Effects of Treatment with Muramyl Dipeptide and Certain of Its Analogs on Resistance to *Listeria monocytogenes* in Mice

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Studies were carried out to determine whether treatment of mice with the synthetic adjuvant muramyl dipeptide (MDP) afforded any resistance to infection with *Listeria monocytogenes*. Regardless of the timing, dose, or route of administration, there was no evidence that treatment with either MDP or two of its analogs (des-MDP or MDP-D-D) induced any resistance to listeria infection in BALB/c, CBA/J, or C57BL/6j mice. In contrast, pretreatment with MDP induced marked protection to infection with *Streptococcus pneumoniae* (type III).

The synthetic compound N-acetyl muramyl-L-alanyl-D-isoglutamine (muramyl dipeptide [MDP]) has been shown to possess the minimal chemical structure for replacing the mycobacterial component in Freund complete adjuvant (1, 6, 20). Administration of MDP to responsive strains of mice in an aqueous solution with the appropriate antigen or treatment of mouse spleen cell cultures in vitro with MDP can result in a marked enhancement of the in vivo (2, 16) and in vitro (7, 14, 15) antibody responses. In addition, treatment of mice with MDP and certain of its analogs induced resistance to challenge infection with *Klebsiella pneumoniae* (4, 23), *Trypanosoma cruzi* (12), *Toxoplasma gondii* (Krahenbuhl et al., submitted for publication), *Pseudomonas aeruginosa*, or *Candida albicans* (T. R. Matthews and E. B. Fraser-Smith, Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 19th, Boston, Mass., abstr. no. 705, 1979).

The present studies were carried out to determine whether treatment with MDP and certain of its analogs enhances resistance of mice to challenge with *Listeria monocytogenes*. Since resistance to listeria is effected solely by the enhanced microbicidal capacity of activated macrophages (18, 22), the present studies also address the hypothesis that enhancement of resistance to infection by treatment with MDP results from the ability of this compound to activate macrophages (9).

**MATERIALS AND METHODS**

**Mice.** Locally bred BALB/c, as well as CBA/J and C57BL/6j, mice from Jackson Laboratories (Bar Harbor, Maine) were employed. All mice were females and weighed 18 to 22 g at the start of each experiment.

**Synthetic glycopeptides.** MDP, N-acetyl muramyl-d-alanyl-d-isoglutamine (MDP-D-D) and N-acetyl desmethylmuramyl-L-alanyl-D-isoglutamine (des-MDP) were provided by Gordon Jones, Synthetic Chemistry Division, Syntex Research Inc., Palo Alto, Calif. A single dose was usually employed (500 μg), but the route of administration (subcutaneous [s.c.], intravenous [i.v.], or intraperitoneal [i.p.]) and timing of treatment were varied as described below.

**Corynebacterium parvum.** A killed suspension of *C. parvum* (lot no. CA 748, 7 mg/ml, dry weight) was kindly provided by Richard Tuttle (Burroughs Wellcome Laboratories, Research Triangle Park, N.C.). To induce nonspecific resistance to listeria, mice were injected i.v. with 700 μg of *C. parvum* 7 days before challenge.

**L. monocytogenes.** The EGD strain of *L. monocytogenes* employed was obtained originally from George McKaness of the Trudeau Institute, Saranac Lake, N.Y. A standard inoculum was prepared as follows: the organisms were grown overnight in Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md.), washed, and resuspended in TSB containing 1% gelatin, and aliquots were frozen at −70°C. Over the course of these experiments, the viability of this inoculum did not vary as determined by plating on tryptic soy agar (TSA). The i.v. 50% lethal dose (LD50) of this inoculum was 6 x 104 colony-forming units (CFU). Unless mentioned otherwise the challenge inoculum consisted of 2 x 104 viable listeria given i.v.

**Determination of listerial CFU in spleen.** At various times after challenge (usually 24 and 48 h) mice were sacrificed by cervical dislocation, their spleens were removed aseptically, and each spleen was homogenized in cold saline with Teflon pestle tissue grinders (Thomas Scientific, Philadelphia, Pa). Serial 10-fold dilutions of each homogenate were made in cold saline, plated on TSB, and incubated at 37°C to determine the number of CFU.

**Streptococcus pneumoniae.** *S. pneumoniae* type III was obtained from L. P. Mallavia, Washington State University, Pullman. The organisms were grown overnight in brain heart infusion (BHI) broth (Difco Labo...
Laboratories, Detroit, Mich.), washed, and frozen at 
−70°C in BHI containing 50% glycerol. The viability 
of this preparation was confirmed at the time of each 
inoculation by determining the number of CFU on 
blood agar plates.

RESULTS

The growth of listeria in the spleens of normal 
mice injected i.v. with $2 \times 10^4$ organisms is 
shown in Fig. 1. The number of CFU per spleen 
increased during the first 3 days of infection. 
However, since the dose of listeria employed was 
sublethal, multiplication ceased at 72 h and de-

clined thereafter. Treatment with C. parvum 7 
days before challenge with listeria induced a 
level of nonspecific resistance to infection which 
was readily apparent by quantitation of CFU in 
the spleen at 24 and 48 h ($P < 0.001$) after i.v. 
challenge with listeria (Fig. 2). To determine 
whether treatment with MDP induced nonspe-
cific resistance to listeria, we carried out a series 
of experiments in which the route and timing of 
treatment with MDP were varied. A single i.v., 
i.p., or s.c. injection of 500 µg of MDP in BALB/
c mice 1 day before challenge with listeria failed 
to induce any detectable resistance to infection 
(Fig. 3A). Treatment by the i.p. or s.c. route on 
each of the 3 consecutive days before challenge 
did not protect against infection (data not 
shown), and variation of the dose of MDP from 
500 to 2,000 µg was also without effect (Fig. 3B). 
Other strains of mice employed (C57BL/6 and 
CBA) were no more responsive to MDP treat-
ment than BALB/c (Fig. 3C), nor did either of 
two analogs of MDP (des-MDP and MDP-D-D) 
prove to be more effective than the parent com-

pound (Fig. 3D). Similarly, when a lethal dose of 
listeria was employed ($10^4$ i.v.), treatment 
with MDP 1 or 7 days before infection failed to 
enhance resistance to infection as measured 
either by growth of the organisms in the spleen 
(Fig. 4A) or by determination of time to death 
(Fig. 4B).

As proof that the preparation of MDP em-
ployed in the listerial studies described above 
was capable of enhancing resistance to infection, 
Fig. 5 shows that BALB/c mice treated s.c. with 
500 µg of MDP on the day before i.v. infection 
with S. pneumoniae were markedly resistant to 
the lethal effects of pneumococcal infection.

DISCUSSION

Although treatment of mice with MDP has 
been shown to afford nonspecific resistance to 
infection with such a diverse selection of patho-
gens as K. pneumoniae (4, 23), T. cruzi (12), T. 
gondii (Krahenbuhl et al., submitted for pub-
lication), P. aeruginosa, and C. albicans (Mat-
thews and Fraser-Smith, Prog. Intersci. 
Agents Chemother., 19th, 

Boston, Mass., abstr. no. 705, 1979) the ability 
of MDP to protect against infection does not 
appear to extend to L. monocytogenes. A sublethal 
dose of listeria was employed, and the growth of 
the organism was quantitated during the first 48 
h of infection to determine whether treatment

![Fig. 1. Growth of listeria in the spleens of normal BALB/c mice. Each point represents the mean ± the standard error of the mean of the CFU from 7 to 10 mice. Inoculum = $2 \times 10^4$ listeria given i.v.](http://iai.asm.org/)

![Fig. 2. Growth of listeria in the spleens of normal (○) and C. parvum-treated (▲) BALB/c mice. Each point represents the mean CFU from five mice. Inoculum = $2 \times 10^4$ listeria given i.v.](http://iai.asm.org/)
with MDP enhances the innate bactericidal capacity of the host's macrophages during the interval which precedes the induction of the specific T-cell-mediated macrophage activation process which subsequently clears the organism from the tissues. In contrast to our negative findings with MDP and listeria, the methods employed clearly detected enhanced nonspecific

**Fig. 3.** Growth of listeria in the spleens of normal or MDP-treated mice. Each point represents the mean CFU from five mice. (A) Effects of route of MDP (500 µg) treatment on day -1. •, i.p.; ▲, i.v.; ■, s.c.; solid line, control; dashed line, treated. (B) Effects of various doses of MDP treatment given s.c. on day -1. •, control; •, 500 µg; ■, 1,200 µg; ▲, 2,000 µg. (C) Effects of mouse strain. MDP (500 µg) injected i.v. on day -1. •, BALB/c; ■, C57BL/6; ▲, CBA; solid line, control; dashed line, treated. (D) Effects of MDP analog (500 µg). Treatment given i.v. on day -1. •, control; •, MDP-D-D; ■, des-MDP; ▲, MDP.

**Fig. 4.** Growth of listeria in normal (•) BALB/c mice and mice treated i.v. with MDP 1 (▲) or 7 (■) days before infection. Growth of listeria in the spleen is shown in (A). Each point represents the mean CFU from five mice. Cumulative percent death is shown in (B) (20 mice per group). Inoculum = 10⁵ listeria given i.v.

**Fig. 5.** Effects of treatment with MDP on infection of BALB/c mice with S. pneumoniae. •, control; ○, MDP given s.c. on day -1. Inoculum = 10⁵ pneumococci given i.v.
resistance to listeria induced by pretreatment with C. parvum.

Our results confirm and extend two recent reports on the effects of MDP on listerial infection. In the study by Chedid et al. (3) they reported that a single i.v. injection of 300 μg of MDP into Swiss mice 4 days before listeria challenge did not significantly prolong their survival. Finger and Von König (8) treated mice i.p. with 100 to 500 μg of MDP 20 min or 5 days before challenge and found no enhanced resistance to listerial infection. In the latter study, no evidence was presented that the strain of mouse employed (NMRI) was responsive to MDP. In the present study the dose of MDP employed was comparable to that previously reported to be effective against a variety of other pathogens (4, 12, 23) and was sufficient to provide marked resistance to pneumococci. Two of the three strains of mice employed in the present study (BALB/c and CBA/J) are known to be responsive to MDP (5, 27). However, regardless of the timing dose, or route of administration, we found no evidence that treatment with either MDP or two of its analogs (des-MDP or MDP-D-D) afforded any protection to listeria, as determined either by measurement of multiplication of a sublethal dose of the organism in the spleen or by measurement of time to death after a lethal challenge dose. The inability of MDP treatment to induce any detectable resistance to listerial infection contrasts with its ability to protect against infection with pneumococci and the protozoan Toxoplasma gondii (Krahenbuhl et al., submitted for publication).

Although the findings of the present report were negative they offer a clue to the mechanism(s) underlying the ability of MDP treatment to protect a host against infection. Resistance to infection with facultative intracellular bacteria such as listeria is not mediated by humoral antibodies (18). Rather, resistance to listeria depends upon the acquisition of an enhanced microbicidal capacity by the host’s macrophages (19, 22). The term “activated macrophage” was employed originally by Mackaness (19) to describe the increased capacity of these cells to kill intracellular organisms. This term is generally considered to be applicable, as well, to the enhanced cytotoxic capacity of macrophages for tumor target cells, although there are a variety of situations in which microbicidal and cytotoxic functions do not coexist in a population of activated macrophage (29).

A number of reports have previously suggested that MDP-induced immunopotentiation stems from the effects of this compound on macrophages. Fevrier et al. (7) implicated the macrophage in the potentiating effect of MDP on anti-sheep erythrocytes responses in spleen cell cultures (23, 24). Wahl et al. (28) cultured oil-induced peritoneal exudate (PE) macrophages from guinea pigs and showed that treatment with MDP induced elevated synthesis of collagenase and production of a fibroblast proliferating factor(s). Takada et al. (26) showed that culture of thioglycolate-induced guinea pig PE macrophages with MDP stimulated their incorporation of [14C]glucosamine, and Nagao et al. (21) employed paraffin oil-induced guinea pig PE macrophages and reported that MDP inhibited their migration from capillary tubes.

Each of the studies described above involved the exposure of macrophages to MDP in vitro and, although the parameters of macrophage function which were measured were enhanced, none is directly linked to the enhanced microbicidal or cytotoxic capacity of the activated macrophage (11). More pertinent to these effector functions of macrophages is the report of Hadden et al. (9) in which oil-induced guinea pig PE macrophages incubated with MDP acquired an enhanced phagocytic and bactericidal capacity for listeria. In a different experimental model Löwy et al. (18) reported that the phagocytic capacity of mouse macrophages was not enhanced by treatment in vitro with MDP. However, the studies by Juy and Chedie (10) and Taniyama and Holden (27) showed that mouse peritoneal macrophages and macrophage cell lines treated with MDP in vitro acquired an enhanced cytotoxic capacity for tumor target cells. Other than the demonstration that treatment with MDP enhances the carbon clearance capacity of the reticuloendothelial system (17, 26), there is a paucity of evidence that altered macrophage function from in vivo treatment with MDP underlies enhanced resistance to infection.

The negative findings of the present report are consistent with those of Juy and Chedie (10) and Leclerc et al. (13), who found that macrophages from mice treated with MDP were not cytostatic for tumor target cells. Recent studies from our own laboratory (Krahenbuhl et al., submitted for publication) also showed that macrophages from mice treated with MDP were neither cytotoxic for tumor cells nor microbicidal for Toxoplasma.

Thus, in the absence of any direct evidence from the work of others that treatment with MDP in vivo enhances the effector functions of macrophages, the negative findings of the present study support the contention that treatment with this compound affords resistance to infection by mechanism(s) other than the enhance-
ment of the microbicidal capacity of the host's macrophages.

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LITERATURE CITED


