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Enhanced Superoxide Anion Release from Phagocytes by Muramyl Dipeptide or Lipopolysaccharide

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Muramyl dipeptide (MDP) and lipopolysaccharide (LPS) from *Escherichia coli* were tested for the ability to influence superoxide anion (O_2^-) release from guinea pig phagocytes. Both MDP and LPS alone did not, by themselves, stimulate O_2^- release by macrophages and polymorphonuclear leukocytes. However, the preincubation of macrophages with MDP or LPS primed the macrophages to release an enhanced amount of O_2^- when stimulated by cytochalasin E and wheat germ agglutinin. When polymorphonuclear leukocytes were treated in the same way, only LPS showed an enhancing effect. MDP enhanced NADPH oxidase activity of macrophages, which is probably the reason for enhanced O_2^- release by MDP.

Peptidoglycans are structures common to bacterial cell walls. Muramyl dipeptide (MDP), *N*-acetylmuramyl-L-alanyl-D-isoglutamine, is a minimal structure in peptidoglycans responsible for many activities of bacteria, including immunoadjuvant effect (7). Although the injection of a Freund-type water-in-oil emulsion alone generally induces only macrophage accumulation or sometimes foreign body granulomas in the draining lymph nodes, the incorporation of MDP in the emulsion causes remarkable qualitative and quantitative changes, such as the development of extensive epithelioid granulomas indistinguishable from those evoked by tubercle bacilli and a marked infiltration of polymorphonuclear leukocytes (PMN) (8, 28).

MDP activates macrophages to become bactericidal (11), and epithelioid cells appear to be bactericidal against tubercle bacilli (3). However, the mechanism causing macrophages to become bactericidal through MDP is not yet known. It is also not known why PMN accumulate in the MDP-induced granulomas and whether PMN are affected by MDP. To understand these unsolved questions, we investigated, in the present study, the effect of MDP on superoxide anion (O_2^-) release from macrophages or PMN, because O_2^- has been known to contribute to microbicidal activity of phagocytes (5, 6, 17) and to cause the generation of chemotactic factor(s) for PMN (15, 25, 31). Guinea pigs were used because this animal species has been mostly used for the study of granuloma formation by MDP. Since bacterial lipopolysaccharide (LPS) shares many biological activities with MDP, LPS was also studied in parallel with MDP.

MATERIALS AND METHODS

Reagents. Cytochalasin E (CyE), superoxide dismutase (type I, from bovine blood), ferricytochrome *c* (type VI, from horse heart), and NADPH were purchased from Sigma Chemical Co., St. Louis, Mo. Wheat germ agglutinin (WGA) was purchased from P-L Biochemicals, Inc., Milwaukee, Wis. Renex 30, polyoxyethylene tridecyl ether, a product of ICI Americas, Inc., Wilmington, Del., was the generous gift of M. Nakamura, Kyushu University; Hanks balanced salt solution (HBSS) was purchased from Nissui Pharmaceutical Co., Ltd., Tokyo, Japan. Medium 199 and fetal bovine serum were purchased from GIBCO Laboratories, Grand Island, N.Y. Lipopolysaccharide (LPS) from *Escherichia coli* (serotype 0.127:B8, type I) was purchased from Sigma Chemical Co. MDP and its analogs were supplied by A. Inoue, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan; S. Kotani and T. Shiba, Osaka University, Osaka, Japan; and E. Lederer, Laboratoire de Biochimie, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France. Medium 199, HBSS phosphate-buffered saline, and stock solutions of MDP (100 μg/ml) and its analogs (100 μg/ml) in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (17 mM HEPES–120 mM NaCl–5 mM KCl–1 mM $CaCl_2$–1 mM $MgCl_2$–5 mM glucose, pH 7.3) were all endotoxin-free as determined by *Limulus* assay. *Limulus* amoebocyte lysate was obtained from the Wako Chemical Co., Osaka, Japan.

Animals. Unsensitized Hartley male guinea pigs, weighing 400 to 500 g each, were used.

Cell preparations. Four days after the injection of 20 ml of liquid paraffin or thioglycolate medium into the peritoneal cavities of guinea pigs, peritoneal exudate cells (PEC) were collected aseptically with HBSS. Liquid paraffin-induced cells were generally used unless otherwise indicated. The cell suspensions in HBSS were centrifuged at 50 × *g* for 5 min to eliminate

contaminating erythrocytes and lymphocytes. Purification of macrophages from PEC or from normal peritoneal cells were performed as described by Kumagai et al. (13). Briefly, the cells were incubated in medium 199 containing 10% heat-inactivated fetal bovine serum for 45 min in fetal bovine serum-coated glass dishes and washed three times with medium 199. The adherent cells were collected by repeated pipetting, using phosphate-buffered saline containing 0.2% EDTA and 5% fetal bovine serum. More than 95% of the adherent cells thus obtained consisted of macrophages, as determined by phagocytosis (2), morphology, and esterase staining (14, 32). The purity of macrophages was elevated to 99% by repeating the adherence procedure twice.

Guinea pig PMN were elicited by the intraperitoneal injection of 20 ml of 10% polypeptone. Ten hours after injection, PMN were collected aseptically with HBSS. More than 90% of the cells thus collected were PMN. These cells were washed once with HEPES buffer.

Contaminating erythrocytes were removed by hemolysis with 0.83% NH_4Cl in Tris buffer, pH 7.2.

Determination of released O_2^-. O_2^- determination was performed by the method of Nakagawara et al. (21). Briefly, 2 ml of a reaction mixture containing 100 μM ferricytochrome *c* and 2×10^6 cells in HEPES buffer was preincubated for different time intervals in a shaking plastic cuvette of a spectrophotometer with or without MDP or LPS. CyE (5 μg/ml) and WGA (40 μg/ml) were then added to the reaction mixture, and the rate of superoxide dismutase-inhibitable reduction of ferricytochrome *c* was measured continuously by recording the absorption increase at 550 to 540 nm (molar absorption coefficient, 19×10^3) with a Hitachi 556 double-beam spectrophotometer. To prevent sedimentation of the cells, a cell mixer was attached to the cuvette as described by Kakinuma et al. (12).

Determination of NADPH oxidase. NADPH oxidase activity was measured by the method of Nakamura et al. (22). Briefly, 2 ml of HEPES buffer containing 2×10^5 macrophages was preincubated with or without MDP for 40 min at 37°C. After preincubation, ferricytochrome *c* was added and the incubation mixture was equilibrated at 25°C. These macrophages were then stimulated by CyE and WGA, and cytochrome *c* reduction was measured. Four minutes later, the macrophages were lysed by the addition of 10 μl of 2% Renex 30. Exactly 50 s later, NADPH was added to the cell lysates, and the NADPH-induced, superoxide dismutase-inhibitable cytochrome *c* reduction was measured.

Measurement of the binding of WGA with macrophages. A total of 10^6 macrophages were preincubated in HEPES buffer with or without MDP or LPS for 40 min, CyE and ^{125}I-labeled WGA (40 μg, 138 and 165 cpm, respectively) were then added, and, after another 7 min of incubation, these cells were washed twice with HEPES buffer. The cell-bound radioactivity was counted in a Packard gamma scintillation counter.

Statistical analyses. Means ± standard deviations were calculated from data obtained from at least three similar experiments. Differences between two means were assessed by Student's *t* test.

RESULTS

Kinetics of the effect of MDP or LPS on O_2^- release. No O_2^- release was observed when macrophages or PMN were stimulated by either MDP or LPS alone. However, the O_2^- release from macrophages preincubated with either MDP or LPS and then stimulated by CyE and WGA was significantly enhanced as compared with that from macrophages preincubated in the absence of MDP or LPS and then stimulated by CyE and WGA. Thus, MDP or LPS did not, by themselves, stimulate phagocytes to release O_2^-, but they primed macrophages for enhanced O_2^- release triggered by CyE and WGA.

The priming effect of the preincubation with MDP or LPS was measured as a function of the preincubation time. The effect was already significant after 15 min of the preincubation, reached a peak after 40 min, and then diminished rapidly to become almost zero after 60 min (Fig. 1). The curves for the effect of MDP and LPS were almost identical.

In contrast to macrophages, the O_2^- release from PMN measured in the same way was enhanced only by preincubation with LPS; preincubation with MDP had no effect on the rate of O_2^- release from PMN stimulated by CyE and WGA (Fig. 2). The time course of the priming effect of the preincubation with LPS on O_2^- release from PMN was similar to that of its effect on macrophages (Fig. 2).

Effect of different concentrations of MDP or LPS on O_2^- release. Cells were preincubated for 40 min with various concentrations of MDP or LPS, and the rate of O_2^- release was measured after stimulation by CyE and WGA. In the case of macrophages, the 50% effective doses were approximately 0.005 and 0.1 μg/ml for LPS and MDP, respectively (Fig. 3). At higher concentrations, MDP increased O_2^- release more effectively than LPS, whereas the reverse was true at

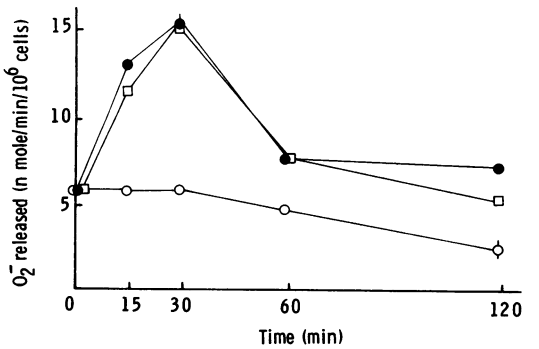

FIG. 1. Time course of the effect of preincubation with MDP (●) or LPS (□). Macrophages (2×10^6) were preincubated with 5 μg of MDP or LPS per ml for various time intervals, and O_2^- release in response to CyE and WGA was measured. Results are shown as means ± standard deviations ($n = 3$). Similar curves were obtained in two additional experiments. ○, Control.

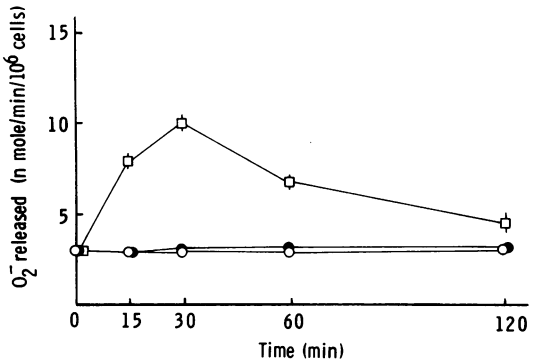

FIG. 2. Time course of the effect of preincubation with MDP (●) or LPS (□). PMN (2×10^6) were preincubated with 5 μg of MDP or LPS for various time intervals, and O_2^- release in response to CyE and WGA was measured. Results are shown as means ± standard deviations (n = 3). Similar curves were obtained in two additional experiments. ○, Control.

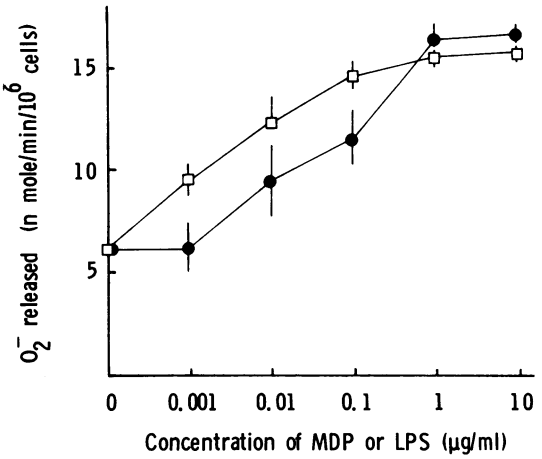

FIG. 3. Dose-response relationship for O_2^- release from macrophages. Macrophages (2×10^6) were preincubated with MDP (●) or LPS (□) at various concentrations for 40 min, and O_2^- release in response to CyE and WGA was measured. Results are shown as means ± standard deviations. A similar curve was obtained in an additional experiment.

lower concentrations. In the case of PMN, LPS showed a dose-dependent capacity to increase O_2^- release, whereas MDP did not show such a capacity at any concentrations tested (Fig. 4).

Effect of MDP analogs on O_2^- release. PEC or resident peritoneal cells were preincubated for 40 min with MDP, MDP analogs, or LPS and then stimulated by CyE and WGA. Preincubation of PEC with MDP or LPS increased O_2^- release to twice that of the control, whereas adjuvant-inactive analogs of MDP, the D-alanine and D-isoasparagine derivatives, did not prime the cells for enhanced O_2^- production (Table 1). These results indicate that the observed priming effect of MDP depends upon the special structure responsible for its activities, such as immunoadjuvant effect. Resident peritoneal cells preincubated with MDP or LPS also released significantly increased amounts of O_2^-, but to a lesser extent than did PEC (Table 1).

Effect of lymphocytes on O_2^- release. PEC were divided into two parts, adherent and nonadherent cells, as described in Materials and Methods. Adherent cells were further purified by the adherence procedure. Purified adherent cells consisted of 99% macrophages and 1% mesothelial cells. Nonadherent cells contained 45% macrophages, 10% PMN, and 45% lymphocytes. The adherent and nonadherent cells were preincubated separately with MDP or LPS for 40 min and then assayed for O_2^- release. The adherent cells, which contained no lymphocyte, produced about twice as much O_2^- as the nonadherent cells after preincubation with MDP or LPS (data not shown). Thus, lymphocytes appeared not to contribute to enhanced O_2^- release.

Effect of MDP on NADPH oxidase activity. NADPH oxidase activity was determined by measuring NADPH-dependent superoxide production of Renex 30-treated macrophages which had been preincubated with or without MDP and then stimulated by CyE and WGA. In parallel with the twofold increase in O_2^- release from macrophages preincubated with MDP, NADPH oxidase activity of the lysates of the same macrophages was enhanced twofold as compared with that of the control (Table 2).

Binding of WGA to macrophages. Preincuba-

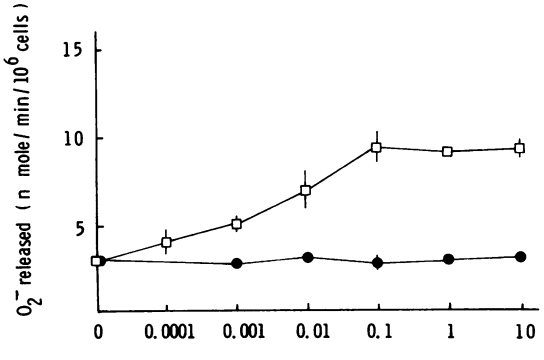

FIG. 4. Dose-response relationship for O_2^- release from PMN. PMN (2×10^6) were preincubated with MDP (●) or LPS (□) at various concentrations for 40 min, and O_2^- release in response to CyE and WGA was measured. Results are shown as means ± standard deviations (n = 6). A similar curve was obtained in an additional experiment.

TABLE 1. Effect of LPS or MDP or its analogs on O_2^- release

Treatment[a]	O_2^- released (nmol/min) per 10^6 cells in[b]:			
	Liquid paraffin-induced macrophage	Thioglycolate-induced macrophage	Resident peritoneal cells	PMN
None	6.1 ± 0.29	5.2 ± 0.21	3.0 ± 0.15	3.1 ± 0.17
LPS	15.0 ± 0.39	19.0 ± 0.40	5.9 ± 0.26	10.0 ± 0.25
MDP	15.4 ± 0.26	18.9 ± 0.12	6.2 ± 0.31	3.1 ± 0.26
D-D	6.3 ± 0.30	5.3 ± 0.38	2.8 ± 0.36	3.1 ± 0.17
D-IsoAsn	5.7 ± 0.42	4.5 ± 0.21	2.6 ± 0.36	2.9 ± 0.21

[a] D-D, *N*-Acetylmuramyl-D-alanyl-D-isoglutamine; D-IsoAsn, *N*-acetylmuramyl-L-alanyl-D-isoasparagine.
[b] Assay was performed as described in the text. Numbers are means ± standard deviations ($n = 6$, except for resident peritoneal cells for which $n = 3$). Similar data were obtained in two additional experiments.

tion of macrophages with MDP or LPS did not influence the amounts of WGA bound to the cells (Table 3).

DISCUSSION

MDP in a water-in-oil emulsion causes extensive epithelioid granulomas with PMN infiltrations in guinea pigs, rats, and rabbits, but not in mice (8, 28). LPS in a water-in-oil emulsion also caused epithelioid granuloma in guinea pigs (unpublished data). Epithelioid granuloma is generally considered to be a hallmark of defense mechanism, and epithelioid cells are suggested to be bactericidal against tubercle bacilli (3). MDP and LPS were found to activate macrophages to become bactericidal against *Listeria monocytogenes* in vivo and in vitro (9, 11; unpublished observation).

The present study revealed that the macrophages of guinea pigs preincubated with MDP or LPS released a larger amount of O_2^- upon stimulation by the cell membrane-perturbing agents CyE and WGA than did those preincubated without MDP or LPS. These results suggest that the activation of macrophages results in an enhanced production of the active oxygen. Nathan and Root also found that this activation was necessary for mouse macrophages to release hydrogen peroxide, though the activation was achieved in vivo (23). Since O_2^- and its related reactive oxygen intermediates are known to play an important role in the microbicidal activity of phagocytes (5, 6, 17), the observed ability of MDP and LPS to increase O_2^- release from macrophages seems to at least partly explain its ability to render macrophages or epithelioid cells bactericidal. (Epithelioid cells are generally considered to be derived from macrophages [1].) Increased O_2^- release by MDP or LPS might contribute to their antitumor activity (4, 10, 16, 27, 30).

Since it had recently been found that O_2^- played an important role for the generation of chemotactic factor(s) for PMN, the increase in O_2^- release caused by MDP that we observed might also have some causal relation with the PMN infiltrations into the MDP-induced granulomas (15, 25, 31). This possibility is now being assessed with superoxide dismutase.

The priming effect of MDP for the enhanced release of O_2^- probably does not require an involvement of lymphocytes or lymphokines because, first, macrophage suspensions containing 99% macrophages and 1% mesothelial cells could be primed by MDP or LPS to about the same extent as PEC containing different concentrations of lymphocytes, and, second, preincubation periods as short as 15 min were enough for MDP to prime macrophages significantly. These results are in accord with our previous observations that MDP evokes epithelioid granulomas and activates macrophages without T cell involvement (18–20, 29). Thus, it seems that phagocytes react to the structures characteristic of the surface of bacteria with heightened defense capacity; this occurs before immune cells are recruited.

Pabst and Johnston reported that MDP and LPS primed mouse peritoneal resident macrophages for enhanced O_2^- production (24). There are discrepancies, however, between their results and ours in the kinetics of the priming. In their study, the priming effect of MDP on macrophages was first noted at 4 h of preincubation, reached its peak at 24 h, and then declined

TABLE 2. O_2^- release and NADPH oxidase activity of macrophages[a]

Preincubation	O_2^- release (nmol/min) per 10^5 cells	NADPH oxidase activity
None (control)	0.2 ± 0.1	0.7 ± 0.1
MDP	0.5 ± 0.1[b]	1.3 ± 0.1[c]

[a] O_2^- release and NADPH oxidase activity were measured as described in the text, using a single macrophage population. Numbers are means ± standard deviations ($n = 3$). Similar data were obtained in an additional experiment.
[b] $P < 0.05$.
[c] $P < 0.005$.

TABLE 3. [^{125}I]WGA binding to macrophages[a]

Preincubation	cpm per 10^6 cells
None (control)	12,981 ± 918
MDP	11,865 ± 679
LPS	12,717 ± 910

[a] Assay was performed as described in the text. Numbers are means ± standard deviations (n = 3). Similar data were obtained in an additional experiment.

gradually thereafter, reaching the control level at 96 h. In the present study, the priming effect was already significant at 15 min, reached its peak at 40 min, and then rapidly declined thereafter, reaching the control level at 1 h. There is also a similar discrepancy for LPS. These discrepancies are probably due to the differences in the assay methods, the animal species used, and the way in which macrophages were obtained.

We have further found that MDP primed macrophages for increased activity of NADPH oxidase, in parallel with the increased O_2^- release, after preincubation for 40 min with MDP. This observation strongly suggests that MDP first caused an increase in NADPH oxidase activity, which in turn resulted in increased production and release of O_2^-. MDP differed from LPS in that LPS primed both macrophages and PMN, whereas MDP primed only macrophages. In agreement with this observation, we also observed that LPS inhibited the migration of both macrophages and PMN, whereas MDP inhibited only macrophage migration (unpublished observation).

In contrast to the present results, Proctor reported that preincubation of human PMN with LPS for 30 min produced significant depression of chemiluminescence, oxygen consumption, and O_2^- release (26). This discrepancy might be due to differences in species and experimental conditions.

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