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Inhibition of Macrophage Migration by Muramyl Peptides

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In the capillary tube migration system a synthetic muramyl dipeptide (MDP; *N*-acetylmuramyl-L-alanyl-D-isoglutamine), a part of bacterial cell wall peptidoglycans, inhibited the migration of peritoneal exudate macrophages from normal guinea pigs or rats. The migration inhibition was also caused by some MDP-containing peptidoglycan fragments from cell walls of *Lactobacillus plantarum* and *Staphylococcus epidermidis*. The migration inhibition could not be explained on the basis of macrophage migration inhibitory factor. A stereochemically highly specific structure of MDP required for its adjuvant activity was also required for the macrophage migration inhibition. These findings suggest that MDP and MDP-containing cell wall fragments may activate macrophages and that this activation may be important in the exertion of their adjuvant activity.

We found previously that a synthetic muramyl dipeptide (MDP; *N*-acetylmuramyl-L-alanyl-D-isoglutamine), a part of the bacterial cell wall peptidoglycans, stimulated the phagocytic capacity of the reticuloendothelial system (18, 19), and we suggested that the stimulation of macrophages may play an important role in the expression of adjuvant activity of MDP because only the stereochemically specific structures required for adjuvant activity could stimulate the macrophage function. We also found that MDP inhibited the migration of macrophages from normal guinea pigs in vitro (20). In the present study, we confirmed and extended the previous finding in vitro (20).

MATERIALS AND METHODS

Animals. Female outbred Hartley guinea pigs weighing 400 to 600 g were obtained from a local breeder. WKA female rats weighing 150 to 200 g were obtained from the Institute for Experimental Animals, Kyushu University.

MDP and its analogs. These were synthesized as described previously (10).

Peptidoglycan fragments. Peptidoglycan fragments designated as LPCM-A, SECS (a-1, a-2, b-1), and SEPS were prepared and analyzed by S. Kawata and K. Yokogawa, Research and Development Division, Dainippon Pharmaceutical Co., and M. Tsujimoto, Department of Microbiology, Osaka University School of Dentistry, Osaka, Japan. An LPCM-A specimen, *bis*-disaccharide-stempeptide dimer, was prepared from an M-1 endo-*N*-acetylmuramidase digest of *Lactobacillus plantarum* (ATCC 8014) cell walls as described previously (9). SECS and SEPS specimens were prepared as will be described separately. Briefly, SECS specimens a-1, a-2, and b-1 were obtained by gel filtration, on columns of Sephadex G-50 and G-25 connected in series, of *Staphylococcus epidermidis* (ATCC 155) walls solubilized by the SALE endopeptidase (an endopeptidase capable of hydrolyzing the cross-bridge of staphylococcal cell wall peptidoglycans; Kawata and Yokogawa, unpublished data) and by chromatography with an ECTEOLA-cellulose column of the Sephadex fractions. SEPS, on the other hand, was isolated by gel filtration of the SALE digest of a peptidoglycan fraction which had been obtained by treatment of *S. epidermidis* walls with 10% trichloroacetic acid at 4°C for 48 h to remove a non-peptidoglycan moiety. Average chain length of a glycan portion of these fragments was determined by the method of Kato and Strominger (7), and molecular weights were estimated by the method of Pusztai and Watt (13). Analytical data on these peptidoglycan fragments pertinent to the present study are given in Table 1. Taking these data and the mode of hydrolytic action of the SALE enzyme into consideration, we concluded that the peptidoglycan fragments were the polymers of disaccharide-stempeptide with some fragments of a cross-linkage consisting of 4 mol of glycine and 1 mol of serine, connected through a glycan chain.

PEC. Peritoneal exudate cells (PEC) were harvested from guinea pigs and rats 4 days after intraperitoneal injection of sterilized liquid paraffin (Wako Pure Chemical Industries, Ltd.) as described previously (12). These cells consisted of about 70 to 75% macrophages, 10 to 15% lymphocytes, and 10 to 15% polymorphonuclear leukocytes, as determined morphologically.

Macrophages. Adherent cells (macrophages) were separated from nonadherent PEC populations by adherence to glass dishes at 37°C for 30 min in 199 tissue culture medium (Chiba-ken Serum Institute, Japan), supplemented with 10% heat-inactivated fetal calf se-

TABLE 1. *Summary of chemical analyses of peptidoglycan fragments*[a]

Fragment	Molar ratio								Avg chain length[b]	Mol wt
	Muramic acid	Glucosamine	Alanine	Glutamic acid	Lysine	A_2pm[c]	Glycine	Serine		
LPCM-A	1.07	0.90	1.75	1.00	—	1.17	—	—	1	ND[d]
SEPS	1.29	0.97	2.19	1.00	1.14	—	2.04	0.26	8	2,000–8,000
SECS										
b-1	1.06	1.01	1.95	1.00	1.55	—	1.50	0.30	9	2,800–5,000
a-1	1.03	1.11	2.30	1.00	1.12	—	1.15	0.28	14	10,000–11,000
a-2	1.18	1.07	1.97	1.00	1.42	—	1.40	0.33	30	18,000–50,000

[a] Content of a non-peptidoglycan portion was either low or insignificant.
[b] Average length of a glycan portion (disaccharide unit).
[c] A_2pm, 2,6-diaminopimelic acid.
[d] ND, Not done.

rum, 7 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid; Doujin Yakukagaku Institute, Japan), 50 U of penicillin per ml, and 50 μg of streptomycin per ml as described previously (12). This supplemented 199 medium was used also for migration inhibition testing unless otherwise stated. Adherent cells thus separated consisted of more than 99% macrophages, as judged by morphological criteria, phagocytic property, and rosette-forming capacity with sheep erythrocytes coated with rabbit anti-sheep erythrocyte immunoglobulin G antibody (15). No T lymphocytes were detected by the method of rosette formation with rabbit erythrocytes (16).

Cell migration. The inhibition of cell migration was measured by the conventional capillary tube method (4) as described previously for the assay of migration inhibitory factor (MIF) (11). Briefly, cell migration was determined in duplicate in the 199 supplemented medium containing various amounts of test materials (MDP, its analogs, or peptidoglycan fragments). The migration area was measured at 15 h for PEC and at 24 h for purified adherent cells. Migration index was calculated as follows: migration index = mean migration area with test materials × 100)/ mean migration area without test materials.

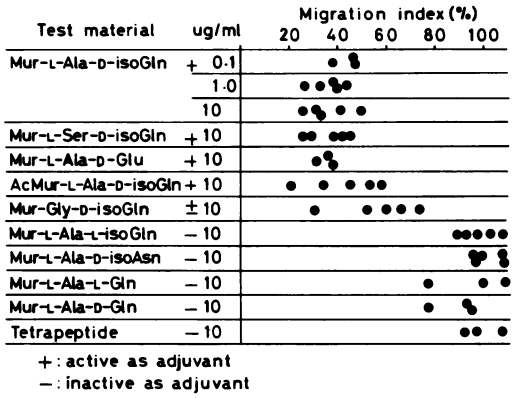

FIG. 1. *Activity of MDP and its analogs as adjuvant in guinea pigs, cited from the literature (1, 7). +, Active as adjuvant; ±, marginally active; −, inactive. Each dot (●) represents a mean of migration indexes in a single experiment. AcMur, 6-O-acetylmuramyl.*

RESULTS

A series of synthetic MDP analogs were tested for their macrophage migration inhibitory capacity. MDP inhibited the migration of PEC from normal guinea pigs at the concentration of 0.1 μg/ml (Fig. 1). Results in Fig. 1 also indicate that a stereochemically highly specific structure was required for MDP to be able to inhibit cell migration. Further, the data revealed a remarkable parallelism between the structure-activity relationships for adjuvant activity and for the migration inhibitory capacity. MDP and its analogs active as adjuvant (1, 8) were also capable of inhibiting definitely the migration of PEC from guinea pigs. A similar inhibition of migration of PEC from WKA rats by MDP was also observed (data not shown).

There is a possibility that the migration inhibition might be due to MIF liberated from lymphocytes stimulated by a mitogenicity or a possible antigenicity of MDP molecules, since MDP itself was found to be mitogenic (3, 17) and bacterial cell wall peptidoglycans were shown to possess antigenicity (14). Reducing the number of nonadherent cells to less than 0.1% did not diminish the extent of inhibition (Fig. 2). Again, the close relationship between adjuvant activity and the macrophage migration inhibition was observed. To examine further the possible involvement of MIF, lymph node cells from normal or tubercle bacilli-sensitized guinea pigs were incubated with MDP or peptidoglycan fragments. No evidence for the liberation of MIF was obtained (Table 2; data for normal cells not shown). It was also found (Table 3) that removal of fetal calf serum from the medium did not affect the inhibition of cell migration.

The inhibitory capacity of MDP was com-

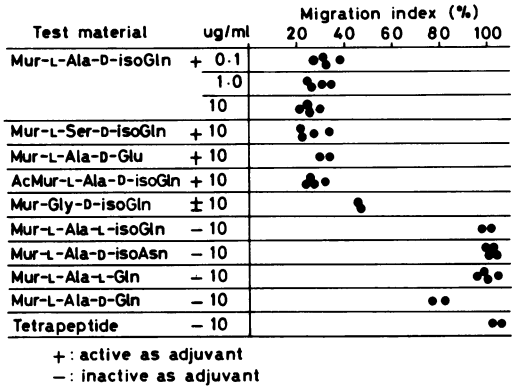

FIG. 2. *See legend under Fig. 1.*

TABLE 2. *Lack of MIF production by stimulation with MDP and peptidoglycan fragments of different sizes*[a]

Test material	Concn (μg/ml)	Migration index (%)
None		100
MDP	10	101 ± 1
	50	93 ± 10
	100	104
LPCM-A	50	95 ± 8
b-1	50	90 ± 11
a-2	50	92 ± 12
PPD[b]	50	47 ± 5
	100	39 ± 29

[a] Lymph node cells (10^7 cells per ml) from a Freund complete adjuvant-immunized guinea pig were incubated with the indicated doses of test materials for 3 h at 37°C, then washed three times. The cell suspensions were further cultured for 21 h at 37°C, and the supernatants, diluted twofold with medium, were tested for MIF activity. Experiments were performed four times, except MDP at the 100-μg dose.

[b] PPD, Purified protein derivative.

pared with that of several bacterial cell wall-derived peptidoglycan fragments containing MDP, which were well characterized with respect to their chemical composition and glycan chain length (Table 1). As shown in Table 4, all these peptidoglycan fragments inhibited macrophage migration. It was noted that MDP showed a stronger inhibitory capacity on a weight basis than the peptidoglycan fragments.

That a very small amount of MDP (0.01 to 0.1 μg/ml) was sufficient but its stereospecificity was required for the migration inhibition of macrophages seems to suggest the existence of receptors for MDP in macrophages. To examine whether or not adjuvant-inactive MDP analogs can compete with MDP for the putative receptor, migration inhibition assays were made with MDP in the presence of adjuvant-inactive MDP analogs (Table 5). The addition of a large excess of such adjuvant-inactive analogs to the medium did not diminish to any extent the migration inhibitory capacity of MDP.

TABLE 3. *Lack of requirement for fetal calf serum in migration inhibition by MDP*[a]

Test material	Concn (μg/ml)	Migration index (%)	
		−FCS	+FCS
Mur-L-Ala-D-isoGln (MDP)	0.1	40	42
	1	24	41
	10	27	30
Mur-L-Ala-L-isoGln	10	102	90
Mur-L-Ala-D-isoAsn	10	104	116
Mur-L-Ser-D-isoGln	10	22	23
6-*O*-Ac-Mur-L-Ala-D-isoGln	10	27	29

[a] Macrophage migration inhibition assays were done either with fetal calf serum (+FCS) or without it (−FCS).

TABLE 4. *Effect of peptidoglycans of different chain lengths on migration of PEC*

Test material	Chain length[a]	Concn (μg/ml)	Migration index (%)
MDP		0.01	62
		0.1	46 ± 10
		1.0	49 ± 11
LPCM-A	1	0.01	94
		0.1	78 ± 6
		1.0	46 ± 14
		10	43 ± 13
SEPS	8	1	57 ± 16
		10	46 ± 9
SECS b-1	9	0.1	85 ± 6
		1.0	53 ± 13
		10	42 ± 7
SECS a-1	14	1.0	63 ± 13
		10	46 ± 12
SECS a-2	30	0.1	92 ± 14
		1.0	54 ± 11
		10	42 ± 12

[a] Average chain length of disaccharide unit. See Table 1 for details. Experiments were performed four times, except MDP and LPCM-A at the 0.01-μg dose.

TABLE 5. *Inability of adjuvant-inactive MDP analogs in competitive inhibition*

Analog[a]	Concn (M)	% Migration
None		100
MDP	2×10^{-7}	57
L-L	2×10^{-4}	105
MDP L-L	2×10^{-7} 2×10^{-4}	53
MDP D-Gln	2×10^{-7} 2×10^{-4}	58

[a] L-L, *N*-Acetylmuramyl-L-alanyl-L-isoglutamine; D-Gln, *N*-acetylmuramyl-L-alanyl-D-glutamine. Cell migration assays were performed using PEC and medium either alone (None) or containing MDP, L-L, or MDP plus analogs.

DISCUSSION

The present study confirmed our previous finding (20) that MDP (0.01 to 0.1 μg/ml) inhibits the migration of peritoneal macrophages from unsensitized guinea pigs in the capillary tube system. Adam, Souvannavong, and Lederer also observed the same phenomenon (personal communication).

Migration inhibition was due to neither toxicity nor cell-agglutinating ability of MDP (20). We observed recently that macrophages treated with MDP in vitro showed increased glucosamine incorporation, glucose oxidation, spreading, and adherence to glass surfaces (unpublished data). Therefore, we consider that the migration inhibition of macrophages may be an expression of macrophage activation.

A stereochemically specific structure was required for MDP to exert migration inhibitory activity. A remarkable parallelism was noticed between the structure-function relationships for migration inhibitory activity and for adjuvant activity. The same close parallelism was seen between the structure-function relationships for adjuvant activity and for reticuloendothelial system-stimulating ability (18). We infer, therefore, that macrophages may play an important role in the expression of adjuvant activity of MDP.

Migration inhibition cannot be explained by a liberation of MIF through the interaction of MDP with lymphocytes, because reducing numbers of lymphocytes to less than 0.1% did not affect migration inhibition to any extent and lymph node cells obtained from guinea pigs sensitized by tubercle bacilli produced no MIF upon incubation with MDP. The latter finding is in agreement with our previous study that MDP did not elicit any positive corneal or skin reaction in guinea pigs sensitized with tubercle bacilli (5). Therefore, we favor the possibility that migration inhibition may be caused by a direct action of MDP on macrophages.

MDP was shown not only to act as adjuvant but also to enhance nonspecifically the resistance to *Klebsiella pneumoniae* infection (2). The present study revealed that MDP-containing bacterial cell wall fragments of various sizes similarly inhibited macrophage migration. This suggests that (although MDP is the essential structure for activating macrophages), in vivo in the site of bacterial infection, MDP-containing cell wall degradation products of various sizes from invading bacteria may participate in macrophage activation, which might play an important role in their adjuvant activity or an expression of defense mechanism by hosts.

Competitive inhibition of the putative receptor in macrophages by adjuvant-inactive MDP analogs was not successful in the present macrophage migration inhibition system. However, this does not deny the existence of such a receptor. A more suitable approach, including the use of isotope-labeled MDP, would have to be performed.

The inhibition of the migration of PEC from guinea pigs by endotoxin was prevented in decomplemented serum (6). Since complement was not required for MDP to inhibit cell migration (Table 3), different mechanisms appear to operate between these two systems.

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