PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 176, 366–370 (1984)

Muramyl Dipeptide-Induced Enhancement of Phagocytosis of Antibiotic Pretreated *Escherichia coli* by Macrophages (41884)

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Abstract. Treatment of mice with muramyl dipeptide, a known immunoadjuvant, resulted in marked augmentation of the phagocytic activity of peritoneal macrophages incubated *in vitro* with *Escherichia coli.* Even greater phagocytosis occurred when the *E. coli* were pretreated for 2 hr with subinhibitory concentrations of the semisynthetic penicillins cyclacillin or ampicillin, but not penicillin G to which they were resistant. The antibiotic-pretreated *E. coli* were more rapidly ingested by the macrophages derived from MDP-treated mice as compared to similar cells from normal mice. Optimum augmentation of phagocytosis of untreated or antibiotic-pretreated *E. coli* occurred 2 to 3 days after administration of MDP to the mice. Similar augmentation of phagocytosis occurred by treating cultures of peritoneal macrophages from normal mice *in vitro* with MDP prior to incubation with the antibiotic-pretreated bacteria. These results indicate that macrophages from MDP stimulated mice interact with antibiotic-pretreated bacteria to a greater extent than with untreated *E. coli,* resulting in increased phagocytosis and killing of the bacteria.

A wide variety of substances stimulate increased phagocytic activity of macrophages, either *in vivo* or *in vitro* (1–4). In this regard, purified bacterial cell wall products, as well as synthetic analogs of the adjuvants of such cell walls are strong immunologic stimulators and macrophage activators (5, 6). A large number of studies have shown that substances such as muramyl dipeptide (MDP), widely studied as a synthetic analog of the cell wall adjuvant derived from microorganisms such as mycobacteria, activate macrophages and increase their phagocytic activity (4–12). Previous studies in this laboratory, as well as others, have shown that bacteria such as staphylococci or *Escherichia coli,* when incubated *in vitro* with an antibiotic which inhibits cell wall synthesis, become extremely susceptible to killing by phagocytic cells such as macrophages, polymorphonuclear cells, neutrophils, etc. (13–22). In the present study *E. coli,* when incubated with semisynthetic penicillins such as cyclacillin or ampicillin, but not with penicillin G to which they were resistant, became much more susceptible to phagocytosis not only by macrophages from normal mice, but also from animals pretreated with the macrophage stimulator MDP.

Experimental Methods and Procedures. *Bacteria.* A penicillin-resistant strain of *E. coli* was utilized for the study (13, 16). The bacteria were cultured for 18 hr in brain–heart infusion broth at 37°C, washed by centrifugation at 10,000*g* with sterile saline and resuspended to a concentration of 10^8 bacteria per milliliter saline as determined by standard plate counting. The bacteria were resistant to at least 500 U penicillin/ml but were susceptible to cyclacillin and ampicillin, with an MIC of 5–10 μg/ml.

Animals. Inbred BALB/c mice weighing 16–18 g each were obtained from The Jackson Laboratory (Bar Harbor, Me.). They were maintained in groups of five to six in plastic mouse cages and fed Purina mouse pellets and water *ad libitum.*

Phagocytic assay. Cells from the peritoneum of unstimulated mice were obtained by lavage with 5 ml sterile Medium 199. The cells in the lavage fluid were pelleted by centrifugation at 1000*g*, and resuspended in Medium 199 containing 10% fetal calf serum. The percentage of macrophages in the cell suspensions was ascertained by microscopic examination after Giemsa staining. Over 90% of the cells readily phagocytized latex particles within 15 min at 37°C as determined by microscopic examination. For phagocytic studies, a suspension of 10^6 nucleated cells in 0.5 ml medium were incubated for up to 4 hr at 37°C

0037-9727/84 $1.50

with 10^7 or 10^8 bacteria. The number of phagocytizing cells per 100 macrophages was determined by microscopic examination, usually with triplicate samples, and the average number of phagocytizing cells per culture calculated.

Muramyl dipeptide. MDP was obtained as a kind gift from the Pasteur Institute, Paris, France, through the courtesy of Dr. Louis Chedid. The adjuvant was suspended in saline at varying concentrations and injected into animals or added to cultures as needed.

Antibiotics. Cyclacillin, ampicillin, and penicillin G were obtained from Wyeth Laboratories and resuspended in saline in the appropriate concentrations (16).

Experimental design. Dispersed macrophage preparations were obtained from normal or MDP-pretreated mice and suspensions of 10^6 washed cells were placed in test tubes to which were added graded numbers of viable *E. coli,* either untreated or previously treated for 2 hr with the indicated concentration of antibiotic. Following further incubation of the bacteria–cell mixture for 30 min to 4 hr, aliquots of the cell samples were washed, the supernatants plated for bacteria, and the pelleted cells examined microscopically, after staining with gram stain, for the numbers of bacteria per cell. In some experiments the pelleted cells, after washing, were frozen and thawed three times and the numbers of bacteria determined by standard plate counting. In all cases at least 100 macrophages were examined for phagocytic activity per slide.

Experimental Result. As is apparent in Table I, peritoneal macrophages from MDP-treated mice had approximately twice as much phagocytic activity as those from normal mice after incubation with untreated *E. coli.* However, when the *E. coli* were pretreated for 2 hr with graded amounts of cyclacillin, ampicillin, or penicillin, and then washed before incubation with the macrophage cultures, marked differences in the percentage of phagocytizing cells occurred. Those *E. coli* which had been preincubated with cyclacillin or ampicillin were much more readily phagocytized by macrophages from both normal as well as MDP-treated mice. In general, macrophages from the MDP-treated mice phagocytized twice as many antibiotic-pretreated bacteria as did the macrophages from normal control mice. It was apparent, however, that when *E. coli* were pretreated with penicillin, either a high or low dose, there was essentially no difference in their uptake by macrophages as compared to the uptake of untreated bacteria.

TABLE I. EFFECT OF MDP PRETREATMENT OF MICE ON PHAGOCYTIC ACTIVITY OF PERITONEAL MACROPHAGES INCUBATED WITH ANTIBIOTIC-PRETREATED *E. coli*

Antibiotic pretreatment of *E. coli*[a]	Macrophage source for phagocytosis[b]		
	Normal mice	MDP pretreated[c]	P[d]
None (control)	5.2 ± 1.3	11.3 ± 2.1	<0.05
Cyclacillin (μg)			
10.0	15.3 ± 2.4	30.5 ± 2.6	<0.01
5.0	18.5 ± 3.1	32.6 ± 3.8	<0.01
1.0	12.3 ± 1.2	25.2 ± 1.9	<0.01
Ampicillin (μg)			
10.0	12.3 ± 2.1	28.3 ± 3.1	<0.01
5.0	15.9 ± 3.1	31.1 ± 2.7	<0.01
1.0	16.1 ± 2.9	25.2 ± 3.2	<0.01
Penicillin G (U)			
200	6.1 ± 0.8	10.3 ± 1.2	<0.05
50	5.8 ± 0.7	11.5 ± 0.9	<0.05

[a] Indicated antibiotic incubated with 10^8 *E. coli* for 2 hr at 37°C before assay.

[b] Average percentage ± SD of macrophages taking up two or more *E. coli* after incubation for 2 hr at 37°C with 10^8 *E. coli.*

[c] Mice injected 2 days earlier with 50.0 μg MDP.

[d] Probability determined by Student's *t* test (probability between MDP pretreated vs control for each group).

In other experiments (data not shown) it was found that greater killing of the bacteria occurred with macrophages from MDP-treated mice as compared to normal mice. Following incubation with the bacteria for 2 hr fewer organisms were recovered in the supernatant or cell-free lysates from cultures of macrophages derived from MDP-pretreated mice as compared to control cultures, using *E. coli* pretreated with cyclacillin or ampicillin, but not penicillin.

The time of administration of MDP to mice markedly affected the ability of the peritoneal cells to phagocytize either untreated or antibiotic-pretreated *E. coli* (Table II). Maximum augmentation of phagocytosis occurred when peritoneal cells were obtained from mice given MDP 2 days earlier. Increased phagocytosis

TABLE II. EFFECT OF TIME OF ADMINISTRATION OF MDP TO MICE ON ABILITY OF PE MACROPHAGES TO PHAGOCYTIZE UNTREATED OR ANTIBIOTIC-PRETREATED *E. coli*

Day of injection[a]	Phagocytosis of *E. coli*[b] None	Cyclacillin (2.0 μg)	Penicillin (100 U)
None	4.2 ± 0.8	14.1 ± 2.3*	5.3 ± 1.1
MDP			
1	9.1 ± 1.2	22.6 ± 2.1*	8.1 ± 1.2
2	11.3 ± 1.6	30.1 ± 3.2*	12.3 ± 1.9
4	7.2 ± 0.8	17.8 ± 1.9*	6.7 ± 0.7
8	5.1 ± 1.1	15.3 ± 2.1*	8.2 ± 1.1
15	4.3 ± 0.7	13.8 ± 1.7*	5.3 ± 0.8

[a] Mice injected ip with 50 μg MDP on day indicated before assay for phagocytosis.

[b] Average percentage ± SD of PE macrophages phagocytizing two or more *E. coli* after 2-hr incubation at 37°C with 10^8 *E. coli* either untreated or after 2-hr pretreatment with indicated antibiotic.

* $P < 0.02$ determined by Student's t test for phagocytosis of cyclacillin vs untreated bacteria.

also occurred, but to a lesser extent, when the macrophages were obtained 1 day after MDP administration. However, by the third to fourth day after MDP administration there was only a slight or marginal effect on phagocytic activity. By 8 to 15 days after MDP pretreatment there was essentially no detectable effect.

MDP addition to cultures of normal macrophages increased their ability to phagocytize *E. coli,* either untreated or pretreated with cyclacillin (Table III). Addition of 1–10 μg MDP to cultures of normal macrophages 2 hr prior to addition of the *E. coli* resulted in marked stimulation of the ability of the macrophages to phagocytize the bacteria. In other experiments (data not shown) addition of MDP at the time of culture initiation had marginal, if any effect on the uptake of the bacteria.

Discussion and Conclusions. Results of this study indicate that macrophages obtained from the peritoneum of mice stimulated with a known macrophage activator, i.e., MDP, showed enhanced ability to phagocytize and kill *E. coli in vitro.* The total number of phagocytized *E. coli* in the macrophages and the percentage of macrophages showing uptake of two or more *E. coli* increased markedly when these cells were obtained from MDP-treated mice. Furthermore, addition of MDP to cultures of macrophages *in vitro* resulted in markedly stimulated phagocytosis of both untreated and cyclacillin or ampicillin-pretreated *E. coli.*

Previous studies had shown that semisynthetic penicillins, i.e., cyclacillin and ampicillin, markedly increased the susceptibility of *E. coli* to killing by antibody and complement *in vitro* or by macrophages (13–16). It seemed likely that a subinhibitory dose of the anti-

TABLE III. EFFECT OF MDP ADDITION TO CULTURES OF NORMAL MOUSE PERITONEAL MACROPHAGES ON PHAGOCYTOSIS OF ANTIBIOTIC-PRETREATED *E. coli*

MDP addition to cultures[a]	Antibiotic pretreatment of *E. coli*[b] None	Cyclacillin[c] 5.0 μg	Cyclacillin[c] 1.0 μg	Penicillin (100 U)[c]
None	5.2 ± 1.2	15.1 ± 2.5*	17.3 ± 2.1*	6.1 ± 0.9
MDP (μg)				
100	12.1 ± 2.2	24.4 ± 1.9*	31.4 ± 2.4*	11.3 ± 1.2
5.0	11.3 ± 1.9	28.5 ± 2.6*	30.5 ± 3.1*	10.1 ± 2.2
1.0	6.5 ± 0.5	20.1 ± 2.5*	27.6 ± 1.3*	8.3 ± 1.3
0.5	7.2 ± 0.9	14.5 ± 2.2*	20.3 ± 2.1*	7.5 ± 0.9

[a] Indicated amount of MDP added to cultures of 10^6 peritoneal macrophages from normal mice 2 hr before assay for phagocytosis.

[b] Average percentage ± SD of macrophages in 3–4 cultures phagocytizing two or more *E. coli* after incubation for 2 hr with 10^8 *E. coli* at 37°.

[c] *E. coli* pretreated for 2 hr culture with indicated antibiotic at 37°C before washing and phagocytosis assay.

* $P < 0.02$ for phagocytosis of cyclacillin vs untreated bacteria.

biotic altered the surface of the bacteria sufficiently so they would be more readily recognized by host immune factors such as antibody or phagocytes. Studies from a number of laboratories have shown that filaments are formed when *E. coli* or other bacteria are exposed *in vitro* for even relatively short periods of time with a subinhibitory concentration of an antibiotic (26). Furthermore, other studies in this laboratory showed a greater immunogenicity of antibiotic-pretreated *E. coli* as determined by appropriate injection of extracts of macrophages cultured *in vitro* with the bacteria (6).

The results of this study support the view that even a subinhibitory concentration of an antibiotic, which does not kill the bacteria, may alter the susceptibility of a bacterium to host factors, possibly by exposure of new antigenic determinant(s) on the surface or increasing the amount of somatic antigens. Such bacteria undoubtedly become much more readily recognizable by cells such as macrophages. The results of the present study further show that activated macrophages, i.e., those obtained from MDP-pretreated mice, can recognize such antibiotic-pretreated bacteria to a greater extent than untreated bacteria and thus result in increased phagocytosis and killing of the bacteria. Such synergism between antibiotic pretreatment of bacteria and enhancement of phagocytic activity by an adjuvant may be a useful strategy for increasing the immunologic competence of immunosuppressed individuals who are susceptible to opportunistic microorganisms such as gram-negative bacteria. The combination of antibiotic therapy and immunostimulation should be useful for overcoming heightened susceptibility to infection by individuals with immunologic deficiencies.

1. Klebanoff SJ, Haman CB. Antimicrobial systems of mononuclear phagocytes. In: von Furth R, ed. Mononuclear Phagocytes in Immunity, Infection and Immunopathology. Oxford, pp507–529, 1975.
2. Nelson DS, ed. Immunobiology of Macrophages. New York, Academic Press, 1978.
3. Cohn ZA. The activation of mononuclear phagocytes: Fact, fancy and future. J Immunol **121**:813–820, 1978.
4. Sone S, Fidler IJ 1981. In vitro activation of tumoricidal properties in rat alveolar macrophages by synthetic muramyl dipeptide encapsulated in liposomes. Cell Immunol **57**:42–50, 1981.
5. Friedman H, Specter S, Butler C. Stimulation of immunostimulatory factors by bacterial endotoxin or nontoxic polysaccharides. In: Nowotny A, ed. Beneficial Aspects of Endotoxin. New York, Plenum, pp273–282, 1983.
6. Friedman H. Immunomodulation by small molecular weight bacterial products. Adv Exp Biol Med **166**:199–214, 1983.
7. Lederer E. Immunomodulation by muramyl peptides; Recent developments. Clin Immunol Newslett **3**:83–85, 1982.
8. Yamamura Y, Zuma I. Immunostimulation in cancer patients by biological response modifiers. Adv Exp Med Biol **166**:1–13, 1982.
9. Chedid L, Audibert F, Johnson AG. Biological activities of muramyl dipeptide, a synthetic glycopeptide analogous to bacterial immunoregulating agents. Progr Allergy, **25**:13–79, 1978.
10. Chedid L, Parant M, Audibert F, Lefrancier F, Chocy J, Sela M. Enhancement of certain biological activities of muramyl dipeptide derivatives after conjugation to a multipoly (DL-alanine)-poly (L-lysine) carrier. Proc Natl Acad Sci USA **76**:6557–6561, 1979.
11. Humphres RC, Henika PR, Ferraresis RW, Krahoubuhl JC. Effects of treatment with muramyl dipeptide and certain of its analogs on resistance to Listeria monocytogenes in mice. Infect Immun **30**:462–466, 1981.
12. Kierszenbaum F, Ferraresis RW. Enhancement of host resistance against *Trypanosoma cruzi* infection by immunoregulatory agent muramyl dipeptide. Infect Immun **25**:273–278, 1979.
13. Friedman H, Warren GH. Antibacterial activity of cyclacillin in vivo and in vitro: Effects on gram-negative organisms. Progr Chemother **2**:119–126, 1974.
14. Friedman H, Warren GH. Enhanced susceptibility of penicillin-resistant staphylococci to phagocytosis after in vitro incubation with low doses of nafcillin. Proc Soc Exp Biol Med **146**:707–711, 1974.
15. Friedman H, Warren GH. Antibody mediated bacteriolysis: Enhanced killing of cyclacillin-treated bacteria. Proc Soc Exp Biol Med **153**:301–304, 1976.
16. Friedman H, Warren GH. Cyclacillin-induced potentiation of *Escherichia coli* immunogenicity in vivo and in vitro. Chemotherapy **23**:324–336, 1977.
17. Friedman H, Warren G. Increased phagocytosis of *Escherichia coli* pretreated with subinhibitory concentrations of cyclacillin or ampicillin. Proc Soc Exp Biol Med **169**:301–305, 1982.
18. Ofeki I, Beeckery EH, Seisenstein BI, Alkan ML, Shann N. Suppression of bacterial adherence by sublethal concentration of B-lactan and aminoglycoside antibiotics. Rev Infect Dis **1**:832–837, 1979.
19. Germmell CG, Petersen PK, Schmalling D, Kim Y, Malteus J, Wannanaker L, Quie PG. Potentiation of opsonication of phagocytosis of *Streptococcus pyogenes* following growth in the presence of clindamycin. J Clin Invest **67**:1249–1256, 1981.

20. Vosbeck K, Handschin H, Merge EB, Zak G. Effects of subinhibitory concentration of antibiotics on adhesiveness of *Escherichia coli* in vitro. Rev Infect Dis **1**:845–851, 1979.
21. Forsgen AG, Banck H, Beckman A, Belhoshene. Antibiotic–host defense interactions in vitro and in vivo. Scand J Infect Dis Suppl **24**:195–203, 1980.
22. Mandell LA. Effects of antimicrobial and antineoplastic drugs on the phagocytic and microbicidal function of the polymorphonuclear leukocyte. Infect Dis Rev **4**:683–687, 1982.
23. McDonald PJ, Wetherall BL, Prinel H. Post antibiotic leukocyte enhancement. Increased susceptibility of bacteria pretreated with antibiotic to activity of leukocytes. Rev Infect Dis **3**:38–44, 1981.
24. Horne D, Tomasz D. Hypersusceptibility of penicillin treated group B streptococci to bactericidal activity of human polymorphonuclear leukocytes. Antimicrob Agents Chemother **19**:745–753, 1981.
25. Root RK, Istnonz R, Molavs A, Metcalf JA, Maleck HL. Interaction between antibiotics and human neutrophils in the killing of staphylococci; studies with normal and cytochalasin B treated cells. J Clin Invest **67**:247–259, 1981.
26. Gillissen G. Antibiotics and immune responses, concomitant effects of chemotherapy. Immun Infek **8**:79–88, 1980.
27. Munster AM, Loodholdt CB, Leary AG, Barnes MA. Effect of antibiotics on cell-mediated immunity. Surgery **81**:692–695, 1977.

Received January 30, 1984. P.S.E.B.M. 1984, Vol. 176.
Accepted April 18, 1984.