ACTIVATION OF MACROPHAGES FOR ENHANCED RELEASE OF SUPEROXIDE ANION AND GREATER KILLING OF *CANDIDA ALBICANS* BY INJECTION OF MURAMYL DIPEPTIDE*

By NANCY P. CUMMINGS, MICHAEL J. PABST, and RICHARD B. JOHNSTON, Jr.

From the National Jewish Hospital and Research Center and the Departments of Pediatrics and Biochemistry, University of Colorado School of Medicine, Denver, Colorado 80206

Preparations of mycobacteria have been used as adjuvants for many years. The glycopeptide muramyl dipeptide, *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP),[1] which was synthesized to correspond to a component of mycobacterial cell wall, represents the minimal chemical structure required for the adjuvant activity of complete Freund's adjuvant (1, 2). The adjuvanticity of MDP in the primary antibody response has been attributed to stimulation of either T or B lymphocytes, or both, or to stimulation of macrophages, resulting in increased antigen processing or increased lymphocyte stimulation by monokines (3). When added to cultured cells in vitro, MDP also stimulates a variety of macrophage functions that are not necessarily related to the response to specific antigens, namely, inhibition of macrophage migration (4), release of endogenous pyrogen (5), production of lymphocyte-activating factor (6), enhanced production of collagenase and prostaglandins (7), increased uptake of glucosamine (8), inhibition of growth of mastocytoma cells (9), and augmented direct cytolytic activity against tumor target cells (10). We have recently shown that the addition of MDP to mouse peritoneal macrophages in vitro primed these cells to produce increased amounts of superoxide anion (O_2^-) during stimulation by phorbol myristate acetate (PMA) or phagocytosis of opsonized zymosan. MDP appeared to affect the macrophages directly, without a requirement for lymphocyte involvement (11). This enhanced ability to secrete microbicidal and tumoricidal oxygen metabolites appears to be a fundamental component of macrophage activation (12, 13).

We have extended these in vitro observations by studying the effects of MDP on macrophages in the intact animal. We report here that subcutaneous administration of MDP to mice induces in their peritoneal macrophages, morphologic, metabolic, and microbicidal properties of "activated" macrophages. Injection of MDP also prepares the mice to withstand more effectively a lethal infectious challenge by *Candida albicans*.

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[1] *Abbreviations used in this paper:* BCG, bacillus Calmette-Guérin; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MDP, *N*-acetylmuramyl-L-alanyl-D-isoglutamine; O_2^-, superoxide anion; PMA, phorbol myristate acetate.

Materials and Methods

Macrophage Cultures. Peritoneal leukocytes were harvested from 4- to 8-wk-old Swiss-Webster mice (13). Nude mice were purchased from Taconic Farms, Germantown, N. Y. Cells were washed and allowed to adhere for 2 h to tissue culture dishes in medium containing penicillin, streptomycin, and 20% heat-inactivated (56°C for 30 min) fetal calf serum (FCS; Sterile Systems, Logan, Utah), as previously described (13). Adherent cells were washed vigorously twice with Hanks' balanced salt solution (HBSS; Microbiological Associates, Walkersville, Md.). After the initial 2-h adherence, cells were assayed or incubated overnight in medium with 20% FCS and antibiotics. All solutions were free from bacterial lipopolysaccharide (LPS), as tested by the limulus amebocyte lysate assay (E-toxate; Sigma Chemical Co., St. Louis, Mo.), except that FCS contained 125 pg/ml LPS. Therefore, medium with 20% FCS contained 25 pg/ml of LPS, which is less than the minimum concentration of LPS (100 pg/ml) that we have found capable of detectably affecting macrophages overnight in vitro (11). Cells adherent after 2 h were >97% macrophages, as determined by examination of stained cells and by phagocytosis of *Candida* (14); after overnight culture, <1% of adherent cells were polymorphonuclear.

Determination of Cell Number. Cells adherent to culture dishes were lysed with Zap-Oglobin II (Coulter Diagnostics, Inc., Hialeah, Fla.). The residue was scraped off the dish with a rubber policeman, diluted 1:20 in HBSS, and nuclei were counted with a Coulter counter Model ZB (Coulter Diagnostics, Inc.). In early experiments, nuclei counts were confirmed by counting with a hemocytometer.

O_2^- *Release.* Adherent cells were washed twice with HBSS, then assayed for production of O_2^- measured as superoxide dismutase-inhibitable reduction of ferricytochrome *c* (13). The stimulus was PMA (Consolidated Midland Corp., Brewster, N. Y.), 0.5 μg/ml. After removal of the O_2^- assay mixture, the cells were washed three times with HBSS; and protein was determined by the Lowry method (11). In some experiments, three to five separate dishes were assayed for protein after washing but without addition of the O_2^- reaction mixture; protein contents were equivalent with the two procedures.

Enzyme Assays. *N*-acetyl-β-glucosaminidase was assayed by a modification of the method of Woolen et al. (15). Results are expressed as nanomoles of substrate hydrolyzed in 1 min. Lactate dehydrogenase (LDH) was assayed by determining the rate of oxidation of reduced nicotinamide-adenine dinucleotide (Sigma Chemical Co.) at 340 nm (15). The amount of LDH that was released into the culture supernate was expressed as the percentage of total cell LDH, determined by lysis of the cultures with 0.05% Triton X-100. 1 U of LDH activity was defined as a decrease in optical density of 0.001/min.

Phagocytosis of Candida. *C. albicans* (ATCC 18804) was used for phagocytosis and phagocytic killing assays, as previously described (14). *Candida* (1×10^6 in 0.5 ml HBSS with 20% fresh FCS) were added to $\sim 3 \times 10^5$ washed cells adherent after overnight culture to 13-mm-diameter cover slips. After incubation, cover slips were washed, then double-stained with Wright's and Giemsa stains. Approximately 95% of cell-associated *Candida* could be seen within a detectable phagocytic vacuole (14). Slides were coded and read without knowledge of macrophage origin. The percentage of 200 macrophages that ingested one or more *Candida*, and the number of *Candida*/200 macrophages, were determined.

Phagocytosis was also studied using *Candida* labeled with fluorescein isothiocyanate (Calbiochem Behring Corp., American Hoechst Corp., San Diego, Cal.) (16), as described (14). Crystal violet, 0.5 mg/ml, abolished fluorescence of extracellular but not intracellular *Candida*, allowing the differentiation of ingested from attached fungi. In three comparative experiments, results at 60 min with this technique were identical to those obtained with stained cells (above).

Candidacidal Assay. Peritoneal cells were plated on 16-mm-diameter dishes to yield equivalent numbers of adherent macrophages after overnight culture, regardless of the source of the cells, based on counts of macrophage nuclei in separate dishes and on counting cells in representative fields of the dishes to be used in the assay (14). Macrophages were washed, then incubated with 10^5 *Candida* in 0.5 ml HBSS with 20% fresh FCS at 37°C in 5% CO_2-95% air. The macrophage: *Candida* ratio was ~3:1. The reaction was stopped by sonication for 30 s, and diluted suspensions were cultured in pour plates made with Sabouraud's agar (14). The number of colonies formed was determined in plates from triplicate reaction mixtures.

Protection against Death by Candida Infection. *C. albicans* isolated from the blood of a patient

with disseminated candidiasis was grown in Sabouraud's broth for 18 h at 37°C. *Candida* were washed twice with HBSS and counted with a hemocytometer. Each animal was given an injection of 2×10^6 fungi into the lateral tail vein.

Injection of MDP. MDP and the D-D stereoisomer of MDP, obtained from Calbiochem-Behring Corp., were diluted in nonpyrogenic saline to a concentration of 500 μg/ml. Injections were made into the flank using a No. 26 needle. In most experiments, saline was injected as control. However, values with cells from saline controls were no different than those obtained with cells from animals given the D-D stereoisomer (as noted) or no injection. This preparation of MDP, a second MDP preparation generously given us by Dr. S. Kotani, Osaka University, Japan, and a third preparation purchased from the Pasteur Institute, Paris, when incubated in vitro with resident macrophages, primed them to an almost identical extent to release increased amounts of O_2^- when stimulated with PMA (11). All MDP solutions were negative for LPS by the limulus assay (11).

The MDP obtained from Calbiochem-Behring Corp., at concentrations of 5 and 30 μg/ml, did not activate the alternative pathway during incubation for 15 or 30 min in either pooled human serum or serum from Swiss-Webster mice, using a sensitive kinetic hemolytic assay (17). Phenol-extracted LPS (11), 30 μg/ml, vigorously activated the alternative pathway in both types of sera. MDP, 5 and 30 μg/ml, also did not activate the classical pathway in human serum when incubated at 37°C for 30 min, using antibody-coated sheep erythrocytes in the same kinetic system.

Results

Macrophage Spreading, Protein Content, and Enzyme Activities. Table I summarizes the effects of injection of MDP on certain properties of peritoneal macrophages that have been associated previously with macrophage activation (13, 18–20). Macrophages from MDP-treated mice showed enhanced spreading during 2 h of adherence, increased protein content, greater total cell activity of the lysosomal hydrolase β-glucosaminidase, and increased total cell activity of LDH. β-glucosaminidase re-

TABLE I

*Effect of Injection of Mice with MDP on Peritoneal Macrophages**

Macrophage function or constituent	Pretreatment of mice‡	
	Saline	MDP
Spreading (% of cells elongated)§	3.6 ± 0.7	11.8 ± 1.2 (33)
Protein content (μg/10^6 adherent cells)	93 ± 19	134 ± 36 (7)
β-glucosaminidase activity (nmol/min per mg protein)	116 ± 16	173 ± 12 (7)
Lactate dehydrogenase activity (U/mg protein)	1,143 ± 181	1,791 ± 130 (3)

* Mice were injected subcutaneously with 25–100 μg MDP or saline ~18 h before collecting cells from the peritoneal cavity. After incubation on dishes for 2 h, nonadherent cells were washed away, and the adherent cells were studied as described. Cells studied for β-glucosaminidase and LDH activities were from mice given 100 μg MDP.

‡ Mean ± SEM. Number of paired experiments is given in parentheses in the MDP column. Each experiment was performed with cells pooled from 2–10 mice, and tested in duplicate or triplicate. Results were significantly greater with cells from MDP-treated mice ($P < 0.01$ for protein content, $P < 0.001$ for the other three variables, paired t test).

§ Elongated cells had a length:width ratio of >1.5, as detected with phase-contrast microscopy (11).

mained significantly higher in cells from MDP-treated mice after 24 or 48 h in culture (five experiments). Release of LDH, an indicator of cell death, was equivalent in the two cell populations after culture for 24 and 48 h ($n = 5$). Treatment of mice with MDP also increased the number of peritoneal cells obtained ~18 h later (3.6 ± 0.3 and 5.7 ± 0.2 × 10^6 cells/mouse for control and MDP-treated mice, respectively; mean ± SEM for six pools of cells). The percentage of these cells that adhered to culture dishes as macrophages was equivalent in the two groups of mice (~30%, $n = 7$).

In view of the possible relationship of macrophage stimulation by MDP to the adjuvanticity of this compound (3), additional experiments were performed testing the effects of injection of the D-D stereoisomer of MDP, which is inactive as an adjuvant. In two paired experiments, results with macrophages pooled from six to eight mice given D-D MDP were almost identical to those with macrophages from saline-injected animals for each of the four variables of Table I and for the number of peritoneal cells obtained per mouse. Results with cells from animals given saline were equivalent in these experiments and the paired experiments of Table I.

Generation of O_2^-. Macrophages obtained from inflammatory exudates or from BCG-infected mice, or macrophages incubated with MDP in vitro, are primed to release greater amounts of O_2^- when stimulated with PMA than are resident cells (11, 13). MDP, 25–100 μg administered subcutaneously 18 h before macrophage harvesting, primed peritoneal macrophages to produce up to five times more O_2^- than control macrophages produced (Fig. 1). A dose of 50 μg MDP was employed to evaluate the time course of the O_2^- effect (Fig. 2). Some enhancement in PMA-stimulated O_2^- release was noted 1 h after injection. The enhanced O_2^- response reached optimal levels by 3 h after MDP injection and remained at such levels for at least 48 h.

The results shown in Figs. 1 and 2 were obtained with macrophages allowed to adhere for 2 h; in six experiments with mice given 50 μg MDP 18 h before macrophage harvest, the release of O_2^- from macrophages cultured overnight was almost identical to release from macrophages stimulated after 2-h adherence (645 ± 67 and 651 ± 49 nmol/mg, respectively). Injection of the D-D stereoisomer of MDP did not prime

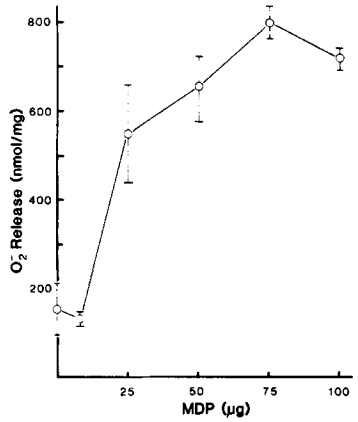

FIG. 1. Effect of pretreatment of mice with varying amounts of MDP on the release of O_2^- by cultured peritoneal macrophages stimulated by 0.5 μg/ml PMA. The mean ± SEM of three to five experiments is shown at each amount given; each of these experiments was performed with peritoneal cells pooled from four to six mice, and placed into triplicate dishes.

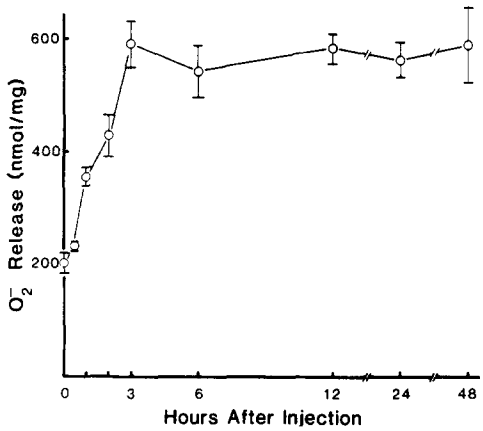

FIG. 2. The extent of PMA-stimulated O_2^- release by cultured peritoneal macrophages plotted as a function of the time at which macrophages were harvested after injection of 50 μg of MDP. The mean ± SEM of four to six experiments is shown at each time point.

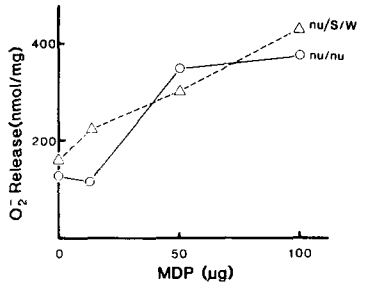

FIG. 3. Extent of O_2^- release by peritoneal macrophages from nude mice (nu/nu) and normal littermates on a Swiss-Webster background (nu/S/W) treated with MDP 18 h prior to macrophage harvesting. The means of triplicate values are shown for one representative experiment of three performed with varying amounts of MDP. In each, the response to injection of MDP was equivalent in nude mice and littermates, and two- to fivefold greater than the PMA-stimulated O_2^- response of control mice.

peritoneal macrophages for enhanced O_2^- response (165 ± 20 nmol/mg, compared with 148 ± 12 nmol/mg for control cells; $n = 2$).

We explored the question of whether MDP might act directly on the macrophage or whether it interacts with lymphocytes to cause production of lymphokines, which would, in turn, activate the macrophages. Nude mice (nu/nu, on Swiss-Webster background), which lack mature T cells, were pretreated with MDP in varying amounts. Normal littermates (nu/S/W) were used as controls. Fig. 3 shows that both nu/nu mice and littermates responded in a similar fashion to MDP, suggesting that mature T-cell function is not essential for the MDP-mediated enhancement of O_2^- release.

Phagocytosis and Killing of Candida. The capacity of control and MDP-treated macrophages to ingest *Candida* was compared. As noted in Fig. 4 A, at all time points a slightly higher percentage of macrophages from MDP-treated mice were phagocytic, and MDP macrophages ingested slightly more *Candida* (Fig. 4 B).

The killing of *C. albicans* by adherent macrophages is demonstrated in Fig. 5. Studies were performed at a ratio of ~1 *Candida*:3 macrophages in the presence of 20% fresh

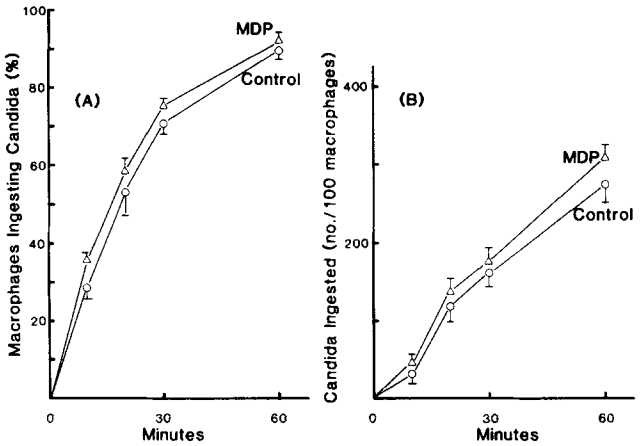

FIG. 4. Phagocytosis of *C. albicans* by cultured macrophages from control mice and mice given 50 μg MDP 18 h previously. The candida:macrophage ratio was ~3:1. The percentage of macrophages ingesting at least one organism (A), and the number of candida ingested by 100 macrophages (B) are plotted. The mean ± SEM of six experiments are shown. The difference in phagocytosis shown in panel A is significant at the level of $P < 0.01$ (analysis of variance, repeated measures) and in panel B at the level of $P < 0.05$.

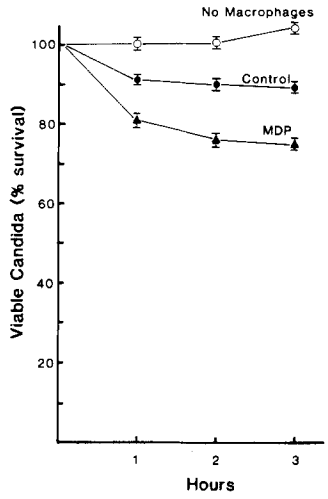

FIG. 5. The killing of *C. albicans* by macrophages from control or MDP-treated mice. Means ± SEM of six matched experiments are shown. Control cells killed 10.6 ± 2.3% of the fungi after 3 h; macrophages from mice given 50 μg MDP killed 21.5 ± 1.5%.

FCS. Under these conditions, >99% of *Candida* on stained cover slips were ingested by 60 min. Crystal violet did not extinguish the fluorescence of fluorescein-labelled *Candida*, confirming the ingestion of all *Candida* by 60 min. *Candida* were not easily killed by the macrophages, in agreement with the killing of only 15–36% of *C. albicans* by human monocytes and neutrophils (21) and rabbit peritoneal macrophages (22). However, macrophages from MDP-treated mice killed approximately two times more *Candida* than did control cells at each time point studied, and this difference was significant at each time point ($P < 0.001$, t test). The killing of *Candida* by cells from

mice given the D-D stereoisomer (10.0 ± 0.2%) was equivalent to killing with cells from control mice (12.9 ± 0.3%, mean ± SEM, $n = 2$ at 1 h).

Protection of Mice against Candida Challenge. 5- to 6-wk-old mice were given 50 μg of MDP subcutaneously either 18 h before, immediately after, or 24 h after being infected intravenously with 2×10^6 *C. albicans*. Mortality was recorded for 16 d. As shown in Fig. 6A, MDP given to 10 mice 18 h before challenge resulted in 60% survival of the mice by day 7, by which time all 10 control mice had died. In a second experiment with 15 7-wk-old mice (data not plotted), injection of 50 μg MDP 18 h before infection permitted 50% survival by day 12, by which time all 15 control mice had died. In Fig. 6B, similar results were obtained when injection of MDP immediately followed infectious challenge in 12 mice, survival being 58% by day 7. Even when MDP was given 24 h after the *Candida* challenge (15 mice), some protection was still observed in MDP-treated mice as compared with control mice (Fig. 6C).

Discussion

The designation "activated" was originally applied to macrophages obtained from animals that had enhanced ability to resist infection (19). Biochemical, morphologic, and functional characteristics of macrophages from such animals have been described subsequently (12, 13, 18, 19). By several of these criteria, we have demonstrated activation of mouse peritoneal macrophages after subcutaneous injection of the

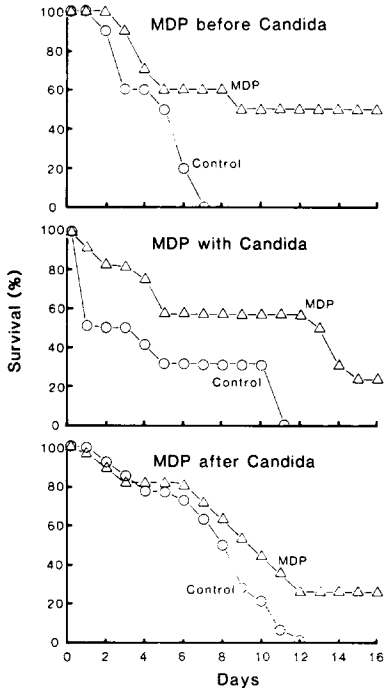

FIG. 6. Effect of survival from infection with *C. albicans* of mice treated with MDP. (A) MDP, 50 μg, was administered subcutaneously to 10 mice 18 h before administration of candida intravenously. (B) MDP, 50 μg, was administered to 12 mice within 1 min after candida challenge. (C) MDP, 50 μg, was administered to 15 mice 24 h after candida challenge. In each experiment the number of control mice (no MDP) was identical to or greater than the number receiving MDP.

adjuvant MDP: Mice treated with MDP showed increased numbers of peritoneal macrophages, and when cultured, these macrophages showed more spreading and increased total cell protein. Total cellular content of the lysosomal hydrolase β-glucosaminidase and of the cytoplasmic enzyme LDH was increased, as previously reported with macrophages elicited by thioglycollate, streptococcal protein A, or protease-peptone (20). Enhancement of the phagocytosis-associated burst of oxidative metabolism also appears to be a manifestation of macrophage activation (12, 13). Macrophages obtained from animals infected with BCG have been shown to have increased potential for generating O_2^- (13) and hydrogen peroxide (12) when stimulated by phagocytosis or contact with PMA. A similar priming for enhanced O_2^- generation was achieved by incubating MDP with mouse peritoneal macrophages in vitro (11). In the present report, we have shown that MDP given in vivo also markedly enhanced PMA-stimulated O_2^- release. The absolute levels of O_2^- release achieved were equivalent to those obtained previously with macrophages from animals infected with BCG (13).

MDP treatment enhanced phagocytosis of *C. albicans* only slightly. Previous studies have shown that the rate of clearance of carbon particles from the blood stream, presumably a phagocytosis-dependent event, was enhanced in MDP-treated mice (23, 24).

The enhanced microbicidal potential of macrophages from MDP-treated animals was confirmed by direct testing. Macrophages from mice pretreated with MDP consistently killed over two times more *C. albicans* than did resident macrophages. This doubling of candidacidal activity is almost identical to that observed with LPS-elicited or BCG-activated mouse peritoneal macrophages (14) and Freund's adjuvant-elicited rabbit peritoneal macrophages (22). The extent of killing of *C. albicans* by mouse macrophages is equivalent to that reported with human neutrophils or monocytes (21).

The molecular basis for the enhanced killing capacity of MDP-activated macrophages has not been completely defined. It is unlikely that the slightly increased phagocytosis by MDP macrophages played an important part; moreover, in the candidacidal system, all candida were ingested after 60 min by both types of macrophages. We have previously reported data to suggest that the enhanced candidacidal capacity of BCG-activated and LPS-elicited macrophages is dependent on their increased oxidative metabolic response (14), and it seems highly likely that the enhanced candidacidal activity of macrophages from MDP-treated animals results at least in part from this enhancement. Others have reported a similar association with the enhanced killing of *Toxoplasma gondii* (25, 26) and *Trypanosoma cruzi* (27). A higher content in MDP macrophages of hydrolytic enzymes, as represented by β-glucosaminidase, could also be relevant.

The enhanced phagocytic killing ability of macrophages from MDP-treated mice presumably played an important role in protecting the mice against lethality from *C. albicans* infection, although other immunostimulatory effects of MDP also could have been involved. MDP and certain MDP analogs have been reported to protect both adult and neonatal mice challenged with lethal *Klebsiella* infection (24, 28) or adult mice infected with *T. cruzi* (29). Direct injection into tumors of certain MDP analogs, along with trehalose dimycolate, has been associated with tumor regression in guinea

pigs (30). In contrast, 100–500 μg of MDP given intraperitoneally 20 min or 5 d before infection did not protect against lethal challenge by *Listeria monocytogenes* (31).

The mechanism for the rapid, apparently T-cell-independent activation of peritoneal macrophages by MDP injected at a distant site is not known. MDP did not activate complement in vitro by either the classical or alternative pathways, but stimulation of the formation or release of other humoral factors or interaction with complement components on the surface of the macrophage is possible. It is clear that MDP can induce functional changes in adherent macrophages when added to these cells in vitro (4, 5, 8–11), and vigorous efforts to exclude lymphocytes as mediators of these effects (4, 11) have indicated that MDP can act directly on the macrophage. Moreover, some of these changes are induced within a few hours after contact with MDP (4, 11), which is more prompt than would be expected for a lymphokine-dependent effect (27). Thus, extrapolation from these in vitro data favors a direct effect of injected MDP on the macrophage.

The experiments reported here show clearly that injection of MDP activates macrophages by morphologic, metabolic, and microbicidal criteria. These results indicate that MDP has important potential as a tool for the study of macrophage physiology. Our studies also indicate that MDP offers promise as a means of enhancing host defense. The adverse side effects of BCG and larger cell wall extracts, such as granuloma formation and adjuvant polyarthritis (3, 32), and the potential danger from administering a viable organism like BCG, are lacking when MDP is given in saline. In fact, the only deleterious effect thus far reported with MDP has been mild pyrogenicity in rabbits (5). The apparently direct effect of MDP on the macrophage, bypassing the T lymphocyte, suggests particular usefulness of this compound for treating the immunocompromised host.

Summary

The adjuvant muramyl dipeptide (MDP) has been shown to affect a number of macrophage functions in vitro. We studied the effect of subcutaneous injection of MDP into mice. Cultured peritoneal macrophages from treated mice displayed increased spreading, total cell protein, and specific activity of β-glucosaminidase, a constituent of macrophage lysosomes, and of lactate dehydrogenase. Generation of superoxide anion (O_2^-) by MDP-treated macrophages stimulated by contact with phorbol myristate acetate was enhanced by over fivefold to levels achieved by macrophages from bacillus Calmette-Guérin-infected mice. The enhancement in stimulated O_2^- release was noted by 1 h after injection of MDP, peaked by 3 h, and remained high for at least 48 h. Priming for enhancement of O_2^- release by MDP was similar in athymic nude mice and in normal littermates, suggesting that mature T lymphocytes are not involved in this MDP effect. Priming for enhanced stimulated O_2^- release, and morphologic and enzymic changes, were not achieved by injection of the D-D stereoisomer of MDP. Phagocytosis of *Candida albicans* was only slightly greater by macrophages from mice given MDP, but MDP-stimulated cells killed two times more *C. albicans* in vitro than did cells from untreated animals. When MDP was given 18 h before, simultaneously with, or 24 h after lethal infectious challenge with *C. albicans*, treated mice were protected compared with controls. These results suggest that injection of MDP effectively and rapidly activates macrophages in the recipient

animal. This agent should serve as an important probe of macrophage physiology and, perhaps ultimately, as a means of enhancing host defense in humans.

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