# INCREASED PRODUCTION OF SUPEROXIDE ANION BY MACROPHAGES EXPOSED IN VITRO TO MURAMYL DIPEPTIDE OR LIPOPOLYSACCHARIDE*

By MICHAEL J. PABST and RICHARD B. JOHNSTON, Jr.

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

In response to an appropriate stimulus such as phagocytosis of an opsonized particle, macrophages reduce oxygen, first to superoxide anion ($O_2^-$),[1] and then to hydrogen peroxide ($H_2O_2$) (1, 2). These metabolites of oxygen are involved in the killing of phagocytosed microorganisms (3). More recently, $H_2O_2$ has also been implicated in macrophage killing of lymphocyte tumor lines (4, 5). Macrophages obtained from the peritoneal cavity of mice infected with *Bacille Calmette-Guérin* (BCG) or injected intraperitoneally with bacterial lipopolysaccharide (LPS) display a greatly enhanced ability to generate $O_2^-$ in response to stimulation by opsonized zymosan or the membrane-reactive agent phorbol myristate acetate (PMA) (6). Such activated macrophages also exhibit enhanced $H_2O_2$ release (2). These observations correlate with studies showing that activated macrophages are better able to kill microorganisms (7, 8) and tumor cells (9, 10).

Treatment of macrophages in vitro with LPS has been shown to enhance their ability to kill tumor cells (11–14), and to increase their secretion of collagenase (15) and other hydrolytic enzymes (16). *N*-Acetylmuramyl-L-alanyl-D-isoglutamine, or muramyl dipeptide (MDP), also enhances macrophage tumor cell killing in vitro (17). This segment of bacterial cell wall peptidoglycan is the minimal fragment that retains adjuvant properties (18). This same material has been shown to cause macrophage migration inhibition when incubated with mononuclear cells (19, 20), to induce granuloma formation in vivo (21), and to confer resistance to death by infection with *Klebsiella pneumoniae* in mice (22). Macrophages exposed to MDP in vitro produced increased amounts of collagenase, prostaglandin, and a fibroblast proliferation factor (23). We report here that direct contact of cultured peritoneal macrophages with MDP or LPS primes the cells to respond with increased $O_2^-$ after stimulation with PMA or opsonized zymosan.

## Materials and Methods

*Chemicals.* PMA was obtained from Consolidated Midland Corp., Brewster, N. Y.; a stock solution of 2 mg/ml in dimethylsulfoxide (DMSO) was stored at −70°C. 1–10 μl of this stock

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[1] *Abbreviations used in this paper:* BCG, *Bacille Calmette-Guérin*; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; LAF, lymphocyte-activating factor; LPS, bacterial lipopolysaccharide; MDP, *N*-acetylmuramyl-L-alanyl-D-isoglutamine (or muramyl dipeptide); $O_2^-$, superoxide anion; PMA, phorbol myristate acetate; SRBC, sheep erythrocyte(s).

solution was added directly to the $O_2^-$ assay mixture just before use. An equivalent volume of DMSO had no effect.

The LPS used in this study was a gift from Dr. Floyd McIntire of the University of Colorado School of Dentistry, Denver, Colo. The preparation was phenol-extracted from *Escherichia coli* K235, treated with detergent, and purified by gel filtration (24). This LPS was free from the lipid A-associated endotoxic protein. Examination of the UV spectrum of the LPS showed no detectable ubiquinone contamination (<1 part per 1,000) (25). Less than 0.1 ng of this LPS was detectable by the limulus amebocyte lysate assay. Limulus lysate (E-toxate) was obtained from Sigma Chemical Co., St. Louis, Mo., and was used according to the instructions in Sigma Technical Bulletin No. 210. A butanol-extracted LPS that contained lipid A-associated protein (26) was a gift from Dr. Sally Betz of the Institut de Biochimie, Université de Lausanne, Epalinges, Switzerland.

MDP was purchased from the Pasteur Institute, Paris, France. The D-D isomer was a gift from Dr. Louis Chedid of the Pasteur Institute. The L-L isomer was a gift from Dr. Shozo Kotani of the Osaka University Dental School, Osaka, Japan. MDP and its isomers, which were synthesized chemically, were negative for LPS by the limulus assay; stock solutions at 10 μg/ml (20 μM) were tested.

*Mice.* Swiss-Webster mice were either bred at National Jewish Hospital or purchased from Charles River Breeding Laboratories, Wilmington, Mass., or from Bio-Lab Corp., St. Paul, Minn. Nude mice were purchased from Timco Breeding Laboratories, Houston, Tex. C3H/HeJ and C3Heb/FeJ mice were purchased from The Jackson Laboratories, Bar Harbor, Maine. All mice were 1- to 2-mo-old females.

*Macrophage Harvesting and Cultivation.* Peritoneal cells were harvested as previously described (6), washed, and allowed to adhere for 2 h to 35-mm diameter tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM) that contained penicillin, streptomycin, and 20% heat-inactivated fetal calf serum (FCS) (6). Nonadherent cells were washed away with fresh medium. After the 2-h adherence, 96–98% of adherent cells phagocytosed antibody-coated sheep erythrocytes (SRBC). Erythrocytes were treated with a 1:300 dilution of rabbit anti-SRBC IgG, then washed three times with Hanks' balanced salt solution (HBSS). Erythrocytes, 1 ml of a 2.5% cell suspension in HBSS, were added to the macrophage cultures for 60 min at 37°C in 5% $CO_2$. The cultures were washed vigorously three times with HBSS, then treated with distilled water for 30 s to lyse external erythrocytes, followed by a further washing with HBSS. The macrophage cultures were then examined by phase contrast microscopy, and the percentage of cells that had phagocytosed erythrocytes was determined by two independent observers. Many of the nonphagocytosing cells appeared to be of the monocyte-macrophage line, because they bore small pseudopods. Approximately 2% of the adherent cells appeared to be lymphocytes, perhaps adherent B cells, as previously described (27). After the initial 2-h adherence, cells were incubated overnight in DMEM (with antibiotics but without FCS) and with either LPS, MDP, or saline as control. The number of granulocytes that survived overnight culture, and which could theoretically interfere in the $O_2^-$ assay, was <2% (6).

All results reported here have been confirmed with solutions that were LPS-free, as tested by the limulus assay. The FCS routinely used for adherence contained LPS. However, results identical to those reported were obtained with cells cultured in 1% heat-inactivated autologous mouse serum, which was negative for LPS by the limulus assay. Mouse serum was not used routinely because it permitted an increased percentage of lymphocytes to adhere. The effect of LPS or MDP reported here could be achieved in the presence of FCS, but FCS was not routinely present during the LPS or MDP treatment, because it tended to elevate to a variable degree the $O_2^-$ response of control cells. This effect may have been a result of LPS contamination of the serum or to factors inherent in the serum itself.

*$O_2^-$ Assay.* After overnight exposure to LPS or MDP, the cells were washed twice with HBSS, then assayed for $O_2^-$ production in response to stimulation for 60 min by 0.5 μg/ml PMA, using superoxide dismutase-inhibitable reduction of ferricytochrome *c*, as previously described (6). Results are presented as nanomoles of $O_2^-$ released in 60 minutes per milligram of cell protein. Because $O_2^-$ generated by cells without PMA treatment was <2 nmol/60 min per mg protein, individual assays in the absence of PMA were not routinely performed.

*Protein Determination.* Protein was determined by the Lowry method, with bovine serum albumin as standard. After the $O_2^-$ assay, culture dishes were washed three times with HBSS,

copper-tartrate reagent was added directly to the culture dishes, and the remainder of the procedure was performed directly in the dishes.

*J774.1 Cell Line.* J774.1 cells were obtained originally from Dr. Peter Ralph, Sloan-Kettering Institute, Rye, N. Y. (6). The cells were grown in suspension in 10-cm microbiological plastic Petri dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) in DMEM that contained 20% heat-inactivated FCS and antibiotics. Cells did not adhere, or adhered very weakly, to the plastic dishes that had not been treated for tissue culture by the manufacturer. Cultures were established by adding five drops of cell suspension from a previous culture to 15 ml of the medium, followed by incubation at 37°C in 95% air–5% $CO_2$ for 5 d. The cells were harvested, washed, and resuspended in fresh DMEM with FCS and antibiotics at $2.5 \times 10^6$ cells/ml. 1 ml of the cell suspension was added to each 35-mm dish and allowed to adhere for 2 h at 37°C in 95% air–5% $CO_2$. Essentially all cells adhered. After adherence, the cells were washed, then incubated in 2 ml of DMEM without serum, and with either saline, LPS, or MDP for 4 h. The 4-h time period was selected because the cell line did not tolerate long-term culture in the absence of serum, nor did the cells respond with increased PMA-stimulated $O_2^-$ generation after incubation with MDP or LPS in the presence of 20% heat-inactivated FCS, 1% heat-inactivated mouse serum, or 20% horse serum. The dishes were washed twice with HBSS, then assayed for $O_2^-$ production in response to 1 μg/ml PMA. Because of the low amounts of $O_2^-$ produced by J774.1 cells, results with each concentration of MDP and LPS were compared with those obtained with the same reaction mixture in the absence of PMA.

## Results

*Effect of LPS and MDP on Cultured Macrophages.* Resident mouse peritoneal macrophages which were cultured overnight in the presence of MDP (Fig. 1 b) consistently showed markedly enhanced pseudopod formation compared with control cells cultured in medium alone (Fig. 1 a). Cells treated with LPS (Fig. 1 c) were characterized by long, thin pseudopodia, which were not found in control or MDP-treated cells under these conditions.

The enhanced spreading produced by overnight incubation with LPS and MDP is summarized for a typical cell preparation in Table I. This effect correlated with an increased $O_2^-$-generating capacity of the treated cells (Table I). When stimulated with PMA, cells treated with LPS produced almost seven times more $O_2^-$ than did cells preincubated with saline, and cells treated with MDP produced twice as much $O_2^-$ as did control cells. In contrast, cells treated with stereoisomers of MDP, which have been shown to be inactive as adjuvants (18, 28), were not primed to produce more $O_2^-$.

Exposure to MDP or LPS did not, in itself, cause the production of $O_2^-$ by macrophages. Macrophages treated only with LPS or MDP but not stimulated by PMA produced <2 nmol $O_2^-$/mg ($n = 6$). Opsonized zymosan (13 μg/ml) was also effective as a stimulus. However, zymosan elicits more $O_2^-$ from resident (control) macrophages than does PMA (6), and the differences between control (176 ± 12 nmol/mg protein, mean ± SEM, $n = 4$) and MDP- or LPS-treated cells (228 ± 12 or 320 ± 10, respectively, $n = 4$) were less than the differences observed with PMA.

MDP and LPS were effective at enhancing $O_2^-$-generating capacity of macrophages over a wide range of concentrations, as shown in Fig. 2. Increased $O_2^-$-generating capacity was achieved after exposure to submicrogram quantities of either material. LPS at 0.01–1 μg/ml appeared to be optimal; LPS at 10 μg/ml was less effective, perhaps a result of toxic effects on the cells (29). MDP was most effective at concentrations >0.5 μg/ml, although activity persisted down to 0.05 μg/ml. The inactive stereoisomers of MDP had no effect at 0.05, 0.5, or 5 μg/ml.

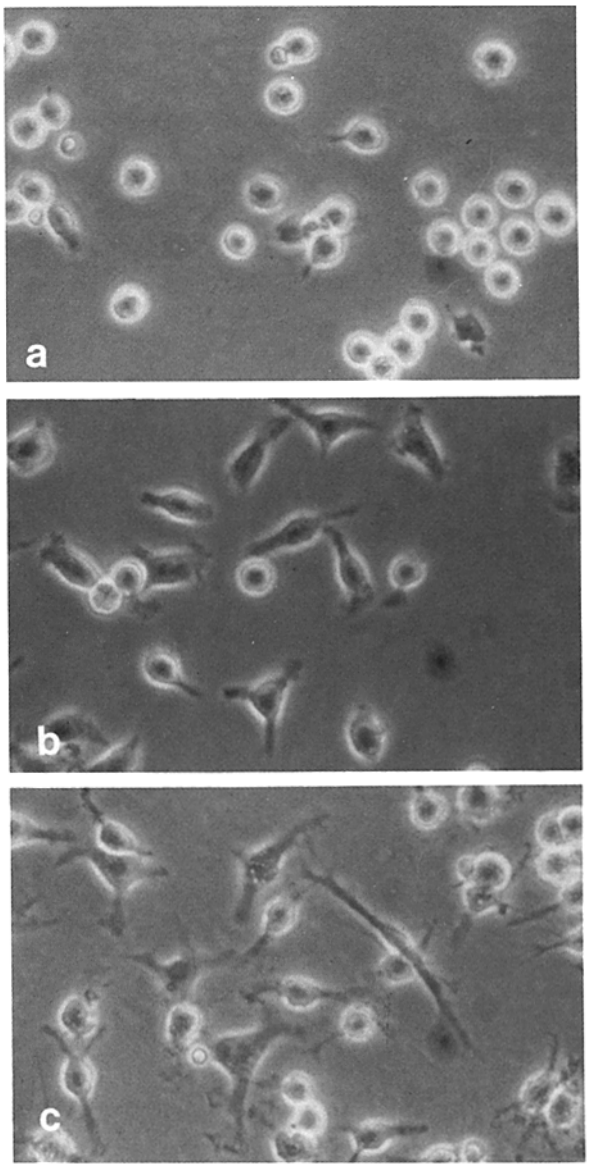


FIG. 1. Phase contrast micrographs of resident mouse peritoneal macrophages. Cells were allowed to adhere to tissue culture dishes for 2 h in DMEM with 20% heat-inactivated FCS and antibiotics, then washed and cultured overnight in DMEM without serum. Cultures were washed again before photography. (a) Control macrophages cultured overnight in medium alone. (b) Macrophages incubated overnight with 1 μM (0.5 μg/ml) MDP. (c) Macrophages incubated overnight with 1 μg/ml LPS.

The time-course of the effect of LPS and MDP is shown in Fig. 3. A significant effect of LPS was observed within 30 min. In contrast, the first effect of MDP was noted at 4 h. The effect of LPS was greater than that of MDP at all time points; with continuous exposure, the response to MDP declined more rapidly than did the

TABLE I

*Effect of Priming with MDP or LPS on Spreading and on PMA-stimulated $O_2^-$ Release by Mouse Peritoneal Macrophages*

| Treatment* | PMA-stimulated $O_2^-$ release‡ | Spreading§ |
|---|---|---|
| | *nmol/mg protein* | % |
| Control (saline) | 67 ± 12 (6) | 25 |
| LPS (1 μg/ml) | 450 ± 53 (6) | 86 |
| MDP (1 μM) | 140 ± 10 (6) | 37 |
| MDP, L-L stereoisomer (1 μM) | 71 ± 13 (3) | — |
| MDP, D-D stereoisomer (1 μM) | 78 ± 15 (3) | — |

* After adherence for 2 h to culture dishes in DMEM-20% FCS with antibiotics, resident mouse macrophages were preincubated overnight in medium alone or medium with MDP, MDP analogue, or LPS. The cells were washed twice before being assayed for $O_2^-$ release or examined for spreading. 1 μM of MDP is ~ 0.5 μg/ml.

‡ $O_2^-$ was quantitated by its capacity to reduce ferricytochrome *c*, as described in Methods. Cells were stimulated with 0.5 μg/ml PMA for 60 min. The values shown represent mean ± SEM. The number of experiments, done in duplicate or triplicate, is given in parentheses.

§ Culture dishes were coded, and ~200 cells were counted with phase microscopy on each of two culture dishes by two independent observers. The values shown represent the mean percentage of cells that show definite pseudopod formation. Although pseudopod formation was not counted in the case of the L-L and D-D analogues, these cells did not differ in appearance from control cells.

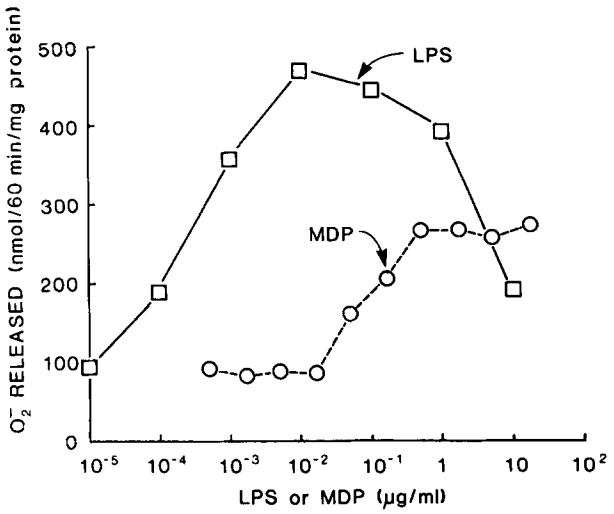


FIG. 2. Effect of concentration of MDP or LPS on enhancement of the $O_2^-$-generating capacity of resident macrophages cultured overnight in medium without serum. Values shown represent the mean $O_2^-$ produced in response to stimulation with 0.5 μg/ml of PMA after priming with LPS or MDP (six replicates each). The standard error of all points was 15 or less. The $O_2^-$ response of untreated control cultures was 84 ± 7 (SEM, $n = 6$). MDP from three different sources (Pasteur Institute, Dr. Kotani, and Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) was used (two replicates from each source, yielding $n = 6$); the three sources of MDP gave almost identical results at all concentrations (SEM <15).

response to LPS. After 4 d, all cells appeared healthy under phase contrast microscopy, and the $O_2^-$ response of the control cells was undiminished.

*Role of Lymphocytes in the Response of Macrophages to LPS and MDP.* We explored the question of whether, in the preceding experiments, MDP and LPS had acted directly

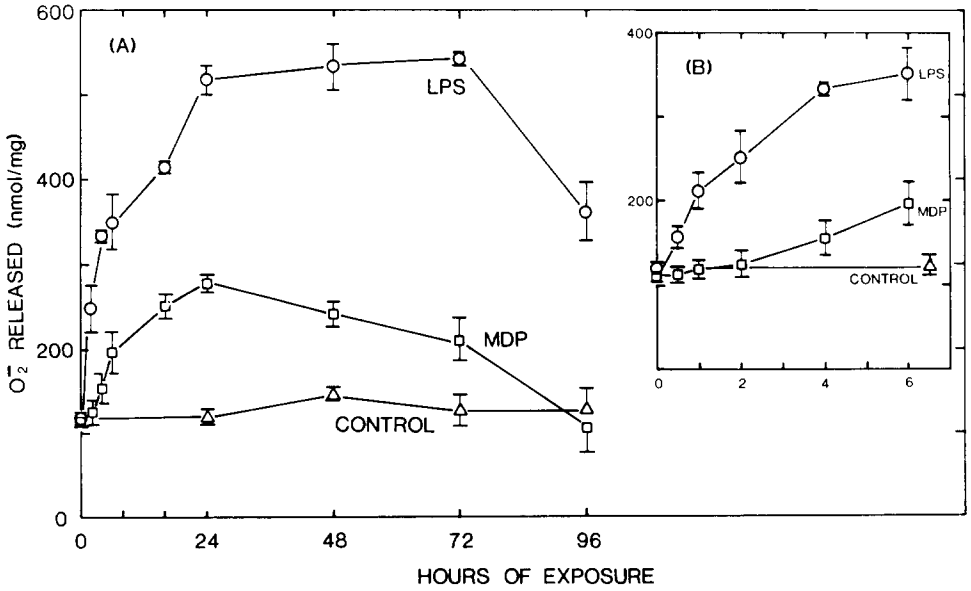


FIG. 3. Effect of the time of exposure to LPS or MDP on the $O_2^-$-generating capacity of macrophages. All cultures were started after an initial 2-h adherence (time 0). Results with cultures exposed to LPS or MDP for periods of time up to 24 h were compared with those of control (untreated) dishes cultured for 24 h in DMEM alone. Cultures exposed to LPS or MDP for 48, 72, or 96 h were compared with control cells incubated for the corresponding period of time. Means for duplicate cultures are plotted. The bars represent 1 SEM. Results are representative of those of four experiments. The insert (B) is an expansion of Fig. 3A at earlier time points.

on the macrophages themselves, or had interacted with adherent lymphocytes to cause the production of lymphokines, which, in turn, had primed the macrophages. In the experiments represented in Table II, cells that were nonadherent after the initial 2-h incubation were removed and washed, then re-added to one-half of the macrophage cultures. Thus, the cultures to which nonadherent cells were re-added contained between one and two times the number of nonadherent cells that were originally present in the peritoneal lavage ($1-2 \times 10^6$ nonadherent cells per dish). The cells were then incubated overnight with MDP and LPS. On the next day the cultures were washed and assayed for $O_2^-$ production. The presence of the nonadherent cells during the LPS and MDP treatment produced an enhancement of $O_2^-$ production in both control and MDP-treated cells. However, the presence of the nonadherent cells did not enhance the relative response of the macrophages to either LPS or MDP.

Macrophages maintained in culture for 24 or 48 h, to further reduce the possibility of lymphocyte contamination, responded as well to subsequent priming by LPS and MDP as did cells whose priming began after the initial 2-h adherence (four experiments, data not shown). Addition of freshly harvested nonadherent cells at $10^4$, $10^5$, or $10^6$ cells per dish had no significant effect on the priming response of macrophages cultured for 24 or 48 h before priming.

To evaluate further whether macrophages respond directly to LPS and MDP, cells were prepared from nude mice (Swiss-Webster nu/nu), which lack mature T lympho-

TABLE II

*Evaluation of the Role of Lymphocytes in the Macrophage Response to MDP and LPS: Effect of the Addition of the Nonadherent Cell Population*

| Treatment | PMA-stimulated $O_2^-$ release* | |
|---|---|---|
| | Macrophages alone | Macrophages + nonadherent cells |
| | *nmol/mg protein* | |
| Control (saline) | 74 ± 10 | 146 ± 5 |
| LPS (1 μg/ml) | 437 ± 13 | 439 ± 16 |
| MDP (1 μM) | 140 ± 9 | 202 ± 10 |
| MDP, D-D isomer (1 μM) | 82 ± 4 | 153 ± 11 |

* Results are means ± SEM for triplicate assays. Dishes that contained nonadherent cells alone produced no detectable $O_2^-$. These results are representative of those obtained in four experiments.

TABLE III

*Priming of Macrophages from Nude Mice by MDP and LPS*

| Treatment | PMA-stimulated $O_2^-$ release* |
|---|---|
| | *nmol/mg protein* |
| Control (saline) | 78 ± 17 |
| LPS (1 μg/ml) | 340 ± 16 |
| MDP (1 μM) | 145 ± 11 |
| MDP, D-D isomer (1 μM) | 83 ± 11 |

* Results are means ± SEM of two experiments done in duplicate.

cytes. As shown in Table III, peritoneal macrophages from nude mice responded normally to MDP and LPS. Thus, mature T-cell function does not appear to be essential for the response of macrophages to either LPS or MDP in this in vitro system.

The effect of LPS and MDP was also tested on the C3H/HeJ strain of mice, which has been reported to have both B lymphocytes and macrophages that are defective in their response to LPS with respect to B-cell mitogenesis, macrophage tumor lysis, LPS-induced lethality, and production of prostaglandin and lymphocyte-activating factor by macrophages (30–37). As shown in Fig. 4, at all concentrations of LPS tested, macrophages from the C3H/HeJ strain gave a definite, but only partial, response to phenol-extracted LPS. In other systems, cells from C3H/HeJ mice have responded much better to butanol-extracted LPS (34, 36), which contains lipid A-associated protein (26), than to phenol-extracted LPS. Butanol-extracted LPS also primed HeJ macrophages to produce more $O_2^-$ than did phenol-extracted LPS (Fig. 4). With macrophages from a closely related normal strain of mice, C3Heb/FeJ, the two forms of LPS had equal priming capacity at all concentrations tested from 0.1 ng/ml to 10 μg/ml; the results being similar to those observed with Swiss-Webster cells in Fig. 2.

Macrophages from C3H/HeJ mice responded normally to MDP (Table IV). The levels of $O_2^-$ released after PMA stimulation were equivalent to those achieved with MDP-primed Swiss-Webster or C3H/FeJ macrophages.

When nonadherent cells from C3Heb/FeJ cultures were added to cultured C3H/HeJ macrophages, no appreciable change in response to either purified LPS or MDP

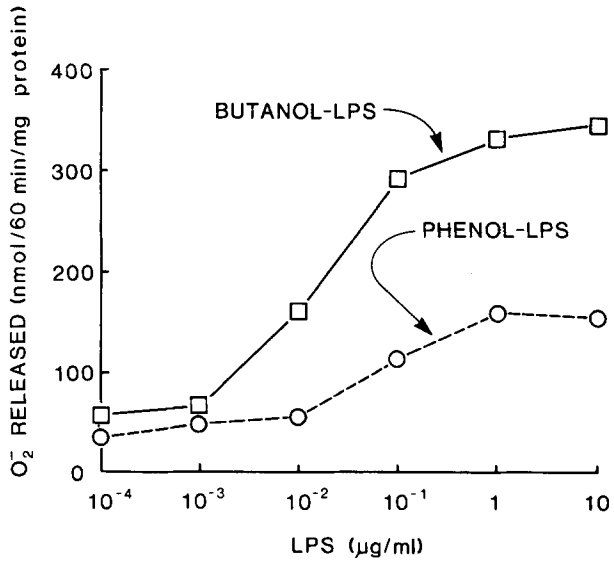


FIG. 4. Priming of C3H/HeJ macrophages by phenol-extracted LPS compared with butanol-extracted LPS. Values shown are means of four replicates. Standard error of all points was <15. $O_2^-$ release of control (untreated) cultures was 78 ± 7 SEM (four replicates). This experiment is representative of two performed.

TABLE IV

*Priming of Macrophages from C3H/HeJ and C3Heb/FeJ Mice by MDP*

| Treatment | PMA-stimulated $O_2^-$ Release* | |
|---|---|---|
| | HeJ | FeJ |
| | *nmol/mg protein* | |
| Control (saline) | 72 ± 7 | 68 ± 16 |
| MDP (0.01 μM) | 71 ± 4 | 82 ± 3 |
| MDP (0.1 μM) | 80 ± 1 | 96 ± 4 |
| MDP (1.0 μM) | 272 ± 25 | 191 ± 1 |

* Results are means ± SEM for duplicate assays. Results are representative of those obtained in three experiments.

was observed (Table V), which indicates that normal lymphocytes from the FeJ strain could not repair the weak HeJ macrophage response to purified LPS. (The FeJ nonadherent cells had a slight stimulatory effect compared with HeJ nonadherent cells, but this was the same for both LPS-treated and control cells.) Nor did the HeJ nonadherent cells appear to significantly suppress the response of FeJ macrophages to LPS. These results suggest that MDP and LPS act directly on the macrophages themselves.

To further evaluate the role of lymphocytes, the effect of MDP and LPS was tested on the macrophage-like cell line, J774.1. For technical reasons (see Methods), this cell line was treated with LPS or MDP for only 4 h, the minimal time required to observe a response to MDP by normal peritoneal macrophages (Fig. 3). The $O_2^-$ response of these cells was ~10% that of peritoneal cells, but the cells remained >98% viable by

TABLE V

*Effect of HeJ and FeJ Nonadherent Cells on the Response of HeJ and FeJ macrophages to MDP and LPS*

| Macrophage | Nonadherent cell | PMA-stimulated $O_2^-$ release* | | |
|---|---|---|---|---|
| | | Control (saline) | LPS (1 μg/ml) | MDP (1 μM) |
| | | *nmol/mg protein* | | |
| HeJ | HeJ | 17 ± 7 | 107 ± 1 | 114 ± 28 |
| HeJ | FeJ | 35 ± 6 | 139 ± 4 | 116 ± 8 |
| FeJ | HeJ | 145 ± 7 | 306 ± 21 | 255 ± 8 |
| FeJ | FeJ | 176 ± 26 | 316 ± 13 | 283 ± 14 |

* Results are means ± SEM of duplicate assays. Dishes that contained nonadherent cells alone produced no detectable $O_2^-$. These results are representative of those obtained in three experiments.

TABLE VI

*Priming of J774.1 Macrophage-like Cell Line by LPS and MDP*

| Treatment | PMA-stimulated $O_2^-$ release* |
|---|---|
| | *nmol/mg protein* |
| Control (saline) | 6.7 ± 0.7 |
| LPS (1 μg/ml) | 13.3 ± 0.8‡ |
| MDP (1 μM) | 10.0 ± 0.9‡ |
| MDP, D-D isomer (1 μM) | 6.2 ± 1.4 |

* Values are means ± SEM for seven to nine cultures incubated for 4 h in the presence of saline (control) or the substance shown, then stimulated by incubation with PMA for 60 min.

‡ Significantly different from control ($P < 0.002$, Student's one-tailed $t$ test for independent variables).

a trypan blue exclusion test. As shown in Table VI, a significant enhancement of $O_2^-$ production by both MDP and LPS was observed.

## Discussion

In vitro treatment of peritoneal macrophages with LPS or MDP, compared with preincubation in medium alone, results in the macrophages becoming primed to release several times more $O_2^-$ after stimulation by PMA or opsonized zymosan. This priming process occurs within a matter of hours after exposure to LPS or MDP. It is concentration dependent and associated with other manifestations of activation such as increased pseudopod formation.

Because peritoneal macrophages become primed after exposure to as little as 0.1 ng/ml of LPS, the presence of LPS in reagents becomes a serious problem. In the case of MDP, which was synthesized chemically, priming was not a result of contamination by LPS, because MDP was negative in the limulus assay (a 10-μg/ml test solution had <0.1 ng/ml of LPS); MDP from three independent laboratories gave superimposable dose-response curves (Fig. 2); and analogues of MDP synthesized in the same laboratories did not prime macrophages. However, the presence of LPS must be considered in testing the effects on macrophages of commercial reagents and products isolated from natural sources (33).

It is difficult to prove that adherent lymphocytes are not essential for the priming response, but the weight of evidence presented here suggests that MDP and LPS achieve their effect through direct interaction with macrophages. Greater than 95%

of the cells in preparations routinely used were capable of phagocytosing antibody-coated erythrocytes. Deliberate addition of nonadherent cells made no difference to the response of macrophages to LPS or MDP, although it could be argued that sufficient lymphocytes were already present to produce the maximal response. Macrophages from nude mice, which lack mature T lymphocytes, responded normally to LPS and MDP. Macrophages from C3H/HeJ mice were tested because cells from this strain have responded abnormally to LPS in several systems (30–37). A partial defect in response to LPS was exhibited by C3H/HeJ macrophages; the defect remained regardless of whether C3H/HeJ or C3H/FeJ (normal) lymphocytes were present. This result indicated that LPS interacts directly with the macrophages. The observation that the macrophage-like cell line responds to LPS and MDP also provides evidence that these compounds directly affect macrophages. Finally, the onset of priming after 30-min or 4-h of exposure to LPS or MDP, respectively, is a more prompt response than that expected from exposure to lymphokines (8). This conclusion is further supported by data obtained in a tumor-cell killing system. With pure populations of cloned macrophages from bone marrow as effector cells, Weinberg et al. (33) showed that LPS could increase cytotoxicity in the complete absence of lymphocytes.

The partial response of C3H/HeJ macrophages to purified phenol-extracted LPS with respect to priming for $O_2^-$ generation is interesting in view of reports that this type of LPS does not stimulate either the production of the macrophage product, lymphocyte-activating factor (LAF) (36), or glucose utilization (37) by cultured HeJ macrophages. Addition of FeJ lymphocytes to the HeJ macrophages had no effect on LAF production (36) but did enhance glucose utilization (37). Apparently, the need for lymphocyte involvement may vary depending upon the particular macrophage function being studied. The normal response of C3H/HeJ macrophages to MDP was not surprising, because MDP, unlike LPS, appears to have a normal adjuvant effect in C3H/HeJ mice (18).

The normal response of macrophages from nude mice to priming by LPS and MDP may be a phenomenon which contributes to the surprising ability of nude (nu/nu) mice to survive a challenge infection that is lethal to normal (nu/+) littermates (38–40). Although the reason for the enhanced survival of nude mice remains unclear (40), one hypothesis is that, in the absence of T-cell-mediated immunity, nude mouse macrophages are exposed to increased amounts of bacterial products, and are thereby chronically activated. These activated macrophages then provide an increased level of nonspecific resistance to infectious challenge.

In a normal intact animal, LPS would be expected to interact with B (41) and T cells (30), as well as with macrophages as shown here. MDP has also been shown to interact with both B and T cells (42–44). A combination of direct effects on macrophages and indirect effects via lymphokines could raise the macrophages to a higher state of activation than that achieved by the direct effect on macrophages alone. Such a combined effect of LPS and lymphokines has been shown in vitro with a tumor lysis assay (32). Nevertheless the in vitro priming to greater stimulated $O_2^-$ response represents at least one step in the process of macrophage activation (32). In fact, the degree of stimulation of $O_2^-$-generating capacity achieved in vitro with LPS (from 67 to 450 nmol $O_2^-$/mg) is equivalent to the difference in $O_2^-$-generating capacity observed between resident macrophages and macrophages that were sub-

jected to LPS or BCG activation in vivo (from 49 to 590 nmol/mg for LPS; 493 nmol/mg for BCG) (6). It remains to be determined whether cells primed in vitro manifest greater microbicidal or tumoricidal activity, or whether further differentiation, perhaps under the influence of lymphokines, is required for efficiency of these critical functions.

The complement system may play a role in macrophage activation by LPS in vivo. Although cultured macrophages may synthesize components of the complement system, experiments with MDP suggest that complement activation is probably not a requirement for priming in vitro, at least with MDP. We found that MDP, at concentrations up to 50 μM, did not activate the alternative pathway in serum, as tested by a sensitive kinetic hemolytic assay (45), and activation of complement by the classical pathway would require antibody and C1, which should not be present in macrophage cultures.

Although the in vitro priming effect of LPS and MDP may represent only one step or aspect of the process of macrophage activation as it occurs in vivo, this system should provide a useful tool. The addition of chemical effectors and inhibitors to cells undergoing priming by LPS or MDP may reveal some of the mechanisms that are involved in the process of macrophage activation.

The initial product of the respiratory burst that characterizes phagocytic cells appears to be $O_2^-$ (1). Spontaneous dismutation of $O_2^-$ leads to $H_2O_2$ formation, and ensuing molecular interactions generate hydroxyl radical and, perhaps, singlet oxygen (1). These toxic metabolites of oxygen are required for efficient killing of many microorganisms and, at least in some systems, for lysis of tumor cells (1, 3–5, 8). Our studies suggest that certain microbial products can interact directly with macrophages to accentuate their oxidative metabolic response to plasma membrane perturbation. Thus, in appropriate situations, exposure of macrophages to LPS, MDP, or related fragments of microbial cell surface constituents could result in an increased capacity to kill infecting microorganisms. On the other hand, this same mechanism could also permit macrophages to mediate oxidative tissue injury.

## Summary

After in vitro exposure to lipopolysaccharide (LPS) or muramyl dipeptide (MDP), cultured resident mouse peritoneal macrophages were primed to display enhanced generation of superoxide anion ($O_2^-$) in response to stimulation by phorbol myristate acetate (PMA) or opsonized zymosan. Priming with LPS (1 μg/ml) produced a sevenfold enhancement of PMA-stimulated $O_2^-$ generation; priming was detected within 30 min and persisted for at least 4 d. Exposure to MDP (1 μM) primed the macrophages to double their $O_2^-$ release; the response was first observed after 4 h and persisted for at least 3 d. The priming response was not observed with stereoisomers of MDP, which are inactive as adjuvants.

LPS and MDP appeared to work directly on the macrophages rather than indirectly by interacting with adherent lymphocytes: (*a*) Addition of nonadherent cell populations that contained lymphocytes had no effect on the response. (*b*) The response was normal with cells from nude mice, which lack mature T lymphocytes. (*c*) Macrophages from C3H/HeJ mice, whose B lymphocytes fail to respond to LPS, were weak in their response to priming by LPS; the addition of normal (C3Heb/FeJ) nonadherent cells

had no effect on this weak response. (*d*) The macrophage-like cell line J774.1 also showed enhanced $O_2^-$-generating capacity after a 4-h exposure to LPS or MDP.

The $O_2^-$-generating capacity of macrophages primed with LPS in vitro was equivalent to that previously observed with cells elicited in vivo by injection of LPS or activated by infection with *Bacille Calmette-Guérin*. The data suggest that previous exposure to bacterial products could prime macrophages to respond with increased production of toxic oxygen metabolites on contact with invading microorganisms or tumor cells.

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