that did not contain liposomes were tumoricidal. In addition, in studies of Daemen et al. (42) rat Kupffer cells that had taken up most of the liposomes encapsulating MDP appeared to be the least tumoricidal. These in vivo data and our observations that murine peritoneal macrophages in vitro could not be stimulated by LE-MTPPE to an increased uptake and killing of *K. pneumoniae* (data not shown) suggest that other cell types besides the macrophages that have taken up LE-MTPPE are involved in the induced resistance. Since it is known that MDP-derivatives are able to stimulate macrophages to produce M-CSF, TNF, IL-1 and IL-6 (1,70,187), it seems likely that LE-MTPPE stimulates macrophages to produce cytokines which stimulate other macrophages to take up and kill the bacteria. In future studies it will be investigated which cytokines and celltypes are involved in the by LE-MTPPE induced resistance against *K. pneumoniae*.

CHAPTER 8

FREE VERSUS LIPOSOME-ENCAPSULATED MURAMYL TRIPEPTIDE PHOSPHATIDYLETHANOLAMIDE AND INTERFERON-γ IN EXPERIMENTAL INFECTION WITH *LISTERIA MONOCYTOGENES*

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Abstract

The effect of free and liposome-encapsulated muramyl tripeptide phosphatidylethanolamide (MTPPE) and interferon-γ (IFN-γ) on the resistance against *Listeria monocytogenes* infection in mice was investigated. It was shown that administration of MTPPE or IFN-γ at 24 h before bacterial inoculation led to increased resistance against *L. monocytogenes* infection in terms of a decrease in bacterial numbers in liver and spleen. Encapsulation of MTPPE and IFN-γ in liposomes increased their efficacy 33- or 66-fold, respectively. In addition, liposomal encapsulation led to a more rapid decrease in bacterial numbers. The immunmodulator to lipid ratio appeared to be very important in the antibacterial effect of LE-MTPPE and LE-IFN-γ. When nontherapeutic doses of liposome-encapsulated MTPPE or IFN-γ were administered in a larger amount of lipid (so at higher lipid:immunomodulator ratio), these doses became effective. Exposure of macrophages in monolayer infected with *L. monocytogenes* in vitro to MTPPE had no effect, whereas exposure to IFN-γ only led to growth inhibition of the intracellular bacteria. However, incubation of macrophages with a combination of MTPPE and IFN-γ resulted in killing of the intracellular bacteria. Exposure of macrophages in vivo to both immunomodulators in combination can be effected by using liposomes as carrriers. It was observed that administration of MTPPE and IFN-γ co-encapsulated in liposomes resulted in a synergistic enhanced antibacterial resistance against *L. monocytogenes*. Both reactive oxygen and nitrogen intermediates seemed to play a role in the killing of *L. monocytogenes* by macrophages activated with a combination of MTPPE and IFN-γ.

Introduction

Antibiotic treatment of infections in the immunocompromised host does not always have a satisfactory therapeutic effect. The failure of the host system to give adequate support to antibiotic treatment may contribute to this lack of success. In this respect it would be of great value to activate the nonspecific antimicrobial defenses of the host, particularly, the mononuclear phagocyte system (MPS). The cells of the MPS can be activated by

immunomodulators, either from bacterial origin, such as LPS or muramyl peptides, or cytokines such as interleukin-1, colony stimulating factor, tumor necrosis factor or interferon-γ (IFN-γ).

Muramyl peptides stimulate macrophages to morphological and biochemical changes, increased production of monokines and enzymes as well as enhanced tumoricidal activity (61,65,134,172,180). In addition, muramyl peptides have been proved to be effective in stimulating the host defense against several bacterial infections (158,167). However, reports about the efficacy of muramyl peptides in experimental infections with *Listeria monocytogenes*, an intracellular bacterium which is able to survive in macrophages, are contradictory (65,92,154,158). We observed that exposure of macrophages infected with *L. monocytogenes* to MTPPE in vitro had no effect on intracellular survival of the bacteria. However, administration of MTPPE to mice infected with *L. monocytogenes* led to increased antibacterial resistance.

The macrophage is also one of the main target cells for IFN-γ. IFN-γ stimulates macrophages to increased production of enzymes, monokines as well as enhanced tumoricidal activity (13,95). In addition, it is shown that IFN-γ increased the antibacterial defenses against several infections (87,132). With respect to *L. monocytogenes* again the reports about the efficacy of IFN-γ in stimulating host defenses are contradictory (31,108,117,203,204). We observed that exposure of macrophages infected with *L. monocytogenes* to IFN-γ in vitro led to growth inhibition of intracellular bacteria, whereas administration of IFN-γ to mice infected with *L. monocytogenes* led to bacterial killing and increased antibacterial resistance.

Since our previous studies showed that treatment with MTPPE or IFN-γ in the free form resulted in increased antibacterial resistance against *L. monocytogenes*, in the present study the effect of liposomal encapsulation of MTPPE (LE-MTPPE) or IFN-γ (LE-IFN-γ) on the therapeutic effect was investigated. Since, immunocompromised patients are prone to infections during a prolonged period of time, repeated administration of immunomodulating agents is expected to be needed. Repeated administration of MTPPE or IFN-γ has been shown to result in toxic side effects (18,49,58,118,188). Encapsulation of MTPPE or IFN-γ in liposomes may therefore be of importance since, in the liposomal form, a reduction in MTPPE toxicity (118,188) or IFN-γ toxicity (90) has been demonstrated. In addition, due to liposomal encapsulation MTPPE or IFN-γ can be effectively targeted to the cells of the MPS (123).

The influence of the immunomodulator to liposomal lipid ratio on the antibacterial efficacy of LE-MTPPE or LE-IFN-γ is also investigated in the present study. In tumorcytotoxicity experiments it was found that this ratio in the liposomal formulation used was an important determinant in the degree of induced tumorcytotoxicity (41,186).

In the last part of the present study the effect of MTPPE and IFN-γ co-encapsulated in liposomes on *L. monocytogenes* infection was investigated. With the use of liposomes as carrriers is possible to target two different immunomodulators to the same cell, probably resulting in maximum activation in terms of antibacterial effect. Data from tumorcytotoxicity studies suggest that co-encapsulation of MTPPE and IFN-γ in liposomes led to an increased effect compared to the that obtained with MTPPE and IFN-γ encapsulated in separate liposomes (144,186).

Materials and methods

Animals. Specific pathogen free, 11 to 13 weeks old female C57Bl/Ka mice were used (ITRI-TNO, Rijswijk, The Netherlands).

Reagents. N-acetyl-muramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-[1,2-dipalmitoyl-sn-glycero-3-(hydroxy-phosphoryloxy)]ethanolamide (MTPPE) was kindly provided by Ciba-Geigy Ltd. (Basel, Switzerland). Recombinant rat IFN-γ with a specific activity of 4.4×10^6 U/mg protein was kindly provided by Dr P. van der Meide (ITRI-TNO, Rijswijk, The Netherlands) based on collaboration between the two laboratories. Superoxide dismutase (SOD), catalase (CAT), and dimethyl sulfoxide (DMSO) were purchased from Sigma Diagnostics (St Louis, Missouri, USA), sodiumazide (SA) from Brithish Drug Houses Ltd (Poole, England) and N^G-monomethyl-arginine acetate (NMMA) from ICN Biomedicals Inc. (Cleveland, Ohio, USA)

Preparation of liposomes. Liposomes were prepared from a dry lyophilisate composed of 250 mg phosphatidylcholine and phosphatidylserine in a molar ratio of 7:3 with or without MTPPE. Preparation of LE-MTPPE by shaking the lyophilisate with MTPPE in 2.5 ml of phosphate buffered saline (PBS). Preparationof LE-IFN-γ by shaking the lipid lyophilisate without MTPPE in PBS containing IFN-γ. Free IFN-γ was removed by washing with PBS at 44,000xg for 1 h at 4 °C. By means of trace amounts of ^{125}I-labeled IFN-γ, 22% of the IFN-γ added was appeared to be encapsulated in the liposomes. The average diameter of the constituted liposomes was between 2.0 and 3.5 μm and at least 80% of the liposomes were larger than 1.5 μm (205).

Bacteria. A strain of *Listeria monocytogenes* type 1/2 was used. Bacteria were grown for 16 h at 37°C in Todd-Hewitt broth (Oxoid Ltd, Basingstoke, England) and preserved on ice. Directly before the inoculation of mice, the bacteria were washed twice with PBS.

Experimental infection caused by L. monocytogenes. Infections were induced by intravenous inoculation of 5×10^4 CFU *L. monocytogenes* into C57Bl/Ka mice. At different intervals after inoculation, blood samples were taken from the retroorbital plexus and twofold diluted in 3.8% sodium citrate (BDH Chemicals Ltd, Poole, England). Serial 10-fold dilutions in PBS were prepared and volumes of 0.2 ml of each dilution were spread on tryptone soya agar (TSA) plates (Oxoid Ltd, Basingstoke, England). Mice were then sacrificed and spleen and liver were removed and each was homogenized in 20 ml of PBS for 30 s at 10,000 rpm in a VirTis homogenizer (The VirTis Co. Inc., Gardiner, New York). Serial 10-fold dilutions of homogenate in PBS were prepared. Volumes of 0.2 ml of each dilution and 2 ml volumes of the undiluted homogenate were spread on TSA plates. The remainder of the homogenate together with an equal volume of double concentrated TSA was poured in plastic plates. All plates were incubated for 48 h at 37°C. The number of viable bacteria recovered from liver, spleen and blood was used as a parameter of therapeutic efficacy.

Monolayers of peritoneal macrophages infected with L. monocytogenes. Monolayers of peritoneal macrophages from C57Bl/Ka mice were cultured at 37°C in chamber-slides (Miles laboratories Inc., Naperville, Illinois) in an humidified atmosphere of 7.5% CO_2 in air in culture medium containing Dulbecco modified Eagle medium (D-MEM, Flow Laboratories, Irvine, Scotland) with 1% glutamine and 15% fetal bovine serum (FBS, HyClone Laboratories Inc., Logan, Utah, USA) for 48 h before incubation with bacteria. After the first

2 h of incubation fresh culture medium was added to the monolayer. The cells were exposed 24 h before incubation with bacteria to MTPPE or IFN-γ, or to PBS. At zero time bacteria were added in a ratio of 16 bacteria per macrophage. After an uptake period of 30 min the noningested bacteria were removed by washing the monolayer three times with culture medium. The macrophages were reincubated for 6 h. In some experiments scavengers of reactive oxygen and nitrogen intermediates were added during the uptake of bacteria and the reincubation of the macrophages. At different time intervals during the period of reincubation, monolayers were washed with icy-cold culture medium and the macrophages were disrupted by quickly freezing and thawing and vigorously mixing of the cell suspension. The intracellular numbers of viable bacteria were determined by preparing tenfold serial dilutions. Volumes of 0.2 ml of each dilution were spread on TSA plates. All plates were incubated for 48 h at 37°C.

Statistical analysis. Statistical evaluation of differences in numbers of bacteria between MTPPE-treated, LE-MTPPE-treated, IFN-γ-treated, LE-IFN-γ-treated and PBS-treated groups of animals was performed by using the Mann-Whitney test.

Results

Effect of free MTPPE and liposome-encapsulated MTPPE (LE-MTPPE) on L. monocytogenes infection. After intravenous injection of $5x10^4$ CFU *L. monocytogenes*, most bacteria are taken up by liver and spleen. Numbers increased rapidly until 72 h after bacterial inoculation. Thereafter, bacterial numbers in the liver remained stable until 120 h after bacterial inoculation, and numbers in the spleen slightly decreased. Figure 8.1 shows that administration of a dose of 100 μg free MTPPE at 24 h before bacterial inoculation resulted in significantly decreased bacterial numbers in liver and spleen at 120 h after inoculation. Administration of a dose of 50 μg was only effective in the spleen not in the liver, and a dose of 25 μg had no effect anymore on bacterial numbers in liver and spleen. However, when a dose of 25 μg MTPPE was given in the liposome-encapsulated form, bacterial numbers in liver and spleen were significantly decreased. From a twofold dose range, 3.1 μg LE-MTPPE was the lowest dose that resulted in decreased bacterial numbers in liver and spleen. Administration of a dose of 1.6 μg LE-MTPPE or lower was not effective anymore. In addition, a dose of 6.3 mg placebo liposomes (the equivalent of the amount of lipid in which 25 μg MTPPE was encapsulated) had no effect on bacterial numbers (data not shown).

Administration of the doses of LE-MTPPE that resulted in significant lower numbers of bacteria in liver and spleen at 120 h after bacterial inoculation (25 μg, 6.3 μg or 3.1 μg LE-MTPPE) also led to decreased bacterial numbers at 72 h (Figure 8.2). Administration of free MTPPE (100 μg) only resulted in decreased bacterial numbers at 120 h, not at 72 h.

Effect of free IFN-γ and liposome-encapsulated IFN-γ (LE-IFN-γ) on L. monocytogenes infection. Figure 8.3 shows that administration of a dose of 12500 U IFN-γ at 24 h before bacterial inoculation resulted in significantly decreased bacterial numbers in liver and spleen at 120 h after bacterial inoculation. From a twofold dose range this appeared to be the lowest effective dose, since administration of a dose of 6250 U IFN-γ or lower was not

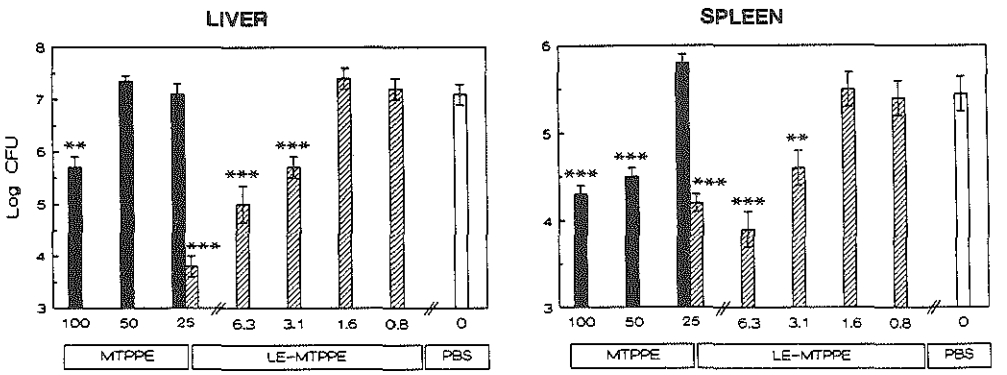

FIGURE 8.1 Effect of free MTPPE and liposome-encapsulated MTPPE (LE-MTPPE) on numbers of *L. monocytogenes* in liver and spleen. At 24 h before i.v. inoculation with $5x10^4$ CFU *L. monocytogenes*, mice were i.v. treated with one dose from a twofold dose range (25 μg - 100 μg) of free MTPPE, or with one dose from a twofold dose range (0.8 μg - 25 μg) of LE-MTPPE, or with PBS. Each point represents the geometric mean for six mice $\pm$ SEM at 120 h after bacterial inoculation. Significance *$p \leq 0.05$ **$p \leq 0.02$ ***$p \leq 0.002$ versus PBS-treated mice.

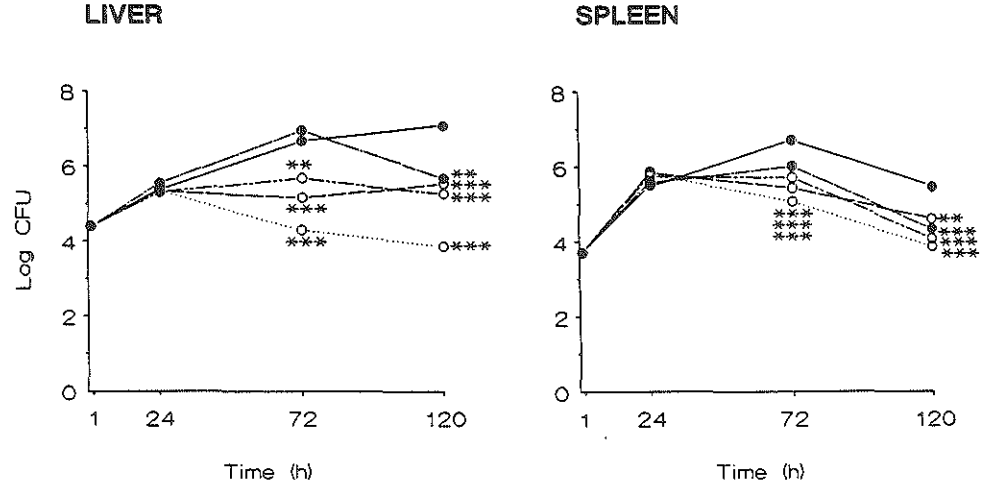

FIGURE 8.2 Effect of free MTPPE and liposome-encapsulated MTPPE (LE-MTPPE) on numbers of *L. monocytogenes* in liver and spleen. At 24 h before i.v. inoculation with $5x10^4$ CFU *L. monocytogenes*, mice were i.v. treated with 100 μg of free MTPPE (●— - —●), or with 3.1 μg (o— - —o), 6.3 μg (o— —o), or 25 μg (o····o) of LE-MTPPE per mouse, or with PBS (●——●). Each point represents the geometric mean for six mice. Significance *$p \leq 0.05$ **$p \leq 0.02$ ***$p \leq 0.002$ versus PBS-treated mice.

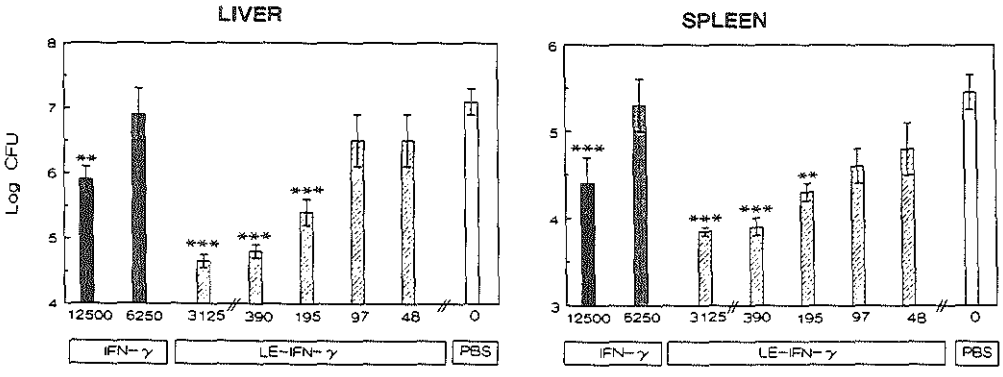

FIGURE 8.3 Effect of free IFN-γ and liposome-encapsulated IFN-γ (LE-IFN-γ) on numbers of *L. monocytogenes* in liver and spleen. At 24 h before i.v. inoculation with $5x10^4$ CFU *L. monocytogenes*, mice were i.v. treated with one dose from a twofold dose range (6250 U - 12500 U) of free IFN-γ, or with one dose from a twofold dose range (48 U - 3125 U) of LE-IFN-γ, or with PBS. Each point represents the geometric mean for six mice ± SEM at 120 h after bacterial inoculation. Significance $*p \leq 0.05$ $**p \leq 0.02$ $***p \leq 0.002$ versus PBS-treated mice.

not effective anymore. However, when a dose of 3120 U IFN-γ was given in the liposome-encapsulated form, bacterial numbers in liver and spleen were significantly decreased. From a twofold dose range 195 U LE-IFN-γ was the lowest dose that resulted in decreased bacterial numbers in liver and spleen. Administration of a dose of 97 U LE-IFN-γ or lower was not effective anymore.

Administration of the doses of LE-IFN-γ that resulted in significant lower numbers of bacteria in liver and spleen at 120 h after bacterial inoculation (3120 U, 390 U or 195 U LE-IFN-γ) also led to decreased bacterial numbers at 72 h (Figure 8.4). Administration of free IFN-γ (12500 U) only resulted in decreased bacterial numbers at 120 h, not at 72 h.

Influence of the MTPPE to lipid ratio on the antibacterial resistance against L. monocytogenes. Administration of nontherapeutic doses of LE-MTPPE became effective when encapsulated in increasing amounts of lipid (Table 8.1). Administration of 0.8 μg MTPPE in 1.6 mg lipid had no effect on bacterial numbers in liver and spleen at 120 h after bacterial inoculation, compared to numbers in PBS-treated mice as shown in Figure 8.1. Increasing the amount of encapsulating lipid led to significantly decreased numbers of bacteria in liver and spleen. The same results were found for 1.6 μg MTPPE encapsulated in increasing amounts of lipid.

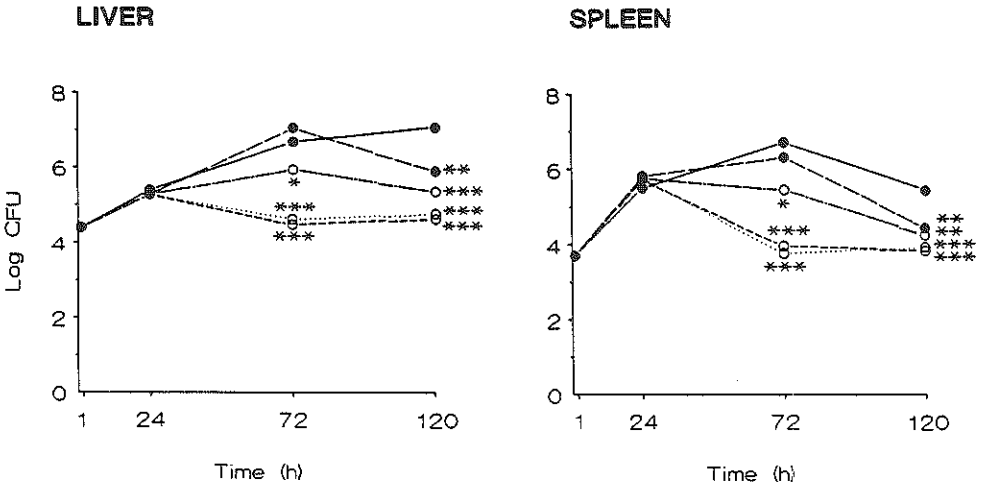

FIGURE 8.4 Effect of free IFN-γ and liposome-encapsulated IFN-γ (LE-IFN-γ) on numbers of *L. monocytogenes* in liver and spleen. At 24 h before i.v. inoculation with $5x10^4$ CFU *L. monocytogenes*, mice were i.v. treated with 12500 U of free IFN-γ (●— —●), or with 195 U (○— - —○) or 390 U (○· ····· ·○) or 3125 U of LE-IFN-γ (○- - - - -○), or with PBS (●———●). Each point represents the geometric mean for six mice. Significance *$p \leq 0.05$ **$p \leq 0.02$ ***$p \leq 0.002$ versus PBS-treated mice.

TABLE 8.1 Influence of the MTPPE to lipid ratio on the antibacterial resistance against *L. monocytogenes*[a]

Dose of MTPPE (μg)[a]	Dose of lipid (mg)[b]			
	0.8	1.6	3.1	6.3
0	-[c]/-[d]			-/-
0.4			-/-	-/-
0.8		-/-	-/+	+/+
1.6	-/-	+/-	+/+	
3.1	+/+	+/+		

[a] At 24 h before i.v. inoculation with $5x10^4$ CFU *L. monocytogenes*, mice were treated with one dose from a twofold dose range of MTPPE encapsulated in liposomes.

[b] The amount of lipid in which MTPPE was encapsulated.

[c/d] Numbers of bacteria in the liver/spleen of mice treated with LE-MTPPE (n=6) compared to bacterial numbers in the liver/spleen of PBS-treated mice (n=12) at 120 h after bacterial inoculation. Significance: + = $p \leq 0.05$, - = not significant.

Influence of the IFN-γ to lipid ratio on the antibacterial resistance against L. monocytogenes. Administration of nontherapeutic doses of LE-IFN-γ became effective when encapsulated in increasing amounts of lipid (Table 8.2). Administration of 48 U IFN-γ in 0.2 mg lipid had no effect on bacterial numbers in liver and spleen at 120 h after bacterial inoculation, compared to numbers in PBS-treated mice as shown in Figure 8.3. Increasing the amount of encapsulating lipid led to significantly decreased numbers of bacteria in liver and spleen. The same results were found for 97 U IFN-γ encapsulated in increasing amounts of lipid.

TABLE 8.2 Influence of the IFN-γ to lipid ratio on the antibacterial resistance against *L. monocytogenes*[a]

Dose of	Dose of lipid (mg)[b]					
IFN-γ (U)[a]	0.2	0.4	0.8	1.6	3.1	6.3
0			-[c]/-[d]			-/-
24		-/-	-/-	-/-	-/-	
48	-/-	-/+	-/-	+/+	+/+	+/+
97	-/-	-/+	+/+			
195	+/+					

[a] At 24 h before i.v. inoculation with $5x10^4$ CFU *L. monocytogenes*, mice were treated with one dose from a twofold dose range of IFN-γ encapsulated in liposomes.

[b] The amount of lipid in which IFN-γ was encapsulated.

[c/d] Numbers of bacteria in the liver/spleen of mice treated with LE-IFN-γ (n=6) compared to bacterial numbers in the liver/spleen of PBS-treated mice (n=12) at 120 h after bacterial inoculation. Significance: + = $p \leq 0.05$, - = not significant.

Effect of MTPPE and IFN-γ co-encapsulated in liposomes on L. monocytogenes infection. Administration of 1.6 μg MTPPE and 97 U IFN-γ co-encapsulated in 0.2 mg of liposomal lipid led to significantly decreased bacterial numbers in liver and spleen at 120 h after bacterial inoculation (Table 8.3). A dose of 1.6 μg LE-MTPPE alone, or 97 U LE-IFN-γ alone had no effect on bacterial numbers when administered in 0.2 mg of liposomal lipid. Also twofold lower doses of MTPPE and IFN-γ, 0.8 μg and 48 U, respectively, co-encapsulated in liposomes resulted in significantly decreased numbers of bacteria.

Effect of MTPPE and IFN-γ on the uptake and intracellular survival of L. monocytogenes in macrophages in monolayer. Table 8.4 shows that in macrophages not exposed to MTPPE or IFN-γ numbers of intracellular bacteria increased 7-fold within 6 hours after bacterial uptake. In macrophages exposed for 16 h to MTPPE at a concentration from a fourfold dose range, ranging from 0.1-100 μg/ml, bacterial numbers increased in the same way as in untreated macrophages, (the data obtained at 1.6 μg/ml are shown). Incubation of the macrophages with a dose of IFN-γ from a fourfold dose range, ranging from 15 U- 1000 U/ml, led to inhibition of bacterial growth (the data obtained at the lowest effective dose of

TABLE 8.3 Effect of a combination of MTPPE and IFN-γ co-encapsulated in liposomes on antibacterial resistance against *L. monocytogenes*[a]

Dose of IFN-γ (U)[a]	Dose of MTPPE (μg)[a]		
	0	0.8	1.6
0	ND	-[b]/-[c]	-/-
48	-/-	+/+	ND
97	-/-	ND	+/+

[a] At 24 h before i.v. inoculation with $5x10^4$ CFU *L. monocytogenes*, mice were treated with 0.2 mg liposomal lipid per mouse in which IFN-γ or MTPPE or both IFN-γ and MTPPE were encapsulated.

[b/c] Numbers of bacteria in the liver/spleen of mice treated with LE-MTPPE (n=6) compared to bacterial numbers in the liver/spleen of PBS-treated mice (n=12) at 120 h after bacterial inoculation. Significance: + = $p \leq 0.05$, - = not significant.

250 U/ml are shown). Incubation of the macrophages with a combination of 1.6 μg MTPPE/ml and 250 U IFN-γ/ml led to killing of the intracellular bacteria from 4 h after bacterial uptake.

Effect of scavengers of reactive oxygen and nitrogen metabolites on the killing of L. monocytogenes. Table 8.5 shows that incubation of macrophages, previously activated with a combination of 1.6 μg MTPPE/ml and 250 U IFN-γ/ml, in the presence of scavengers of reactive oxygen metabolites: SOD (300 U/ml) or SA (1 μmol/ml) or CAT (15000 U/ml) or DMSO (300 μmol/ml), or with scavengers of reactive nitrogen metobolites (NMMA, 100 μg/ml) led to inhibition of the killing of intracellular *L. monocytogenes*.

TABLE 8.4 Effect of MTPPE and IFN-γ on uptake and intracellular survival of *L. monocytogenes* in peritoneal macrophages in monolayer[a]

Treatment	Log CFU *L.monocytogenes* per 10^6 macrophages at times (h) after bacterial uptake[b]		
	0	4	6
MTPPE	5.84±0.15	5.86±0.17	6.32±0.16
IFN-γ	5.72±0.07	5.64±0.07*	5.72±0.08**
MTPPE+IFN-γ	5.43±0.09	5.18±0.08**	4.82±0.06**
PBS	5.55±0.05	5.97±0.08	6.34±0.05

[a] Before incubation with bacteria, macrophages in monolayer culture were exposed for 16 h to MTPPE (1.6 μg/ml), IFN-γ (250 U/ml), or MTPPE (1.6 μg/ml) plus IFN-γ (250 U/ml) or PBS.

[b] Data represent the geometric mean ± SEM for six observations or sixteen observations (control). Significance: *$p \leq 0.05$ **$p \leq 0.02$ vs PBS.

TABLE 8.5 Effect of scavengers of reactive oxygen intermediates and reactive nitrogen intermediates on the intracellular survival of *L. monocytogenes* in peritoneal macrophages in monolayer exposed to a combination of MTPPE and IFN-γ[a]

Treatment[a]	Log CFU *L. monocytogenes* per 10^6 macrophages at times (h) after bacterial uptake[b]		
	0	4	6
MTPPE+IFN-γ+SOD	5.73±0.09	6.16±0.04	6.32±0.09
MTPPE+IFN-γ+SA	5.42±0.12	6.14±0.22	5.60±0.10
MTPPE+IFN-γ+CAT	5.37±0.09	6.08±0.17	6.55±0.07
MTPPE+IFN-γ+DMSO	5.24±0.13	6.03±0.02	5.59±0.20
MTPPE+IFN-γ+NMMA	5.71±0.05	5.84±0.10	6.10±0.08
MTPPE+IFN-γ	5.43±0.09	5.18±0.08**	4.82±0.06***
PBS	5.55±0.05	5.97±0.08	6.34±0.05

[a] Before incubation with bacteria, macrophages in monolayer culture were exposed for 16 h to MTPPE (1.6 μg/ml) plus IFN-γ (250 U/ml) or PBS. Uptake and intracellular survival of bacteria was studied in the presence of scavengers: superoxide dismutase (SOD, 300 U/ml), sodiumazide (SA, 1 μmol/ml), catalase (CAT, 15000 U/ml), dimethyl sulfoxide (DMSO, 300 μmol/ml) or N^G-monomethyl-arginine acetate (NMMA, 100 μg/ml).

[b] Data represent the geometric mean ± SEM for sixteen observations (control) or twelve observations (MTPPE+IFN-γ) or six observations (all other treatments). Significance: **$p \leq 0.02$ ***$p \leq 0.002$ versus PBS.

Discussion

An increased resistance against various bacterial infections due to administration of muramyl peptide derivatives was found by several investigators (158,167). However, the reports about the effects of administration of muramyl peptides on resistance against *L. monocytogenes* infection are contradictory. Some investigators (92,154,65,158) found an increased resistance, whereas others (65,158) concluded that muramyl peptide derivatives were not effective in enhancing the host resistance against *L. monocytogenes* infection. This study shows that administration of MTPPE led to increased antibacterial resistance against *L. monocytogenes* infection in mice, in terms of killing of bacteria in liver and spleen. Due to liposomal encapsulation the efficacy of MTPPE was 33-fold increased. These observations are in agreement with those of other investigators who found that the resistance against *K. pneumoniae* infection in mice (136), and herpes simplex virus type 2 infection in mice (112) was increased after encapsulation of MTPPE in liposomes. In tumorcytotoxicity studies it was often found that liposome-encapsulated muramyl peptide derivatives had increased efficacy compared to compounds in the free form (42,172).

This study also shows that administration of another immunomodulator, IFN-γ, led to increased antibacterial resistance against *L. monocytogenes* infection. This effect was 66-fold increased due to liposomal encapsulation. An increased resistance due to administration of

IFN-γ against various bacterial infections was also found by other investigators (95,132,142). However, the reports about the effects of administration of IFN-γ on resistance against *L. monocytogenes* infection show great discrepancy (31,108,203,204). Mellors et al. observed an increased efficacy of IFN-γ after it was encapsulated in liposomes, the efficacy of LE-IFN-γ was 10-fold higher as that of free IFN-γ in in vitro growth inhibition of *Toxoplasma gondii* in macrophages (135). In addition, Smith et al. (194) found an increased efficacy of LE-IFN-γ compared to free IFN-γ in stimulating macrophages to tumorcytotoxic activity.

Liposomal encapsulation of MTPPE or IFN-γ not only allowed a 33- to 66-fold decrease in the dose to obtain the same effects of the immunomodulator in the free form, but it also led to a more rapid decrease in numbers of *L. monocytogenes* in liver and spleen. Probably this is the result of higher MTPPE and IFN-γ concentrations in the liver and spleen,since as a result of liposomal encapsulation the immunomodulators have been targeted to these organs. Liposomes can interact with cells in several ways. Important is the phagocytosis of liposomes by the cells of the MPS, resulting in high intracellular concentrations (156). With respect to this Fidler et al. (59,60) showed that liposomal encapsulation allowed IFN-γ and MTPPE to bind to intracellular receptors of macrophages. It is also expected that liposomes can be adsorbed to cells so that the immunomodulator can slowly leak out, resulting in high local concentrations (156).

We have also shown in this study that the immunomodulator to lipid ratio seemed to be of relevance in the by LE-MTPPE and LE-IFN-γ induced antibacterial resistance. When nontherapeutic doses of LE-MTPPE or LE-IFN-γ were administered in a larger amount of encapsulating lipid (so at a higher lipid:immunomodulator ratio), these doses became effective. Future studies will be necessary to elucidate whether encapsulating a certain amount of immunomodulator in increasing amounts of lipid results in activation of macrophages more efficiently or activation of higher numbers of macrophages. The influence of the immunomodulator:lipid ratio upon the efficacy was also described by other investigators (41,186), who showed a higher level of tumorcytoxicity when a same dose of muramyl dipeptide was administered in more encapsulating lipid.

When macrophages in monolayer were exposed to MTPPE and IFN-γ in combination we have found a synergistic effect of MTPPE and IFN-γ on the bactericidal activity of the macrophages. Incubation of macrophages in vitro with MTPPE alone had no effect on the intracellular growth of *L. monocytogenes*, incubation with IFN-γ alone resulted in growth inhibition, whereas incubation with a combination of MTPPE and IFN-γ led to killing of the intracellular bacteria. Other investigators found that incubation of macrophages with IFN-γ alone resulted in an increased bactericidal (104,179), antifungal (21,138) and antiparasitic (12,48) activity. A synergistic antiparasitic activity of macrophages in vitro against *Leishmania donovani* was shown by Hockertz et al. (89) after incubating the macrophages with a combination of MTPPE and IFN-γ. Exposure of macrophages in vivo to MTPPE and IFN-γ in combination may be realized by injection of liposomes in which both immunomodulators were co-encapsulated. It was shown that co-encapsulation of IFN-γ and MTPPE in liposomes was synergistic in enhancing the antibacterial resistance against *L. monocytogenes* infection. Synergism due to co-encapsulation of muramyl peptide derivatives and IFN-γ was demonstrated in vitro in tumorcytotoxicity studies by Saiki et al. (186) and Fidler et al. (57). They found synergistic effects of MDP and IFN-γ in activating

macrophages in vitro to tumorcytotoxicity. Hockertz et al. (89) found synergistic effects of IFN-γ and MTPPE co-encapsulated in liposomes in stimulating the resistance of mice against *Leishmania donovani* infection.

We found that reactive oxygen as well as nitrogen metabolites seem to play a role in the by MTPPE and IFN-γ induced bacterial killing. Other investigators only have studied the antimicrobial mechanisms of macrophages activated with IFN-γ alone. In these studies reactive oxygen metabolites or reactive nitrogen metabolites played a role in the killing or growth inhibition of microorganisms by IFN-γ activated macrophages. They have demonstrated this by finding an increased production of the metobolites themselves: O_2^-, H_2O_2 (47,101,104) or NO_2^- (47,64) or by showing a prevention of killing of growth inhibition of microorganisms due to incubation of the IFN-γ activated macrophages with scavengers of the metabolites (21,64,170).

In summary, this study shows that administration of MTPPE and IFN-γ led to increased resistance against *L. monocytogenes* infection in terms of decreased bacterial numbers in liver and spleen. Encapsulation of MTPPE or IFN-γ in liposomes increased their efficacy and in addition resulted in a more rapid decrease in bacterial numbers. Exposure of macrophages in vitro to a combination of MTPPE and IFN-γ resulted in a synergistic antibacterial activity. By using liposomes as carriers in which MTPPE and IFN-γ were co-encapsulated also in vivo cells could be exposed to both immunomodulators in combination, resulting in a synergistic antibacterial effect.

CHAPTER 9

GENERAL DISCUSSION

Infections in immunocompromised patients are often treated unsuccessfully as a result of insufficient support from the host defense system to antibiotic treatment (8,164). The cells of the MPS play a major role in the defense against infections. With respect to this, stimulation of these cells might be of great importance for the potentiation of treatment of severe infections. To this aim immunomodulators can be applied. Various experimental studies in animals were performed to investigate the efficacy of immunomodulators to enhance antimicrobial resistance. Models of bacterial infection (83,151,158,207), fungal infection (51,54,100,104,212), viral infection (51,93,112,175), as well as protozoal infection (5,25,45,109,120) were used. The immunomodulators were in most cases administered in the free form via several routes (subcutaneously, intraperitoneally, intravenously or intranasally) and usually a single dose was given. In most studies the resistance against microbial infections was enhanced in terms of increased survival of infected animals. Some immunomodulators, among which MTPPE and IFN-γ are at present under investigation in clinical studies in cancer patients to stimulate cells of the MPS in the removal of metastases (56,70,125). In addition, IFN-γ is successfully used in patients with chronic granulomatous disease, in which the microbial infections could not be cured with antibiotics (11,189).

The **aim of this thesis** was to gain insight into the possibilities and restrictions of clinical application of immunomodulators in the free form as well as in the less toxic liposome-encapsulated form (90,118,188) in order to enhance resistance against bacterial infections. To illustrate this, different models of infection in mice were used.

In one experimental model, infections were induced with *L. monocytogenes*. This bacterium is easily taken up by cells of the MPS and is able to resist killing by these cells.

In other experimental models, infections were induced with *K. pneumoniae*, a bacterium that, when it is not opsonized by specific antibodies, is poorly taken up by cells of the MPS. When *K. pneumoniae* infection was induced by i.v. inoculation, about 80% of the bacteria were cleared from the blood by liver and spleen, resulting in death of all mice due to septicemia (artificially-induced septicemia). In a more clinically relevant model of infection *K. pneumoniae* was inoculated i.p. In this model multiplication of bacteria i.p. resulted in appearance of bacteria in the blood at regular intervals. Eventually all mice died due to septicemia (naturally-induced septicemia). As a parameter for efficacy of treatment, survival of animals was used as well as significant decreases in the numbers of bacteria in the infected organs and blood.

In the first studies, the efficacy of MTPPE and IFN-γ in the free form to induce nonspecific resistance in relation to the genetically determined antimicrobial resistance was investigated. **It appeared that the MTPPE induced resistance against *L. monocytogenes* infection depended on the genetically determined innate resistance to this infection. For the efficacy of IFN-γ no such relationship was observed (Chapter 2, Chapter 3).** Since in man genetic factors also contribute to the susceptibility to intracellular infections caused by *Mycobacterium spp* (22,193) usefulness of MTPPE may be restricted in this type of infection. The MTPPE induced antibacterial resistance seemed to be correlated with the

capacity of MTPPE to recruit leukocytes from the bone marrow. These increased numbers of leukocytes (monocytes, granulocytes, lymphocytes) in the blood after treatment with MTPPE are probably the result of increased levels of CSF, a lymphokine which enhances the proliferation and differentiation of these cells in the bone marrow (34). Increased levels of CSF and increased numbers of leukocytes in the blood were found after administration of another lipophilic muramyl peptide derivative, MDP-Lys(L18) (218). These recruited leukocytes are expected to migrate to the infected organs. The IFN-γ induced resistance against *L. monocytogenes* was not related to the capacity of IFN-γ to recruit leukocytes. The usefulness of IFN-γ to enhance resistance against bacterial infections in man, differing in susceptibility to intracellular infections, has to be investigated.

In the MTPPE and IFN-γ induced resistance against *L. monocytogenes* infection other cell types besides the macrophage are involved. This conclusion derives from our observations that in vitro exposure of macrophages to MTPPE did not result in killing of intracellular *L. monocytogenes*. After exposure of macrophages to IFN-γ intracellular growth was only inhibited. Our in vivo and in vitro observations suggest that the induced antibacterial resistance by MTPPE or IFN-γ is mediated by different mechanisms.

Studies were continued with MTPPE in the free form or in the liposome-encapsulated form in the two models of *K. pneumoniae* infection. The artificially-induced *K. pneumoniae* septicemia could be prevented by administration of a single dose of MTPPE (Chapter 4). LE-MTPPE was also effective even at a 2-fold lower dose. Bacterial numbers were decreased not only in liver and spleen, organs rich in cells of the MPS and primarily important in the clearance of bacteria from the blood, but also in lung and kidney, organs containing fewer cells of the MPS. Whether the decrease in bacterial numbers in these organs is the result of the activation of the macrophages locally, or by a decreased presence of bacteria in the blood due to an increased removal of bacteria from the blood by activated macrophages in liver and spleen, is not yet clear.

To obtain a therapeutic effect in the models of *K. pneumoniae* infection, MTPPE and LE-MTPPE had to be given prophylactically. **In the model of naturally-induced *K. pneumoniae* septicemia a single dose of MTPPE or LE-MTPPE was only partially effective (Chapter 6). Repeated prophylactic administration of LE-MTPPE led to improvement in efficacy.** This emphasizes the importance of liposomal encapsulation, resulting in a less toxic formulation and allowing repeated administration (58,188). As observed in the model of *L. monocytogenes* infection, treatment with MTPPE and LE-MTPPE also resulted in the recruitment of leukocytes in the models of *K. pneumoniae* infection. However, it appeared that, in these infection models, the recruitment of leukocytes did not contribute significantly in the resistance against the infection locally. A discrepancy was observed between the *K. pneumoniae* model and the *L. monocytogenes* model, since in the latter model the MTPPE induced recruitment of leukocytes correlated with the therapeutic effect. In *L. monocytogenes* infection a great influx of monocytes, granulocytes, NK cells and T-cells in the infected liver was observed by other investigators (80), indicating an important role for these cells in the host defense against this infection. **In the MTPPE or LE-MTPPE induced resistance against *K. pneumoniae* infection, the recruitment of leukocytes seemed to be of minor importance. However, the activation of tissue macrophages in liver and spleen by MTPPE or LE-MTPPE was of major importance in the induced resistance**

against this infection (Chapter 5). The clinical relevance of this observation is that, immunocompromised patients, which are often leukopenic, may benifit from treatment with LE-MTPPE. In leukopenic patients, development of septicemia from a local infection occurs at an early stage, and maximal blood clearance capacity of the MPS is of great importance.

A substantial amount of liposomes encapsulating MTPPE was distributed over liver and spleen, the organs which are of primary importance in the uptake of bacteria from the blood (Chapter 7). It was shown that the cells that had taken up *K. pneumoniae* often did not contain LE-MTPPE. Taken also into account the observation that exposure of macrophages to LE-MTPPE in vitro did not result in increased uptake or killing of the intracellular bacteria, **it is suggested that LE-MTPPE does not stimulate macrophages directly to increased uptake or killing of bacteria. Other cell types besides the macrophages are probably involved and communication between the cells by means of cytokines seems necessary (Chapter 7).** Future studies are needed to elucidate which cell types and which cytokines are involved. Such studies will provide further insight into the usefulness of immunomodulators in patients, immunocompromised in different ways.

Administration of MTPPE or IFN-γ in the liposome-encapsulated form also led to resistance against *L. monocytogenes* infection (Chapter 8). The liposomal formulation being effective at a 33-fold or 66-fold lower dose, respectively, compared to the agents in the free form. In this induced antibacterial resistance, **the immunomodulator to lipid ratio appeared to be of importance (Chapter 8).** When nontherapeutic doses of MTPPE or IFN-γ in the liposome-encapsulated form were administered in a larger amount of encapsulating lipid (ie. a lower immunomodulator to lipid ratio), these doses became effective. A possible explanation for this may be that macrophages are more efficiently activated when exposed to more liposomes containing MTPPE or IFN-γ, or that greater numbers of macrophages are activated.

It remains to be elucidated why the liposome-encapsulated form is effective at a lower dose than the agent in the free form. With respect to LE-MTPPE, other investigators have shown that the increased therapeutic efficacy was related to increased levels in lung and spleen, as well as to a much longer presence of relatively high MTPPE concentrations in the liver (66). With respect to LE-IFN-γ, it was shown by others (89) that after liposomal encapsulation more than 60% of the administered IFN-γ was accumulated in liver and spleen, whereas free IFN-γ was distributed equally among all compartments.

It was shown that, exposure of macrophages infected with *L. monocytogenes* in vitro to both MTPPE and IFN-γ at the same time resulted in a synergistic antibacterial effect with the intracellular killing of the bacteria. Reactive oxygen as well as reactive nitrogen metabolites seemed to play a role in the MTPPE plus IFN-γ induced bacterial killing. The value of this synergistic effect for treatment of infections is questionable since exposure of macrophages in vivo to both immunomodulators simultaneously after i.v. administration of the agents is expected to be minimal. However, the synergistic effect in vivo could be realized by administration of the immunomodulators co-encapsulated in the same liposome. **Doses of MTPPE and IFN-γ that were not effective when administered in separate liposomes, resulted in antibacterial resistance against *L. monocytogenes* infection, when administered after co-encapsulation in liposomes (Chapter 8).**

In conclusion, in this thesis it is shown that treatment with MTPPE and IFN-γ in the

liposome-encapsulated form appeared to be useful for enhancing the resistance against bacterial infections. Possibilities as well as restrictions of treatment with MTPPE and IFN-γ, in the free form or in the liposome-encapsulated form, are indicated. For understanding the precise mode of action of LE-MTPPE or LE-IFN-γ more studies have to be performed in the future. In this respect knowledge about which cells and cytokines are involved in the LE-MTPPE and LE-IFNγ induced enhanced antibacterial resistance is needed. This will lead to insight in the usefulness of LE-MTPPE and LE-IFN-γ for treatment of infections in patients, immunocompromised in various ways.

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SUMMARY

From clinical experience it is known that in immunocompromised patients severe infections frequently occur and are a continuing threat to patients. Antimicrobial treatment of these infections is not always successful, despite the use of new and potent antibiotics. Several factors may contribute to the failure of antibiotic treatment. One factor is an impaired host defense, unable to provide adequate support for antibiotic therapy. For the potentiation of treatment of severe infections stimulation of the nonspecific resistance of the host, particularly the cells of the MPS, may be of great value. It is expected that stimulation of the MPS not only leads to an increased resistance against infections involving the MPS but also to infections in general. Immunocompromised patients are often leukopenic, resulting in development of septicemia from a local infection at an early stage. For these patients maximal blood clearance capacity of the MPS is of great importance.

The cells of the MPS can be stimulated by immunomodulators, either from bacterial origin, such as muramyl tripeptide phosphatidylethanolamide (MTPPE), or cytokines, such as interferon-γ (IFN-γ). Since immunocompromised patients are prone to infections for a prolonged period of time, repeated administration of immunomodulators is anticipated. This may result in toxic side effects. To reduce the toxicity the immunomodulating agents can be encapsulated in liposomes. These are microscopic vesicles consisting of one or more lipid bilayers, surrounding an internal aqueous compartment. In liposomes a variety of agents can be entrapped.

In this thesis MTPPE and IFN-γ in the free form or in the liposome-encapsulated form were studied for their efficacy to enhance the nonspecific resistance to bacterial infections. The aim was to obtain an insight in the possibilities and restrictions of treatment with these immunomodulators. This was investigated in experimental bacterial infections in mice.

In one experimental model, infections were induced with *Listeria monocytogenes* after intravenous inoculation. This bacterium is easily taken up by cells of the MPS and is able to resist killing by these cells. It is investigated whether the cells of the MPS, when stimulated by MTPPE or IFN-γ are able to kill *L. monocytogenes*. In other experimental models infections were induced with *Klebsiella pneumoniae*, a bacterium that, when it is not opsonized by specific antibodies, is poorly taken up by cells of the MPS. It is investigated whether in the non-immune host the cells of the MPS, when stimulated by MTPPE are able to effectively phagocytose *K. pneumoniae*. When *K. pneumoniae* infection was induced by intravenous inoculation, about 80% of the bacteria were cleared from the blood by liver and spleen, resulting in death of all mice due to septicemia ('artificially-induced septicemia'). In a more clinically relevant model of infection *K. pneumoniae* was inoculated intraperitoneally. In this model multiplication of bacteria in the peritoneal cavity resulted in appearance of bacteria in the blood at regular intervals. Eventually all mice died due to septicemia ('naturally-induced septicemia').

It is known that genetic factors contribute to the susceptibility of man to infections. Therefore it was investigated whether the difference in susceptibility is a restrictive factor in the usefulness of MTPPE and IFN-γ to induce resistance against *L. monocytogenes* infection (Chapter 2, Chapter 3). For this, mouse strains differing in their genetically determined innate resistance to *L. monocytogenes* were used. It appeared that administration of MTPPE to mice

relatively resistant to *L. monocytogenes* infection resulted in an increased resistance to this infection, whereas in mice, relatively susceptible, a decrease in resistance was observed. These effects appeared to be correlated with the capacity of MTPPE to recruit leukocytes from the bone marrow. In contrast, the IFN-γ induced resistance against *L. monocytogenes* infection seemed not to be related to the genetically determined innate resistance. Administration of IFN-γ led in both mouse strains to an increased antibacterial resistance and was not related to the capacity of IFN-γ to recruit leukocytes.

In the MTPPE and IFN-γ induced resistance to *L. monocytogenes* infection other cell types besides the macrophage are probably involved. This conclusion derives from our observation that in vitro exposure of macrophages to MTPPE had no effect on the intracellular multiplication of *L. monocytogenes*. After exposure of macrophages to IFN-γ, intracellular growth of *L. moncytogenes* was only inhibited. Our in vivo and in vitro observations suggest that the induced antibacterial resistance by MTPPE and IFN-γ is mediated by different mechanisms.

Since in the immunocompromised host protection against infections is needed for a prolonged period of time, it was investigated what the effects were of administration of MTPPE in the less toxic liposome-encapsulated form (LE-MTPPE). This was studied in the models of *K. pneumoniae* infection. The artificially-induced *K. pneumoniae* septicemia could be prevented by a single dose of MTPPE (Chapter 4). LE-MTPPE was also effective even at a 2-fold lower dose. Bacterial numbers were decreased not only in liver and spleen, organs rich in cells of the MPS, but also in lung and kidney, organs containing fewer cells of the MPS. In the naturally-induced *K. pneumoniae* septicemia, administration of a single dose of MTPPE and LE-MTPPE was only partially effective (Chapter 6). The therapeutic efficacy in this model of infection was improved after repeated prophylactic administration of LE-MTPPE. This emphasizes the importance of liposomal encapsulation, resulting in a less toxic formulation and allowing repeated administration.

Particularly in immunocompromised patients, the enhancement of nonspecific resistance against infections by administration of immunomodulators may be of importance. These patients are often leukopenic. It was investigated what the relative roles were of the recruitment of leukocytes, on the one hand, and the activation of tissue macrophages, on the other hand, both of which are induced by MTPPE or LE-MTPPE. In the model of naturally-induced *K. pneumoniae* septicemia it was possible to discriminate between both effects of treatment with MTPPE or LE-MTPPE, since recruitment of leukocytes and their phagocytic capacity now could be quantitated. The recruitment of leukocytes appeared to be of minor importance in the MTPPE or LE-MTPPE induced resistance against *K. pneumoniae* infection (Chapter 5). However, the activation of tissue macrophages in liver and spleen by MTPPE or LE-MTPPE was of major importance. The clinical relevance of this observation is that immunocompromised patients, which are often leukopenic may benefit from treatment with LE-MTPPE.

The observation that the MTPPE and LE-MTPPE induced recruitment of leukocytes in the model of *K. pneumoniae* infection did not contribute significantly to the resistance against the infection locally, is in contrast with findings in the *L. monocytogenes* model in which the MTPPE induced recruitment of leukocytes correlated with the therapeutic effect.

A substantial amount of liposomes encapsulating MTPPE was distributed over liver and

spleen, the organs which are of primary importance in the uptake of bacteria from the blood (Chapter 7). It was shown that the cells that had taken up *K. pneumoniae* often did not contain LE-MTPPE. This indicates that LE-MTPPE does not stimulate macrophages directly to an increased uptake or killing of bacteria. Taken also into account the observation that exposure of the macrophages to LE-MTPPE in vitro did not result in an increased uptake or killing of *K. pneumoniae*, it may be concluded that other cell types besides macrophages appear to be involved and communication between various cells by means of cytokines seems necessary.

Administration of MTPPE or IFN-γ in the liposome-encapsulated form also led to resistance against *L. monocytogenes* infection (Chapter 8). The liposomal formulation was effective at a 33- or 66-fold lower dose, respectively, compared to agents in the free form. In this induced antibacterial resistance the immunomodulator to lipid ratio appeared to be of importance. Nontherapeutic doses of MTPPE or IFN-γ in the liposome-encapsulated form became effective when administered in a larger amount of encapsulating lipid.

Exposure of macrophages infected with *L. monocytogenes* in vitro to both MTPPE and IFN-γ at the same time led to a synergistic antibacterial effect with intracellular bacterial killing. Reactive oxygen as well as reactive nitrogen metabolites seemed to play a role in the MTPPE plus IFN-γ induced bacterial killing. The value of this synergistic effect for treatment of infections is questionable since exposure of macrophages in vivo to both immunomodulators simultaneously after intravenous administration of the agents is expected to be minimal. Therefore it was investigated whether this synergistic effect could be realized by intravenous administration of MTPPE and IFN-γ co-encapsulated in liposomes. Doses of MTPPE or IFN-γ that were not effective when administered in separate liposomes, resulted in antibacterial resistance against *L. monocytogenes* infection, when administered after co-encapsulation in liposomes.

SAMENVATTING

Uit klinische ervaring blijkt dat bij patiënten met een verminderde weerstand ernstige infecties regelmatig voorkomen en een voortdurende bedreiging zijn. Ondanks de toepassing van recent ontwikkelde en in vitro goed werkzame antimicrobiële middelen is de behandeling niet altijd succesvol. Verschillende factoren dragen bij tot het falen van de therapie. Een ervan is het feit dat de afweer van de gastheer verminderd is en onvoldoende ondersteuning kan geven aan de antibioticumbehandeling. Ten behoeve van een verbetering van de behandeling van ernstige infecties zou stimulatie van de niet-specifieke afweer, met name van de cellen van het mononucleaire phagocyten systeem (MPS), van grote waarde kunnen zijn. De verwachting is dat door stimulatie van het MPS zowel de afweer tegen infecties in het MPS zelf, als de afweer tegen infecties in het algemeen versterkt wordt. Patiënten met een verminderde weerstand zijn immers vaak leukopenisch, waardoor een lokale infectie gemakkelijk tot een sepsis kan leiden. Voor deze patiënten is het dan ook van groot belang dat het vermogen van het MPS om bacteriën uit het bloed weg te vangen, maximaal is.

De cellen van het MPS kunnen gestimuleerd worden door immunomodulatoren, hetzij stoffen van bacteriële oorsprong, bijvoorbeeld muramyl tripeptide phosphatidylethanolamide (MTPPE), hetzij cytokinen, bijvoorbeeld interferon-γ (IFN-γ). Omdat patiënten met een verminderde weerstand gedurende een langere periode verhoogd gevoelig zijn voor infecties, zullen naar verwachting de immunomodulatoren herhaalde malen moeten worden toegediend. Dit kan echter toxische neveneffecten tot gevolg hebben. Om de toxiciteit te verminderen kunnen immunomodulerende middelen in liposomen worden ingekapseld. Liposomen zijn microscopisch kleine bolletjes, bestaande uit een of meerdere lipide bilagen die waterige compartimenten omsluiten. In liposomen kunnen allerlei stoffen van verschillende aard worden ingebouwd.

In dit proefschrift wordt beschreven in hoeverre MTPPE en IFN-γ, in vrije vorm of ingekapseld in liposomen (LE-MTPPE, LE-IFNγ), in staat zijn de niet-specifieke afweer tegen bacteriële infecties te versterken. Het doel was om inzicht te krijgen in de mogelijkheden en beperkingen van de behandeling met de twee genoemde immunomodulatoren. Dit werd onderzocht in modellen van bacteriële infecties in muizen.

In één experimenteel model werden infecties geïnduceerd door intraveneuze inoculatie van *Listeria monocytogenes*. Deze bacterie wordt weliswaar gemakkelijk opgenomen door cellen van het MPS, maar kan de intracellulaire killing weerstaan. Onderzocht werd of cellen van het MPS, wanneer gestimuleerd door MTPPE of IFN-γ, de bacterie kunnen doden. In andere experimentele modellen werden infecties geïnduceerd door *Klebsiella pneumoniae*. Dit is een bacterie die, wanneer niet geopsoniseerd door specifieke antilichamen, moeilijk wordt opgenomen door cellen van het MPS. Onderzocht werd of in de niet-immune gastheer stimulatie van de cellen van het MPS door MTPPE, leidde tot effectieve opname en killing van de bacterie. In de *K. pneumoniae* infectie geïnduceerd door intraveneuze inoculatie, werd ongeveer 80% van de bacteriën door de lever en milt uit het bloed weggevangen, hetgeen resulteerde in de dood van alle muizen als gevolg van sepsis ('artificieel-geïnduceerde sepsis'). In een ander klinisch meer relevant infectiemodel, werd *K. pneumoniae* intraperitoneaal geïnoculeerd. Vermenigvuldiging van bacteriën in de peritoneale holte leidde tot een regelmatige strooiing van bacteriën in het bloed. Uiteindelijk stierven alle muizen ten

gevolge van sepsis ('natuurlijk-geïnduceerde sepsis').

Bij de mens wordt de gevoeligheid voor infecties mede bepaald door genetische factoren. In dit proefschrift werd onderzocht of dit verschil in infectiegevoeligheid een beperkende factor is in de bruikbaarheid van MTPPE en IFN-γ om de afweer tegen de *L. monocytogenes* infectie te verhogen (Hoofdstuk 2, Hoofdstuk 3). Hiervoor werden muizestammen gebruikt, met een verschillende genetisch bepaalde weerstand tegen *L. monocytogenes* infectie. Toediening van MTPPE aan muizen die relatief infectieresistent waren, leidde bij deze muizen tot een versterking van de afweer. Daarentegen leidde MTPPE behandeling van muizen die relatief infectiegevoelig waren tot een vermindering van de afweer. Deze effecten bleken te correleren met het vermogen van MTPPE om leukocyten uit het beenmerg te recruteren. In tegenstelling tot de effecten verkregen met MTPPE, bleek er bij de IFN-γ geïnduceerde afweer tegen *L. monocytogenes* infectie geen relatie te zijn met de genetisch bepaalde infectiegevoeligheid. Toediening van IFN-γ leidde in beide muizenstammen, tot een verhoogde antibacteriële bescherming, en was niet gerelateerd aan het vermogen van IFN-γ om leukocyten uit het beenmerg te recruteren.

In de door MTPPE en IFN-γ geïnduceerde afweer tegen *L. monocytogenes* infectie zijn behalve de macrofaag waarschijnlijk ook andere celtypen betrokken. Dit kan geconcludeerd worden uit de waarneming dat blootstelling van macrofagen in vitro aan MTPPE geen effect heeft op de intracellulaire vermenigvuldiging van *L. monocytogenes*. Blootstelling van de macrofagen aan IFN-γ resulteerde slechts in verhindering van de intracellulaire bacteriële vermeerdering. Onze in vivo en in vitro bevindingen suggereren dat de door MTPPE en IFN-γ geïnduceerde verhoogde antibacteriële bescherming tot stand komt via verschillende mechanismen.

Omdat de infectiebescherming in patiënten met verminderde weerstand voor een langere periode noodzakelijk is, werd onderzocht wat de effecten waren van MTPPE in de minder toxische, liposomaal-ingekapselde vorm (LE-MTPPE). De studies werden verricht in de modellen van *K. pneumoniae* infectie. De artificieel-geïnduceerde sepsis kon worden voorkómen middels een enkelvoudige dosis van MTPPE (Hoofdstuk 4). LE-MTPPE was eveneens effectief, zelfs bij een 2-voudig lagere dosis. Niet alleen in de lever en de milt, organen rijk aan cellen van het MPS, werd killing waargenomen, ook in de long en de nier, organen die veel minder cellen van het MPS bevatten. In de natuurlijk-geïnduceerde *K. pneumoniae* sepsis bleek een enkelvoudige dosis MTPPE of LE-MTPPE slechts ten dele effectief (Hoofdstuk 6). Het therapeutisch effect van LE-MTPPE kon worden verhoogd door de stof herhaaldelijk prophylactisch toe te dienen. Dit onderstreept het belang van liposomale inkapseling van MTPPE, aangezien deze minder toxische formulering herhaaldelijk toedienen mogelijk maakt.

Vooral voor patiënten met verminderde weerstand is versterking van de niet-specifieke afweer door toediening van immunomodulatoren van groot belang. Deze patiënten zijn vaak leukopenisch. Onderzocht werd wat de relatieve bijdrage was van enerzijds het recruteren van leukocyten, anderzijds het aktiveren van weefselmacrofagen, beide effecten geïnduceerd door MTPPE of LE-MTPPE. In het model van natuurlijk-geïnduceerde *K. pneumoniae* sepsis waarin bacteriën intraperitoneaal geïnoculeerd worden, was het mogelijk een onderscheid te maken tussen de twee genoemde effecten, omdat de recrutering van leukocyten en het fagocyterend vermogen van deze leukocyten konden worden gekwantificeerd. Het recruteren

van leukocyten bleek van ondergeschikt belang in de door MTPPE- of LE-MTPPE-geïnduceerde bescherming tegen *K. pneumoniae* infectie (Hoofdstuk 5). Daarentegen bleek de activatie van weefselmacrofagen in lever en milt door MTPPE of LE-MTPPE wel van groot belang. De klinische relevantie van deze bevinding is, dat patiënten met een verminderde weerstand die leukopenisch zijn, ook voordeel zouden kunnen hebben van behandeling met LE-MTPPE.

De waarneming dat de door MTPPE- en LE-MTPPE-geïnduceerde recrutering van leukocyten in het model van *K. pneumoniae* infectie niet significant bijdroeg aan de afweer tegen een lokale infectie is tegengesteld aan de bevindingen in het *L. monocytogenes* model, waarin wel een correlatie tussen de door MTPPE-geïnduceerde recrutering van leukocyten en het therapeutisch effect bestond.

Een groot deel van de liposomen die MTPPE bevatten bleek terecht te komen in de lever en de milt, organen die belangrijk zijn voor het wegvangen van bacteriën uit het bloed (Hoofdstuk 7). Aangetoond werd dat cellen die *K. pneumoniae* hadden opgenomen meestal geen LE-MTPPE bevatten. Geconcludeerd kan worden dat LE-MTPPE niet rechtstreeks macrofagen stimuleert tot een verhoogde opname of killing van bacteriën. Mede gelet op het feit dat blootstelling van macrofagen in vitro aan LE-MTPPE niet leidt tot een verhoogde opname of tot killing van *K. pneumoniae*, lijkt het waarschijnlijk dat naast macrofagen ook andere celtypen hierbij betrokken zijn en dat communicatie tussen de verschillende cellen door middel van cytokines daarbij noodzakelijk is.

In het model van de *L. monocytogenes* infectie leidde toediening van MTPPE of IFN-γ in de liposomaal-ingekapselde vorm eveneens tot verhoogde antibacteriële bescherming (Hoofdstuk 8). De liposomale formuleringen van MTPPE en IFN-γ waren effectief bij een respectievelijk 33- en 66-voudig lagere dosis, vergeleken bij de stoffen in de vrije vorm. In deze geïnduceerde antibacteriële bescherming bleek de immunomodulator/lipid ratio van belang. Niet-therapeutische doses van MTPPE of IFN-γ in de liposomaal-ingekapselde vorm hadden wel effect wanneer ze werden toegediend in een grotere hoeveelheid lipid.

Het gelijktijdig blootstellen in vitro van *L. moncytogenes* geïnfecteerde macrofagen aan zowel MTPPE als IFN-γ, resulteerde in een synergistisch antibacterieel effect, namelijk killing van intracellulaire bacteriën. Reactieve zuurstofmetabolieten en stikstofmetabolieten bleken een rol te spelen in de door MTPPE plus IFN-γ geïnduceerde bacteriële killing. De waarde van dit synergistisch effect voor de infectiebehandeling is onduidelijk, omdat gelijktijdige blootstelling van macrofagen aan beide immunomodulatoren na intraveneuze toediening naar verwachting zeer beperkt is. Daarom werd onderzocht of het synergistisch effect benut kan worden na toediening van liposomen waarin zowel MTPPE als IFN-γ zijn ingekapseld. Doses van MTPPE en IFN-γ die niet effectief waren wanneer toegediend in afzonderlijke liposomen, bleken wel effectief in de bescherming tegen *L. monocytogenes* infectie wanneer toegediend na inkapseling in dezelfde liposomen.

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CURRICULUM VITAE

De schrijfster van dit proefschrift werd geboren op 17 maart 1962 te Oudenbosch. Zij doorliep het VWO en begon in 1980 aan de studie Biologie aan de Rijksuniversiteit te Utrecht. Drie jaar later slaagde zij voor het kandidaatsexamen. Daarna werden de hoofdvakken Klinische Immunologie (bij Prof. Dr Ballieux) en Klinische Cytogenetica (bij Prof. Dr van Arkel) en het bijvak Vergelijkende Endocrinologie (bij Prof. Dr Thijssen) gevolgd. In februari 1987 ontving de schrijfster haar doctoraaldiploma. Vervolgens was zij aangesteld als assistent in opleiding bij het Instituut Klinische Microbiologie en Antimicrobiële Therapie van de Faculteit der Geneeskunde en Gezondheidswetenschappen, Erasmus Universiteit Rotterdam, waar zij het in dit proefschrift beschreven onderzoek verrichtte onder begeleiding van Mw Dr Bakker-Woudenberg en Prof. Dr Michel. Tijdens deze periode volgde zij eveneens de postdoctorale opleiding tot immunoloog bij Prof. Dr Benner.

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