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Biphasic Effects of Muramyl Dipeptide or Lipopolysaccharide on Superoxide Anion-Generating Activities of Macrophages

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The superoxide anion (O_2^-)-generating activity of guinea pig macrophages stimulated by wheat germ agglutinin (WGA), immune complexes, or phorbol myristate acetate (PMA) was studied after short- and long-term exposures of the cells to muramyl dipeptide (MDP) or lipopolysaccharide (LPS). Neither MDP nor LPS alone induced O_2^- release in macrophages. Short-term (30 min) exposure to these agents caused the enhanced release of O_2^- in response to WGA or immune complexes, though the PMA-induced O_2^- generation was not affected. On the other hand, long-term exposure (more than 24 h) to MDP or LPS progressively enhanced O_2^- generation of the cells induced by WGA, immune complexes, or even PMA. These results suggest that the mechanism for O_2^- generation of macrophages stimulated by WGA or immune complexes differs from that stimulated by PMA and that the differences also exist between short- and long-term exposure to MDP or LPS.

Although superoxide anion (O_2^-) generation by macrophages and polymorphonuclear leukocytes (2, 5, 8, 19) has been extensively studied, the precise mechanism that causes the cells to produce O_2^- is still unclear. An NADPH oxidase in the plasma membrane of these cells is considered to be responsible for O_2^- generation (4). However, the processes for activation of NADPH oxidase after stimulation of the cell surface by various agents remain an enigma.

It has been previously reported that wheat germ agglutinin (WGA) and phorbol myristate acetate (PMA) affect O_2^- generation of macrophages in different ways (20). Pretreatment of macrophages with prostaglandin E_1, prostaglandin E_2, dibutyryl cyclic AMP, and an inhibitor of transmethylation reaction (3'-deazaadenosine) selectively inhibited O_2^- generation induced by WGA but did not affect that induced by PMA.

Treatment of phagocytes with muramyl dipeptide (MDP) or lipopolysaccharide (LPS) has been shown to enhance their production of O_2^- (10, 14, 15). However, the mechanism that causes macrophages to be activated by MDP or LPS for the enhanced O_2^- generation is not well understood.

In this study, we investigated the effect of MDP or LPS on macrophage ability to produce O_2^- in response to various stimuli, such as WGA, immune complexes, or PMA.

MATERIALS AND METHODS

Reagents. Cytochalasin E (Cyt-E), ferricytochrome *c* (type VI, from horse heart), superoxide dismutase (type I, from bovine blood), NADPH, and egg albumin (EA) were purchased from Sigma Chemical Co., St. Louis, Mo. WGA and PMA were 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. RPMI 1640 medium and fetal bovine serum were purchased from GIBCO Laboratories, Grand Island, N.Y. LPS from *Escherichia coli* (serotype O127:B8, type I) was purchased from Sigma. MDP and *N*-acetylmuramyl-D-alanyl-D-isoglutamine (D-D) were supplied by 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. RPMI 1640, HBSS, and stock solutions of MDP (100 μg/ml) and D-D (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 guinea pigs, weighing 400 to 500 g, were used.

Preparations and incubations of peritoneal macrophages. On days 6 to 8 after the injection of 20 ml of liquid paraffin into the peritoneal cavity of guinea pigs, peritoneal cells were collected with HBSS aseptically. Purification of macrophages from peritoneal exudate cells was performed as described previously (17). Briefly, the cells were incubated at 37°C in glass dishes. After a 45-min incubation, the cells adherent to the surface of the dishes were collected and used as purified macrophages.

For a short-term exposure, 5×10^5 cells suspended in 1 ml of HEPES buffer were incubated with MDP or LPS in a plastic cuvette for spectrophotometry, and then the O_2^- generation of the cells was assayed after the addition of various stimuli. For a long-term exposure, 5×10^5 macrophages per ml of RPMI 1640 supplemented with 10% fetal bovine serum were incubated with MDP or LPS for 24 to 72 h. The cells were harvested by gentle pipetting and washed three times with HEPES buffer, and then the O_2^- generation of the cells was assayed after the addition of various stimuli.

Determination of the amount of released O_2^-. O_2^- determination was performed as described previously (10). Briefly, 1 ml of the reaction mixture containing 100 μM ferricytochrome *c* and 5×10^5 cells in HEPES buffer was preincubated in a plastic cuvette for spectrophotometry at 37°C for 10 min. WGA (40 μg/ml) and Cyt-E (5 μg/ml), insoluble immune complexes (25 μg of antibodies per ml; molar ratio of antigen to antibody, 1:1) and Cyt-E (5 μg/ml), or PMA (10^{-8} to 10^{-9} M final concentration) were then added to the reaction

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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), using a Hitachi 556 two-beam spectrophotometer. To prevent the sedimentation of the cells, a cell mixer was attached to the cuvette (9).

Immune complexes were prepared as described previously (17). Briefly, the anti-EA antibodies were specifically purified from antiserum of guinea pigs that were hyperimmunized with EA by using an immunoadsorbent prepared by coupling EA to cyanogen bromide-activated Sepharose 4B. The antibodies of immunoglobulin G2 were obtained from the purified antibodies by chromatography on DEAE-cellulose.

The immunoglobulin G2 antibodies obtained were used for preparation of immune complexes. For the preparation of insoluble immune complexes, the purified immunoglobulin G2 antibodies and EA were mixed at a molar ratio of 1:1 and incubated for 24 h at 4°C.

Determination of NADPH oxidase activity. NADPH oxidase activity was measured by the method of Nakamura et al. (12). Briefly, 1 ml of HEPES buffer containing 2 × 10^5 macrophages and ferricytochrome *c* was equilibrated at 25°C. These macrophages were then stimulated by various stimuli, 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 protein contents. Protein contents of the cells were determined by the method of Lowry et al. (11) with crystalline bovine serum albumin as a standard.

Measurement of the binding of ^{125}I-labeled WGA or ^{125}I-labeled immune complexes to macrophages. Cells (10^6) suspended in 1 ml of RPMI 1640 medium with 10% fetal bovine serum and 0.05% sodium azide were incubated with ^{125}I-labeled WGA (10 μg, 67,000 cpm) for 30 min or with ^{125}I-labeled immune complexes (2 μg of antibodies, 124,000 cpm) for 4 h at 4°C as described previously (10, 17). Then, the cells were washed three times with 5 ml of HBSS, and the cell-bound radioactivity was measured in a Packard gamma counter. The radioactivity bound to the control cells was ca. 15% of the added radioactivity for WGA and 6% for immune complexes. Binding of ^{125}I-labeled WGA to macrophages was inhibited more than 90% by adding 10 mg of *N*-acetylglucosamine per ml in the reaction mixture, implying that the binding is specific to *N*-acetylglucosamine on the cell membrane. The bound radioactivity of ^{125}I-labeled immune complexes with F(ab')$_2$ fragments of antibodies to macrophages was less than 0.3% of that added.

TABLE 1. Effect of pretreatment of macrophages on O_2^- generation of cells

Stimuli for O_2^-	Culture	O_2^- generation[a] (mean ± SD)	
		30 min	72 h
WGA (40 μg/ml) + Cyt-E	Control	54.6 ± 1.4	170.8 ± 18.2
	LPS	103.6 ± 9.8	496.6 ± 62.4
	MDP	92.4 ± 9.8	460.0 ± 72.8
	D-D	57.4 ± 4.2	260.4 ± 43.4
Immune complexes (25 μg/ml) + Cyt-E	Control	39.2 ± 2.8	152.6 ± 11.2
	LPS	126.0 ± 12.6	354.9 ± 41.6
	MDP	106.4 ± 9.8	320.6 ± 26.6
	D-D	37.8 ± 4.2	208.6 ± 40.6
PMA (10^{-9} M)	Control	58.8 ± 4.2	39.2 ± 4.2
	LPS	65.8 ± 7.0	120.9 ± 15.6
	MDP	71.4 ± 2.8	92.4 ± 9.8
	D-D	58.8 ± 1.4	54.6 ± 14.0
PMA (10^{-8} M)	Control	165.2 ± 12.6	137.2 ± 8.4
	LPS	183.4 ± 18.2	382.2 ± 72.8
	MDP	170.8 ± 9.8	343.0 ± 58.8
	D-D	152.6 ± 16.8	194.6 ± 40.6

[a] Amount (nanomoles per minute per milligram of protein) of O_2^- generated by macrophages after pretreatment for 30 min and 72 h with or without LPS (5 μg/ml), MDP (5 μg/ml), or D-D (5 μg/ml); n = 3.

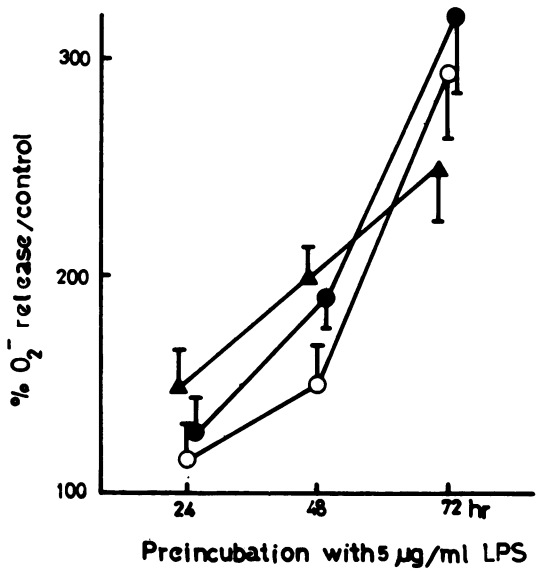

FIG. 1. Effect of long-term exposure of macrophages to LPS on O_2^- generation of cells. Macrophages were incubated with LPS (5 μg/ml) for 24 to 72 h. Then, the O_2^- generation of the cells induced by WGA and Cyt-E (●), immune complexes (▲), or PMA (○) was measured as described in the text. Bars indicate standard deviation (n = 3).

RESULTS

Effect of short-term exposure of macrophages to MDP or LPS on O_2^- generation. Though MDP or LPS alone did not induce O_2^- release by macrophages, a 30-min exposure to those reagents primed the cells to release an enhanced amount of O_2^- when they were stimulated by WGA or by immune complexes in the presence of Cyt-E. The amount of O_2^- released was two- and threefold that of the control with regard to the stimulation by WGA or immune complexes, respectively (Table 1). The enhancing effects of MDP or LPS on O_2^- generation of macrophages peaked at 15 to 30 min and then gradually disappeared (data not shown). However, the exposure of the cells to MDP or LPS for a short period had no effect on PMA-induced O_2^- generation.

Effect of long-term exposure of macrophages to MDP or LPS on O_2^- generation of cells. When macrophages were exposed to MDP or LPS for more than 24 h, the mode of O_2^- generation of the cells was quite different from that of the cells exposed for a short period. Figure 1 shows the enhancing effect of LPS on O_2^- generation of macrophages. When the cells were exposed to LPS for more than 24 h, the O_2^- generation induced by WGA, immune complexes, or PMA increased according to the exposure time (24 to 72 h). The

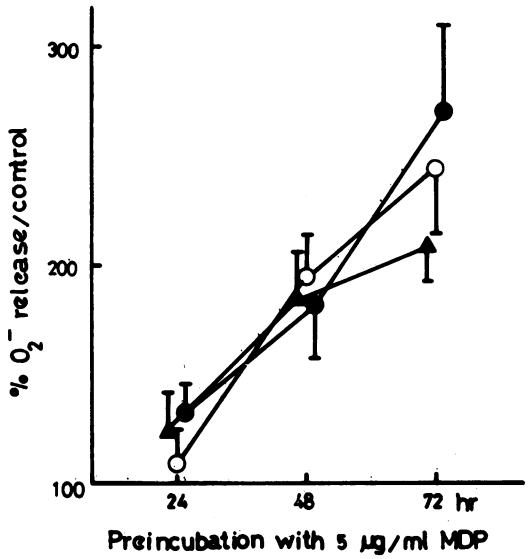

FIG. 2. Effect of long-term exposure of macrophages to MDP on O_2^- generation of cells. Macrophages were incubated with MDP (5 μg/ml) for 24 to 72 h. Then, the O_2^- generation of the cells induced by WGA and Cyt-E (●), immune complexes (▲), or PMA (○) was measured as described in the text. Bars indicate standard deviation (n = 3).

similar kinetics of the enhancing effect on O_2^- generation was observed with MDP (Fig. 2).

Table 1 summarizes the amounts of O_2^- generated from macrophages treated with or without LPS, MDP, or D-D. D-D, which is an adjuvant-inactive analog of MDP, was used as a negative control of MDP because it has no enhancing effect on O_2^- generation of macrophages (10). During long-term exposure, the O_2^--generating activities of the control cells increased gradually when the cells were stimulated by WGA or immune complexes. However, the PMA-induced O_2^- generation was only slightly altered by in vitro culture of the cells for 72 h. Nevertheless, the O_2^--generating activities of the cells exceeded those of controls when the cells were exposed to MDP or LPS and then were stimulated by WGA, immune complexes, or PMA.

Dose-dependent effect of MDP or LPS on O_2^- generation of macrophages. Macrophages were incubated with various doses of MDP or LPS for 72 h, and the O_2^- generation of the cells induced by WGA or PMA was measured. As little as 0.5 ng of LPS per ml or 5 ng of MDP per ml was effective for enhancing O_2^- generation (Fig. 3). These results matched well with dose-response curves for the short-term effects of MDP or LPS reported previously (10).

Effect of long-term exposure of macrophages to MDP or LPS on NADPH oxidase activity. The cells exposed to MDP or LPS for 72 h were stimulated by WGA, immune complexes, or PMA and then lysed by detergent (Renex 30) to measure NADPH oxidase activity in the cell lysate as described above. NADPH oxidase activity was also enhanced more than twofold by pretreatment of the cells with MDP or LPS, and the extent of enhancement paralleled that of O_2^- generation (Table 2).

Effects of MDP or LPS on protein contents and viability of macrophages. To examine whether quantitative changes of O_2^--generating systems in macrophages occur during long-term exposure, we measured the protein contents of these cells in the presence or absence of LPS, MDP, or D-D (Table 3). The results showed that the protein contents were not altered after 3 days of culture with or without LPS, MDP, or D-D and that the viability of the cells was always more than 85%. The total number of viable cells recovered after 5×10^5 macrophages per ml were cultured with medium only (control), LPS, MDP, or D-D was 2.7×10^5 per ml (54%), 3.4×10^5 per ml (68%), 3.1×10^5 per ml (62%), and 2.6×10^5 per ml (52%), respectively.

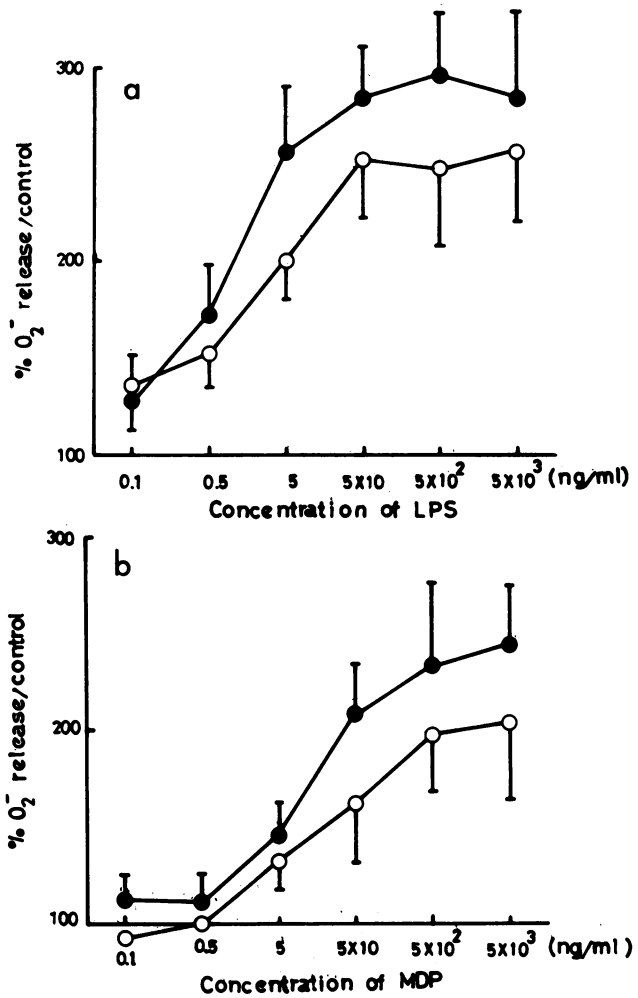

FIG. 3. Dose-dependent effect of LPS or MDP on O_2^- generation of macrophages. Macrophages were incubated with various doses of LPS (a) or MDP (b) for 72 h. Then, the O_2^- generation of the cells induced by WGA and Cyt-E (●) or PMA (○) was measured as described in the text. Bars indicate standard deviation (n = 3).

Effect of MDP or LPS on the binding of WGA to macrophages. The effects of MDP or LPS on the binding of WGA

TABLE 2. NADPH oxidase activity of macrophages after long-term exposure to LPS or MDP

Pretreatment	NADPH oxidase activity with the following stimulus[a] (mean ± SD):	
	WGA	PMA
None	15.4 ± 2.8	11.2 ± 2.8
LPS (5 μg/ml)	35.1 ± 6.5	32.5 ± 5.6
MDP (5 μg/ml)	33.6 ± 5.6	26.6 ± 4.2

[a] NADPH oxidase activity (nanomoles per minute per milligram of protein of macrophages) was measured at 25°C as described in the text; n = 3.

TABLE 3. Protein contents and viability of macrophages after long-term exposure to LPS, MDP, or D-D

Culture	Protein content[a] (mean ± SD)	% Viable cells[b] (mean ± SD)
Control	142 ± 18	91 ± 4
LPS (5 μg/ml)	156 ± 21	95 ± 2
MDP (5 μg/ml)	136 ± 14	93 ± 3
D-D (5 μg/ml)	150 ± 16	89 ± 3

[a] Protein contents (micrograms per 10^6 macrophages) were measured by the method of Lowry et al. (11); $n = 3$.
[b] Viability of the cells was tested by trypan blue exclusion; $n = 3$.

and immune complexes to macrophages were investigated. The binding of ^{125}I-labeled WGA to the cells after 3 days of culture with medium (control), LPS (5 μg/ml), MDP (5 μg/ml), or D-D (5 μg/ml) was (mean ± standard deviation) 10,480 ± 2,180, 12,060 ± 1,480, 9,720 ± 2,350, and 11,360 ± 1,480 cpm per 10^6 cells, respectively ($n = 3$). The long-term exposure of macrophages to MDP or LPS also had no significant enhancing effects on the binding of ^{125}I-labeled immune complexes to the macrophages (data not shown). These results suggest that the increased O_2^- generation of the cells cultured with MDP or LPS was not due to the enhanced binding of the stimuli to the cells.

DISCUSSION

The present study indicates that the mechanism for O_2^- generation by WGA or immune complexes is different from that by PMA. This view is consistent with previous reports that the WGA- or immune complex-induced O_2^- generation of macrophages is regulated by intracellular cyclic AMP and is associated with transmethylation of phospholipid of the membrane, but PMA-induced generation is not (6, 16, 18). The mechanism for O_2^- generation mediated by receptors for WGA or immune complexes is not well understood, but it may be related to various transmembrane events such as an increase in Ca^{2+} influx, methylation of phospholipids, activation of adenylcyclase, increase in intracellular cyclic AMP levels, and activation of phospholipase A_2 (7, 18, 20).

Goodwin and Weinberg (7) reported the presence of a specific receptor for phorbol esters on human monocytes, neutrophils, platelets, and lymphocytes. Furthermore, Niedel et al. (13) showed that the receptor for phorbol esters in rat brain exhibits protein kinase C activity. This enzyme is a Ca^{2+}- and phospholipid-dependent protein kinase that is activated by phorbol esters as well as diacylglycerol, causing phosphorylation of a specific 40,000-dalton protein in the human platelet (3). It has also been shown that phosphorylation of membrane protein of human neutrophils is significantly increased by PMA (1). Therefore, PMA may stimulate steps for O_2^- generation through activation of protein kinase C that are independent of processes regulated by cyclic AMP.

Concerning the enhancing effect of MDP or LPS on O_2^- generation of macrophages during a short period, some regulatory factors for the NADPH oxidase activation system which are linked to O_2^- generation (4) may be responsible. We previously found that preincubation of macrophages with MDP or LPS resulted in enhanced NADPH oxidase activity (10). We further observed that transmethylation of plasma membrane phospholipids and activation of adenyl cyclase occur transiently after exposure of macrophages to LPS (data not shown). The plausible explanation of the enhancing effect of MDP or LPS may be that some factors, for example cyclic AMP or cyclic AMP-dependent protein kinase, prime the activation process for NADPH oxidase with the receptors for WGA or immune complexes but do not affect the process induced by PMA in response to the binding of MDP and LPS to the cell membranes.

On the other hand, O_2^--generating capacities of macrophages increased progressively during long-term exposure to MDP or LPS (24 to 72 h) when the macrophages were stimulated by PMA as well as WGA or immune complexes. These results indicate that the mechanisms involved in O_2^- production differ between short- and long-term exposure to MDP or LPS.

Pabst and Johnston (15) also observed an enhancing effect of MDP or LPS on O_2^- generation in mouse peritoneal macrophages. However, in their study, the priming effect of these compounds on macrophages, initiated after a 4-h exposure, peaked at 24 h and then declined. In human monocytes (14), it was observed that the enhancing effect of LPS on PMA-induced O_2^- generation appeared after 30 min of incubation and increased progressively with preincubation time. The discrepancy between that study and ours may be due to the differences in the animal species used and the method with which cells were obtained.

The present study shows that MDP and LPS biphasically prime macrophages for enhanced O_2^- generation, one phase being observed with the short-term exposure and the other being observed with the long-term exposure, and that the mechanisms of priming for each phase appear to differ from each other.

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