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Macrophages Are Stimulated by Muramyl Dipeptide To Induce Polymorphonuclear Leukocyte Accumulation in the Peritoneal Cavities of Guinea Pigs

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N-Acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide [MDP]) injected intraperitoneally significantly increased the number of cells entering the peritoneal cavity of guinea pigs primed with liquid paraffin or thioglycollate. There was a close relationship between peritoneal polymorphonuclear leukocyte (PMN) accumulation and the uptake of glucosamine by macrophages in guinea pigs treated with a variety of bacterial cell surface components such as cell wall peptidoglycan subunits and bacterial or synthetic lipid A. The PMN accumulation was also facilitated by the intraperitoneal transfer of the peritoneal macrophages that had been stimulated by MDP in vitro. Futhermore, cell-free lavage fluids taken from the peritoneum of MDP-treated guinea pigs also initiated the influx of PMNs when introduced into the peritoneal cavities of liquid paraffin-pretreated guinea pigs. These results suggest that a soluble factor which attracts neutrophils is produced by MDP-treated macrophages. Partial characterization of the factor is described.

Activated macrophages play important roles in the host defense mechanisms against microbial infections (2, 33). Antigen-mediated activation of macrophages is caused by lymphokines which are released by the interaction of sensitized T lymphocytes with the corresponding antigen (26). Macrophages are also activated in the absence of antigens by direct stimulation with various bacterial products (32, 34). Among these bacterial products, *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide [MDP]), a key constituent of bacterial cell wall peptidoglycan, exerts its diverse activities through stimulation of macrophage functions in various ways, without lymphocyte involvement (1, 2, 9, 34). Thus, interaction between macrophages and MDP results in inhibition of macrophage migration (32, 39), suppression of DNA synthesis (31), enhancement of adherence and spreading on plastic or glass surfaces (31), an increase in glucosamine incorporation (37), glucose oxidation via the hexose monophosphate pathway (7), and superoxide anion generation (9). These effects are generally considered to be due to MDP-stimulated monokine production (8).

In this study, we describe the induction of macrophage secretion of a factor which facilitates polymorphonuclear leukocyte (PMN) accumulation.

MATERIALS AND METHODS

Abbreviations. M-1 enzyme, Endo-*N*-acetylmuramidase; AM_3 enzyme, *meso*-2,6-diaminopimelic acid-D-alanine endopeptidase; SEPS, *Staphylococcus epidermidis* peptidoglycan subunit (polymer); SEPS-M, *S. epidermidis* peptidoglycan subunit (monomer); LPCM-A, *bis*disaccharide stempeptide dimer from *Lactobacillus plantarum* cell walls; CMP_3-A, *N*-acetylglucosaminyl-β-(1-4)-*N*-acetylmuramyl-L-alanyl-D-isaglutaminyl-*meso*-2,6-diaminopimelic acid (GlcNAc-MurNAc-L-Ala-D-isoGln-*meso*-A_2 pm); MP_3, *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-*meso*-2,6-diaminopimelic acid; *meso*-A_2PM, *meso*-2,6-diaminopimelic acid; MDP, murumyl dipeptide, specifically, *N*-acetylmuramyl-L-alanyl-D-isoglutamine; MurNAc-L-Ala-D-isoGln-L-Lys(L18), N^{α}-(*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl)-N^{ε}-stearoyl-L-lysine; L2-*O*-Mur Nac-L-Ala-DisoGln, 6-*O*-acetylmuramyl-L-alanyl-D-isoglutamine; L18-*O*-MurNac-L-Ala-D-isoGln, 6-*O*-stearoylmuramyl-L-alanyl-D-isoglutamine; B30-*O*-MurNac-L-Ala-D-isoGln, 6-*O*-(2-tetradecylhexadecanoyl)-muramyl-L-alanyl-D-isoglutamine; LA-14-PP,2,2′-*N*: 3,3′-*O*-tetraquis[(R)-3-hydroxytetradecanoyl]-β(1-6)-D-glucosamide disaccharide-1,4′-bisphosphate, or synthetic lipid A, precursor Ia bisphosphate type; LA-15-PP, 2-*N*-[(R)-3-hydroxytetradecanoyl]2′-*N*[(R)3-dodecanoyl-oxytetradecanoyl]-30-*O*-[(R)-3-hydroxytetradecanoyl]-3′-*O*-[(R)-tetradecanoyloxytetradecanoyl]-β(1-6)-D-glucosamine dissacharide-1,4′-bisphosphate, or synthetic lipid A: *Escherichia coli* bisphosphate type; LA-15-PH, 4′-monophosphate derivative of LA-15-PP, or synthetic lipid A: *E. coli* 4′-monophosphate type; LA-15-HH, dephospho derivative of LA-15-PP, or synthetic lipid A: *E. coli* dephospho type; FMLP, *N*-formylmethionyl-L-leucyl-L-phenylalanine; PEC, peritoneal exudate cells; LPS, lipopolysaccharide; PBS, Dulbecco phosphate buffered saline; PMN, polymorphonuclear leukocyte.

Animals. Outbred Hartley female guinea pigs weighing 400 to 500 g were purchased from Shizuoka Experimental Animal Corporation (Shizuoka, Japan).

Test compounds. (i) Water-soluble peptidoglycan fragments. A polymer of peptidoglycan subunits (SEPS) was isolated by gel filtration of a *Staphylococcus aureus* lytic enzyme, endopeptidase (13), digest of *Staphylococcus epidermidis* (ATCC 155) cell wall peptidoglycan. A monomeric subunit (SEPS-M) was produced by treatment of SEPS with M-1 enzyme (11). Details of the preparation method and chemical properties of the above compounds were described previously (5, 30, 32). An LPCM-A specimen was prepared

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from an M-1 enzyme digest of *L. plantarum* (ATCC 8014) cell walls (10, 19, 32). A disaccharide tripeptide monomer, GMP_3-A, was prepared by combined treatment of *L. plantarum* cell walls with the M-1 enzyme and the AM_3 enzyme, as described previously (14). Treatment of GMP_3-A with an exo-β-*N*-acetylglucosaminidase (3) gave MP_3 (14, 30). Lactyltripeptide was prepared from GMP_3-A by treatment with ammonia water at 30°C for 28 h and subsequent column chromatography of the reaction products. This tripeptide was obtained by treatment of GMP_3-A with *N*-acetylmuramyl-L-alanineamidase (12, 14). These compounds, derived from *L. plantarum* cell walls, were kindly given by S. Kawata, Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan).

(ii) Synthetic muramylpeptides. MDP and two of its analogs were synthesized (21) and generously supplied by Testuo Shiba and Shoichi Kusumoto, Faculty of Science, Osaka University. In one analog, the L-alanine residue of MDP was replaced with L-serine or L-valine, and in the other analog, the D-isoglutamine residue was replaced with the L-isoglutamine, D-isoasparagine, or D-glutamic acid. MurNAc-D-Ala-D-isoGln, one of the first type analogs, was a generous gift from E. Lederer, Laboratoire de Biochimie, Centre National de la Recherche Scientifique, Paris, France. Acyl derivatives of muramyl peptides, MurNAc-L-Ala-D-isoGln-L-Lys, MurNAc-L-Ala-D-isoGln-L-Lys(L18), and its biologically inactive analog, MurNAc-L-Ala-L-isoGln-L-Lys(L18) were kindly supplied by Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan) (30). Murabutide, *N*-acetylmuramyl-L-alanyl-D-glutamine-*n*-butyl-ester, was synthesized according to the method of Lefrancier et al. (23), only for use as a reference compound. 6-*O*-Acyl-MDPs, in which a straight chain fatty acid with 2 or 18 carbon atoms and an α-branched fatty acid with 30 carbon atoms were introduced to the 6-*O*-position of the muramic acid residue of MDP, as described previously, were used to produce L2-MDP, L18-MDP, and B30-MDP, respectively (20, 30). Limulus test by PreGel reagent (Seikagaku Kogyo Co., Ltd., Tokyo, Japan) showed that contamination of all the test MDP and its analogs on derivatives with extraneous LPS was less than 10 pg/mg.

(iii) Bacterial LPS, lipid A (bacterial and synthetic). A specimen of LPS-*W* prepared from *E. coli* O127:B8 by the hot phenol-water extraction method was purchased from Difco Laboratories (Detroit, Mich.). A highly purified bacterial lipid A specimen from *Salmonella minnesota* R595 was generously given by C. Galanos, Max-Planck-Istitut fur Immunbiologie, Frieburg, Federal Republic of Germany. Synthetic *E. coli*-type lipid A, compound LA-15-PP (506), its dephospho- and 4′-monophospho analogs, LA-15-HH (503) and LA-15-PH (504), respectively, and a synthetic counterpart of a biosynthetic disaccharide-type lipid A precursor Ia (IV_A), compound LA-14-PP (406) were synthesized as described previously (16) and generously supplied by Tetsuo Shiba and Shoichi Kusumoto.

(iv) Other materials. Ovalbumin (grade V) and FMLP were obtained from Sigma Chemical Co. (St. Louis, Mo.).

(v) Immune complexes. A soluble immune complex specimen was obtained by the reaction between ovalbumin and anti-ovalbumin rabbit purified immunoglobulin G at a molar ratio of 3:1; the reaction mixture was incubated at 37°C for 1 h and left overnight at 4°C. The resulting insoluble immune complexes were removed by centrifugation at 1,000 × *g* for 30 min, and the supernatant fluid was used as the soluble immune complex without further purification (38).

Assay for PMN accumulation. Guinea pigs were injected intraperitoneally with 20 ml of sterilized liquid paraffin (Wako Pure Chemical Industries Ltd., Tokyo, Japan) or thioglycollate medium (Nissui Pharamaceutical Co., Ltd., Tokyo, Japan). Four days later, the animal received an intraperitoneal injection of 100 μg of test materials per animal in 10 ml of PBS, pH 7.2. Control animals received PBS alone. PEC which accumulated during 24 h in response to the stimulation with test materials were harvested by washing the peritoneal cavity with two 50-ml portions of PBS. The number of viable cells was assessed by trypan blue dye exclusion, and differential cell counts were made on the exudate cell smears stained with the Giemsa stain.

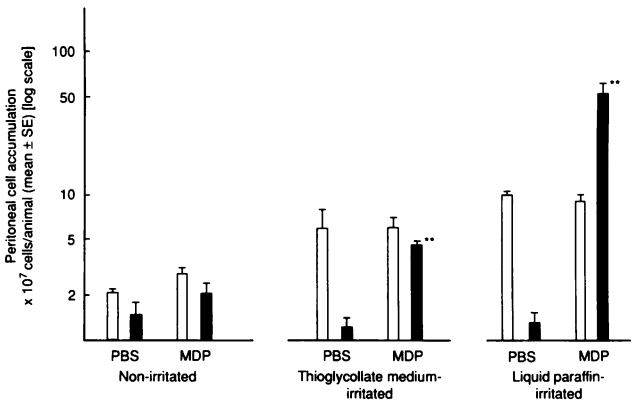

FIG. 1. MDP induction of PMN accumulation in the peritoneal cavity of the guinea pig. Groups (five per group) of guinea pigs received intraperitoneal injections of liquid paraffin (20 ml) or thioglycollate medium (10 ml), respectively (the other group was not injected at this stage). Four days later, each group of animals except the control group received an intraperitoneal injection of 100 μg of MDP in PBS. The control group was injected with PBS alone. The number of viable cells in the peritoneal exudate harvested 24 h later was assessed by trypan blue dye exclusion, and differential cell counts were performed after Giemsa staining. Open and closed columns show the number of viable macrophages and PMN, respectively. Using Student's independent *t* test, significant differences from the respective control value are shown: *, $P < 0.05$; **, $P < 0.01$.

Macrophage transfer. Liquid paraffin-induced PEC were allowed to adhere to glass dishes at 37°C for 1 h in tissue culture medium 199 (TC 199) (Chiba Ken Serum Institute, Chiba, Japan), to separate adherent cells (essentially macrophages) from nonadherent PEC populations. Adherent cells were then removed by using a pipette and a rubber policmen, diluted to cell density of 10^8 cells per ml, incubated in siliconized tubes with or without MDP in PBS (10 μg per ml) for 30 min, and washed three times with PBS. MDP-stimulated macrophages (10^6 to 10^8 cells per animal) were transferred into the peritoneal cavity of a normal guinea pig. Peritoneal cells were harvested, and viable cell and differential cell counts were made 24 h later.

Cell-free peritoneal lavage fluid. Guinea pigs which had been injected intraperitoneally with liquid paraffin 4 days previously received an intraperitoneal injection of MDP (100 μg) dissolved in 10 ml of PBS. The peritoneal exudate was collected 6 h later by washing the peritoneal cavity with PBS (50 ml). The lavage fluid was centrifuged at 150 × *g* for 10 min to remove cellular components, and the supernatant was thoroughly dialyzed against PBS to remove MDP. The protein concentration of the MDP-stimulated peritoneal lavage fluid was measured by the Lowry method (24) by using bovine serum albumin as a reference. A sample of the

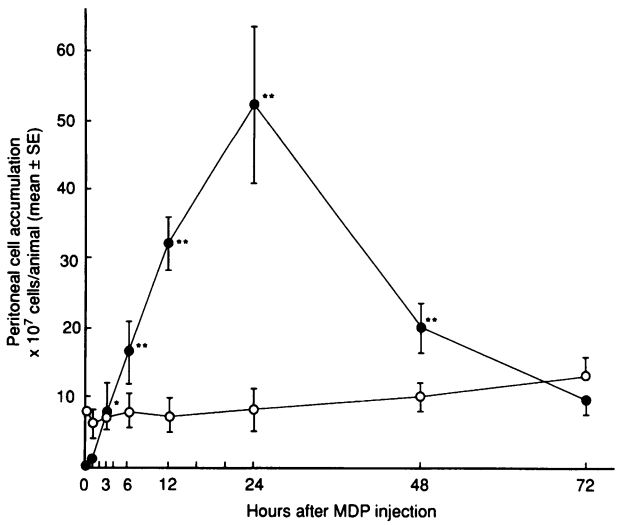

FIG. 2. Time course of PMN accumulation into the liquid paraffin-irritated peritoneal cavities of guinea pigs following intraperitoneal MDP injection. Guinea pigs (five per group) were injected intraperitoneally with liquid paraffin (20 ml). Four days later, the peritoneal cavity was stimulated by injection of 10 μg of MDP. At indicated intervals after MDP injection, PEC were harvested and submitted to viable-cell and differential cell counts. Open and closed circles show the number of viable macrophages and PMN, respectively. Using Student's independent t test, significant differences from the respective control value are shown: *, $P < 0.05$; **, $P < 0.01$.

cell-free lavage fluid was injected into the peritoneal cavity of guinea pigs to assess its ability to induce PMN accumulation.

To determine whether the active component was proteinaceous, the lavage fluid was treated with either polyacrylamide-bound trypsin or agarose-bound protease type VII-A (catalog no. T8386 and P4523; Sigma Chemical Co.). A test sample of peritoneal lavage fluid (containing 0.5 mg of protein per ml) was incubated with 50 μg of each enzyme at 37°C for 1 h, and the enzyme was removed by centrifugation. The heat stability and ability of the PMN attracting factor to pass through a polysulfane membrane (pore size, 0.45 μm; Gelman Sciences, Inc., Ann Arbor, Mich.) were also examined. Effects of lipid extraction of the PEC supernatant on its PMN attracting activity were determined by repeated extraction of the test exudate cell supernatant with chloroform at room temperature for 5 to 10 min.

Incorporation of ^{14}C-labeled glucosamine into macrophage proteins. Monolayers of liquid paraffin-induced guinea pig peritoneal macrophages (10^6 cells per well) were cultured in 1.0 ml of TC 199 medium on plastic culture plates (24 wells; Corning Glass Works, Corning, N.Y.) with test materials for 42 h at 37°C in a humidified 5% CO_2 atmosphere, and 0.2 μCi of D-[U-^{14}C]glucosamine hydrochloride (specific activity 354.0 mCi per mol, Dupont, NEN Research Products, Boston, Mass.) was then added. After incubation for an additional 6 h, trichloroacetic acid was added to the culture to a final concentration of 10% (wt/vol). The cells were detached with a rubber policeman and washed three times with 10% (wt/vol) trichloroacetic acid. The trichloroacetic acid-insoluble fraction was assayed for [^{14}C]glucosamine by using a scintillation counter (Packard Instrument Co., Inc., Rockville, Md.) (7).

Statistical analysis. Student's t test for the independent

TABLE 1. Induction of PMN accumulation and macrophage activation by analogs and derivatives of MDP[a]

Test compound[b]	Total PEC (10^7) (mean ± SE)[c]	(PMN/PEC) (10^2) (mean ± SE)	[^{14}C]Glucosamine incorporation stimulation index[d] (mean ± SE)
1. MurNAc-L-Ala-D-isoGln	53 ± 4**	79 ± 4**	2.5 ± 0.3*
2. MurNAc-L-Ser-D-isoGln	50 ± 3**	81 ± 5**	2.7 ± 0.2*
3. MurNAc-L-Val-D-isoGln	51 ± 4**	80 ± 5**	2.3 ± 0.2*
4. MurNAc-L-Ala-D-isoGln	25 ± 3*	5 ± 3	0.9 ± 0.2
5. MurNAc-L-Ala-L-isoGln	23 ± 3*	2 ± 1	1.0 ± 0.2
6. MurNAc-L-Ala-D-isoAsn	20 ± 2*	4 ± 3	1.1 ± 0.2
7. MurNAc-L-Ala-D-Glu	55 ± 6**	70 ± 7**	2.3 ± 0.3*
8. MurNAc-L-Ala	9 ± 1	1 ± 1	1.1 ± 0.3
9. L2-*O*-MurNAc-L-Ala-D-isoGln	63 ± 6**	82 ± 4**	3.0 ± 0.3*
10. L18-*O*-MurNAc-L-Ala-D-isoGln	52 ± 5**	81 ± 4**	2.6 ± 0.2*
11. B30-*O*-MurNAc-L-Ala-D-isoGln	49 ± 3**	79 ± 6**	2.7 ± 0.4*
12. B30-*O*-MurNAc-L-Ala-D-isoAsn	20 ± 2*	2 ± 1	1.2 ± 0.2
13. MurNAc-L-Ala-D-Glu-O*n*Bu	40 ± 4**	70 ± 3**	2.4 ± 0.4*
14. MurNAc-L-Ala-D-isoGln-L-Lys	54 ± 4**	75 ± 5**	2.7 ± 0.3*
15. MurNAc-L-Ala-D-isoGln-L-Lys (L18)	60 ± 7**	83 ± 6**	2.6 ± 0.5*
16. MurNAc-L-Ala-L-isoGln-L-Lys (L18)	23 ± 2*	4 ± 2	1.1 ± 0.3
17. PBS (control)	10 ± 2	2 ± 1	1.0

[a] Guinea pigs (four per group) were injected intraperitoneally with 20 ml of sterilized liquid paraffin. Four days later, the animals received intraperitoneal administration of 100 μg of MDP (none in the control) in PBS. PEC were harvested 24 h later and were subjected to differential cell counts after staining with Giemsa stain. The data in this and the following tables are shown as the mean and standard error of the mean and were based on the results obtained with three to five different macrophage preparations.

[b] MurNAc, *N*-Acetylmuramic acid; L18, stearic acid; B30, 2-tetradecylhexadecanoic acid.

[c] Using Student's independent t test, significant from the respective control value: *, $P < 0.05$; **, $P < 0.01$.

[d] The ratio of counts per minute of a test culture to counts per minute of the respective control culture without the test specimen (in triplicate cultures). [^{14}C]glucosamine incorporation in five control cultures was 521 ± 28, 473 ± 21, 412 ± 39, 348 ± 25, and 482 ± 26 cpm.

variable was used to assess statistical differences between the means.

RESULTS

Accumulation of peritoneal PMN by MDP stimulation in vivo. Injection of MDP (100 μg) into the peritoneal cavity, which had been treated with liquid paraffin 4 days previously, caused a marked increase in the number of PEC. This increase was predominantly due to the accumulation of PMN. The 4-day time interval between irritation with liquid paraffin and stimulation with MDP was used because pilot assays showed that 4 days after stimulation in the control animals, about 90% of exudate cells are macrophages and fewer than 5% are PMNs. Lymphocytes and eosinophils together accounted for, at most, 5% of the total cells. Prior irritation with liquid paraffin or thioglycollate medium was a prerequiste for the MDP-induced accumulation of PMN, since no significant accumulation of PMN was caused by MDP administration into the nonirritated peritoneal cavity (Fig. 1). The accumulation of PMN by MDP stimulation started around 6 h after MDP injection, reached a maximum after 24 h, and gradually declined during the following 48 h (Fig. 2). No significant accumulation of macrophages was noted during the whole observation period.

Induction of PMN accumulation by native and synthetic bacterial products. A series of synthetic MDP analogs and derivatives were tested for their abilities to induce PMN accumulation in the liquid paraffin-irritated peritoneal cavity (Table 1). The L-alanine residue adjacent to the muramic acid could be replaced with L-serine or L-valine without the loss of the PMN-inducing ability of the parent molecule, while the replacement with D-alanine resulted in a complete disappearance of the activity (rows 1 through 4). The ability of MDP to induce PMN accumulation was retained when the D-isoglutamine residue was substituted with D-glutamic acid but was lost when the residue was renewed or replaced with either L-isoglutamine or D-isoasparagine (rows 5 through 8). These findings indicate the strict stereochemical structural requirements for the expression of the PMN attracting activity of MDP. Substitution of either the hydroxyl group of the C-6 position of the muramic acid residue of MDP (rows 9 through 12) or the *N*-amino group of the L-lysine residue of MDP-L-Lys (rows 15 and 16) with an acyl group did not result in significant changes in PMN accumulatory activity, at least when the effects of 100 μg doses were compared (Table 1). Some of the inactive analogs (rows 4, 5, 6, 12, and 16) caused a significant increase in total PEC counts, without a significant change in the percentage of PMN. Table 1 shows correspondence in the structure-activity relationships between macrophage activation as measured by increased glucosamine uptake and the PMN accumulation caused by test compounds. MDP analogs and derivatives that enhanced glucosamine uptake were capable of inducing PMN accumulation, while those incapable of enhancing glucosamine uptake did not cause the PMN accumulation.

Table 2 summarizes the PMN attracting activity of cell wall peptidoglycan degradation products with different degrees of polymerization of the subunits (rows 1 through 8), LPS, lipid A (bacterial and synthetic), and lipid A analogs (rows 9 through 14). A close correspondence was found between the PMN accumulating activity of these test compounds and their ability to enhance glucosamine uptake by macrophages. All the test compounds capable of activating macrophages in terms of enhanced glucosamine uptake showed the capacity to cause PMN accumulation, while none of the compounds inactive in enhancing glucosamine uptake induced PMN accumulation. Neither soluble immune complexes, 100 μg per animal, nor FMLP, 100 μg per animal, induced the PMN accumulation (Table 2, rows 15 and 16).

TABLE 2. Induction of PMN accumulation and macrophage activation by bacterial peptidoglycan fragments, LPS, lipid A, and its analogs[a]

Test compound[b]	Total PEC (10^7) (mean ± SE)[c]	(PMN/PEC) (10^2) (mean ± SE)	[^{14}C]Glucosamine incorporation stimulation index[d] (mean ± SE)
1. SEPS	46 ± 3**	76 ± 4**	2.7 ± 0.4*
2. SEPS-M	50 ± 4**	80 ± 5**	2.6 ± 0.2*
3. LPCM-A	48 ± 3**	78 ± 6**	2.6 ± 0.3*
4. GMP_3-A	49 ± 5**	80 ± 5**	2.5 ± 0.3*
5. MP_3	51 ± 15**	79 ± 7**	2.6 ± 0.6*
6. MP_2 (MDP)	52 ± 3**	80 ± 5**	2.6 ± 0.3*
7. LP_3	9 ± 2	1 ± 2	1.1 ± 0.2
8. P_3	11 ± 2	2 ± 2	0.8 ± 0.2
9. LPS (*E. coli*)	39 ± 3**	80 ± 5**	3.0 ± 0.4*
10. Lipid A (*S. minnesota* R595); synthetic lipid A	39 ± 5**	78 ± 4**	2.6 ± 0.3*
11. LA-14-PP (406)	37 ± 4**	77 ± 4**	2.6 ± 0.2*
12. LA-L5-HH (503)	8 ± 2	1 ± 1	1.1 ± 0.2
13. LA-15-PH (504)	50 ± 7**	70 ± 2**	2.5 ± 0.2*
14. LA-15-PP (506)	35 ± 3**	75 ± 7**	2.6 ± 0.3*
15. Soluble immune complexes	11 ± 2	8 ± 3	1.2 ± 0.2
16. FMLP	9 ± 1	1 ± 1	1.2 ± 0.2
17. PBS (control)	8 ± 2	1 ± 1	1.0

[a] The experimental conditions and the doses of test materials were the same as those in Table 1.

[b] SEPS and SEPS-M, a polymer and a monomer of the peptidoglycan subunit (L-lysine type), respectively; LPCM-A, a monomer of the subunit (A_2pm type); LP_3, lactyl-L-Ala-D-isoGln-*meso*-A_2pm; P_3, L-Ala-D-isoGln-*meso*-A_2pm; A_2pm, 2,6-diaminopimelic acid.

[c] Using Student's independent *t* test, significant from the respective control value: *, $P < 0.05$; **, $P < 0.01$.

[d] See footnote *d* in Table 1.

The ability of varied concentrations of MDP and LPS to attract PMNs was compared (Fig. 3A and B). A significant PMN accumulation was detected with the administration of test doses higher than 100 ng of MDP and 1.0 ng of LPS, respectively. The maximum accumulation of PMNs occurred at concentrations of 10 μg of MDP and 100 ng of LPS.

Attraction of PMNs by the transfer of macrophages activated by MDP in vitro. Peritoneal exudate macrophages which were induced by peritoneal irritation with liquid paraffin and which were enriched by adherence to plastic were cultured in the presence of MDP for 1 h at a concentration of 10 μg per 10^8 viable macrophages. MDP-treated macrophages (10^6 to 10^8 viable cells per animal) were transferred into the nonirritated peritoneal cavities of guinea pigs. Twenty-four hours after the intraperitoneal injection of more than 5×10^6 MDP treated macrophages per animal, PECs increased, mainly due to a marked PMN accumulation. The administration of untreated macrophages did not cause any significant PMN accumulation, even at the highest cell concentration; however, a significant increase of PEC, exclusively due to macrophage accumulation, was noted (Table 3).

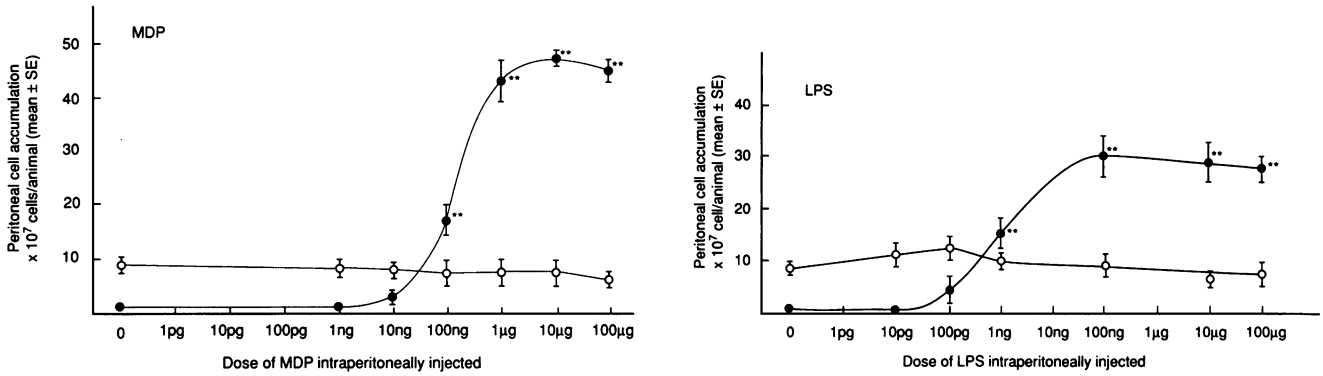

FIG. 3. Dose dependency of PMN accumulation of MDP and LPS. The experimental conditions (except the doses of MDP and LPS) and symbols are the same as those in Table 1 and those described in the legend to Fig. 2, respectively.

PMN properties of the cell-free peritoneal lavage fluid from liquid paraffin-irritated and MDP-stimulated peritoneal exudate. MDP (100 μg per animal) was given to guinea pigs 4 days after the intraperitoneal injection of liquid paraffin. Peritoneal lavage fluid was collected 6 h after MDP stimulation. Cells were removed by centrifugation, and the cell-free fluid was dialyzed to remove MDP and injected intraperitoneally into normal guinea pigs. PMN accumulation was induced by the test-cell-free exudate containing more than 5 mg of protein (Fig. 4). A control supernatant fluid of the liquid paraffin-irritated, PBS-treated peritoneal exudate did not cause any detectable PMN accumulation even at a dose as high as 75 mg of protein per animal (data not shown). A pilot experiment also showed that the analog, MurNAc-L-Ala-L-isoGln was inactive in this respect. Therefore, the PMN accumulation induced by MDP stimulation is due in whole or in part to the release of a soluble factor by liquid paraffin-irritated and MDP-stimulated peritoneal exudate cells.

Finally, Table 4 summarizes the effects of various treatments of PMN attracting activity of a MDP-stimulated PEC supernatant. The PMN attracting activity was significantly decreased by digestion with trypsin and protease and by heating at 80°C for 30 min. The PMN attracting activity after protease treatment was not found to be significantly different from the activity of supernatant from PEC cells elicited with PBS alone. Filtration through a membrane filter (pore size, 0.45 μm) or extraction with chloroform caused no significant change (Table 4).

DISCUSSION

The stimulatory effects of MDP on macrophages had once been assumed to be exclusively mediated through the activation of T cells, partly because a direct action of MDP on macrophages had not been proved. However, subsequent studies demonstrated that macrophages could be directly activated by MDP (32, 34, 39).

In this study, we found that the accumulation of PMN was induced by injection of MDP into the peritoneal cavity of guinea pigs which had been primed with an intraperitoneal administration of liquid paraffin or thioglycollate medium. A close correspondence was found between the ability of a test compound to increase glucosamine uptake (a parameter of macrophage activation) and its ability to increase PMN accumulation, and both activities showed strict structural requirements, as did other immunobiological activities so far reported (17).

TABLE 3. PMN accumulation induced by transfer of peritoneal macrophages stimulated by in vitro incubation with MDP[a]

Incubation with MDP in vitro	PEC of recipient	No. of total and of each PEC cell type (10^7) (mean ± SE) for the following no. of MDP-stimulated macrophages transferred[b]:				
		1×10^6	5×10^6	1×10^7	5×10^7	1×10^8
No	Total PEC	1.0 ± 0.2	1.3 ± 0.3	2.0 ± 0.3	3.4 ± 1.0	7.1 ± 1.3
	Macrophages	0.1 ± 0	1.2 ± 0.1	1.7 ± 0.1	3.2 ± 0	6.8 ± 0.1
	Lymphocytes	ND	ND	ND	0.1 ± 0	0.1 ± 0
	Neutrophil leukocytes	ND	ND	ND	0.1 ± 0	0.1 ± 0
	Eosinophil leukocytes	0.1 ± 0	0.1 ± 0	0.2 ± 0.1	ND	0.1 ± 0.1
	Undefined cells	ND	ND	ND	0.1 ± 0	0.1 ± 0
Yes	Total PEC	2.2 ± 0.2*	4.0 ± 0.5*	6.9 ± 1.8**	9.5 ± 0.4**	17.1 ± 2.1*
	Macrophages	2.0 ± 0.1*	2.2 ± 0.1*	2.9 ± 0.6*	5.2 ± 0.4*	7.4 ± 0.9
	Lymphocytes	ND	ND	ND	0.1 ± 0	ND
	Neutrophil leukocytes	0.1 ± 0	1.7 ± 0.3**	3.6 ± 0.6**	4.3 ± 0.5**	9.6 ± 0.9*
	Eosinophil leukocytes	0.1 ± 0.1	ND	0.3 ± 0.1	ND	ND
	Undefined cells	ND	ND	ND	ND	ND

[a] Guinea pigs were injected intraperitoneally with liquid paraffin. Four days later, macrophages from the irritated peritoneal exudate, enriched by adherence to plastic, were incubated with MDP (10 μg per ml; none in control) in PBS for 30 min. Macrophages (10^6 to 10^8 cells per animal) were transferred into the peritoneal cavities of groups of normal guinea pigs (five per group). PEC of recipient animals were harvested 24 h later, and viable cell and differential cell counts were carried out. ND, Not detected.

[b] Using Student's independent t test, significant difference from the respective control value: *, $P < 0.05$; **, $P < 0.01$.

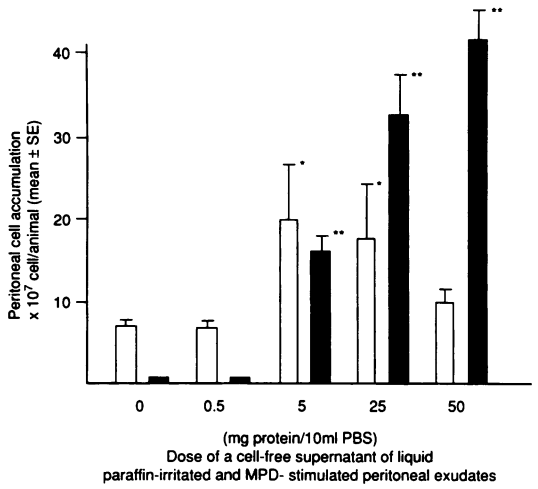

FIG. 4. Induction of PMN accumulation by a cell-free supernatant fluid of liquid paraffin-irritated and MDP-stimulated peritoneal exudate. Guinea pigs (four per group) received an intraperitoneal injection of a cell-free supernatant of the exudate of the peritoneal cavity that had been irritated with liquid paraffin and then stimulated with 100 μg of MDP (none in the control) in PBS. Viable-cell and differential cell counts were made on the peritoneal exudate harvested 24 h later. Open and closed columns show the number of viable macrophages and PMN, respectively. Using Student's independent t test, significant difference from the respective control value are shown: *, $P < 0.05$; **, $P < 0.01$.

A soluble factor produced by macrophages stimulated with MDP seems to be responsible for the observed PMN accumulation for the following reasons. (i) Transfer of macrophages treated with MDP in vitro into the peritoneal cavity of guinea pigs caused PMN accumulation. (ii) Injection of the cell-free exudate supernatant obtained from the peritoneal cavity, which had been irritated with liquid paraffin and then stimulated with MDP, induced PMN accumulation in the peritoneal cavity of normal guinea pigs.

TABLE 4. Effects of different treatments on the ability to induce PMN accumulatory activity of the supernatant of MDP-stimulated peritoneal exudate[a]

Treatment of PEC	Treatment	No. of harvested total PEC per animal (10^7) (mean ± SE)[b]	(PMN/PEC) (10^2) (mean ± SE)
MDP	None	18 ± 1**	60 ± 4**
	Filtration through membrane filter (0.45 μm pore size)	17 ± 3**	70 ± 6**
	Extraction with chloroform	14 ± 3**	81 ± 7**
	Heating at:		
	60°C (30 min)	10 ± 2*	43 ± 4**
	80°C (30 min)	6 ± 3	36 ± 15*
	Digestion with:		
	Trypsin	6 ± 2	25 ± 9
	Protease	7 ± 3	13 ± 5
None	Filtration through membrane filter (0.45 μm pore size)	4 ± 1	9 ± 1

[a] A cell-free supernatant of the liquid paraffin-irritated and MDP-stimulated peritoneal exudate was treated as shown. The treated supernatant fluid (2 mg of protein equivalent per guinea pig) was injected intraperitoneally into normal guinea pigs (four per group). PEC were harvested 24 h later, and viable-cell and differential cell counts were made on the recipient PEC which were collected 24 h later.

[b] Using Student's independent t test, significant difference from the respective control value: *, $P < 0.05$; **, $P < 0.01$.

Macrophage-derived factors such as tumor necrosis factor (TNF), interleukin-1, and leukotriene B4 are known to be chemotactic for PMN (4, 6, 25, 27), although neither recombinant TNF nor interleukin-1 are reported to be chemotactic for neutrophils in vitro (15, 17). In our in vitro experiments, recombinant human TNF (TNF-α, Dainippon Pharmaceutical Company, Ltd.; 100 pg to 10 μg per guinea pig) did not cause PMN accumulation (data not shown). Recently, Peveri et al. reported a novel neutrophil-activating factor produced by human mononuclear phagocytes (35), and Wolpe et al. demonstrated the secretion by a mouse macrophage cell line of a heparin-binding protein having neutrophil chemokinetic properties (40). These macrophage factors were induced by stimulation with various substances, many of which derived from bacterial cell surface layers (18).

While studies on the induction of PMN chemotactic factor by MDP have so far been mainly carried out in vitro, we have demonstrated the in vivo induction of PMN attracting factor by intraperitoneal injection of MDP into appropriately irritated peritoneal cavities of guinea pigs. The in vivo experimental system described here will be useful in studies of the production of macrophage-derived PMN accumulation factor by MDP, because a larger amount of the factor can easily be obtained.

Finally, the biological significance of the phenomena described here is uncertain, since our method of deriving large numbers of macrophages, i.e., irritation of the peritoneal cavity by oil injection, makes it difficult to evaluate or discuss a meaning of the phenomena in natural inflammation.

Further studies on characterization of the present PMN-attracting factor induced by MDP are in progress.

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