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Stimulation of Migration of Human Monocytes by Bacterial Cell Walls and Muramyl Peptides

TOMOHIKO OGAWA,[1]* SHOZO KOTANI,[1] KUNIKO FUKUDA,[2] YOSHIO TSUKAMOTO,[2] MASAKAZU MORI,[2] SHOICHI KUSUMOTO,[3] AND TETSUO SHIBA[3]

Department of Microbiology, Osaka University Dental School, Kita-Ku, Osaka 530[1]; Department of Pharmacology, Osaka Dental University, Higashi-Ku, Osaka 540[2]; and Faculty of Science, Osaka University, Toyonaka, Osaka 560,[3] Japan

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Bacterial cell walls, water-soluble fragments of the wall peptidoglycan, *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP), and 6-*O*-acyl derivatives of MDP were examined for migration-stimulating activity on human peripheral blood monocytes by using a multiwell chemotaxis assembly. Cell walls isolated from 11 bacterial species caused a definite increase in monocyte migration, but the walls of *Micrococcus lysodeikticus* were scarcely active. The migration-enhancing activity of *Staphylococcus epidermidis* cell walls was retained by a monomer as well as a polymer of disaccharide peptides which were prepared by digestion of the peptidoglycan with enzymes. It was finally revealed that the migration of monocytes was enhanced by MDP. 6-*O*-Octadecanoyl-MDP, 6-*O*-(2-tetradecylhexadecanoyl)-MDP, and 6-*O*-(3-hydroxy-2-docosylhexacosanoyl)-*N*-acetylmuramyl-L-seryl-D-isoglutamine were active, but to a lesser extent. A checkerboard assay demonstrated that the increased monocyte migration caused by *S. epidermidis* cell walls was directed toward a positive stimulus (chemotaxis).

Many studies have revealed that bacterial cell walls and their structural units, whether obtained by use of enzymes or synthesized, modify various functions of macrophages in the following ways: enhancement of adherence and spreading on surfaces (28, 42), inhibition of migration from a capillary tube (1, 49), stimulation of differentiation (3), inhibition of DNA synthesis (41), increase of glucosamine uptake (14, 39), induction of lysosomal enzyme release (15), increase of superoxide release (27, 28), stimulation of glucose oxidase level (14), stimulation (6, 10, 24) or inhibition (32) of phagocytosis, induction of cytotoxic or cytostatic activities on tumor cells (17, 31, 37, 43), increased production of collagenase (47), enhancement of monokine production (16, 26, 29, 45), and so on. These studies have also demonstrated that *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP), a common component of the cell wall peptidoglycans of parasitic bacteria, is the unit structure responsible for most of the above activities.

Stimulation of macrophage activities by bacterial cell walls and their components, obtained enzymatically or synthesized, may result in the enhancement of host defense mechanisms to exogenous or endogenous harmful agents. In the present study, we examined possible stimulating effects of cell walls and their fractions upon the migration of blood monocytes, which are generally believed to be precursor cells of tissue macrophages (46), taking into consideration that the accumulation of macrophages should be a prerequisite for the effective manifestation of host defense-enhancing activities of macrophages which are stimulated by cell walls and related compounds.

MATERIALS AND METHODS

Cell walls and peptidoglycans. Test cell wall specimens were prepared by the methods previously described (20, 39, 40) from the following bacteria: *Micrococcus lysodeikticus* NCTC 2665, *Staphylococcus epidermidis* ATCC 155, *Streptococcus mutans* BHT, *Lactobacillus plantarum* ATCC 8104, *Streptomyces gardneri* ATCC 23911, *Rhodococcus rhodochrous* ATCC 184, *Mycobacterium smegmatis* ATCC 19240, *Nocardia corallina* ATCC 14347, *Nocardia corynebacterioides* ATCC 14898, and *Nocardia gardneri* IFO 3385. Briefly, cells grown under appropriate cultural conditions were mechanically disrupted with either a Braun cell homogenizer (model MSK, B. Braun Apparatebau, Melsungen, West Germany) or a Dyno-Mill (type KDL, Willy A. Biochofen Manufacturing Engineers, Basel, Switzerland). A crude wall fraction obtained by differential centrifugation of disrupted cells was purified by digestion with proteolytic enzymes. The cell walls of *Staphylococcus aureus* (Tasaki) and *Streptococcus pyogenes* (type 3, strain 0176) were

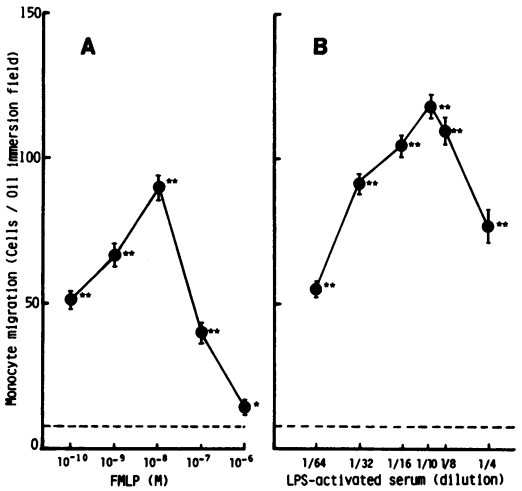

FIG. 1. Enhancement of human monocyte migration by FMLP (A) and LPS-activated human serum (B); dose-response relationship in a multiwell chemotaxis chamber assay. FMLP and LPS-activated serum were prepared as described in the text. Data represent the mean ± standard error of the number of monocytes per oil immersion field in one experiment performed at the same time. Triplicate filters were submitted to assay of each dose of each specimen. (- - -) Monocyte response to Gey BSA-GVB^{2+} without chemoattractant. The difference between the test sample and the negative control was significant at a level of 5% (*) or 1% (**) by Student's *t*-test.

generously supplied by Y. Hirachi, Osaka University Dental School (38), and H. Ohkuni, Nippon Medical School (25), respectively. Peptidoglycan preparations of *S. aureus*, *S. epidermidis*, *S. mutans*, and *L. plantarum* were made by extraction of the respective cell walls with trichloroacetic acid to remove nonpeptidoglycan portions as described previously (20). The peptidoglycan of *S. pyogenes*, a gift of H. Ohkuni, was prepared by the hot formamide method (8).

Monomeric and polymeric peptidoglycan subunits. The "polymer" (designated SEPS) and "monomer" (designated SEPS-M) were obtained by treatment of *S. epidermidis* wall peptidoglycan with the M-1 endo-*N*-acetylmuramidase (50) and the SALE endopeptidase (*S. aureus* lytic enzyme produced by a *Cytophaga* sp. B30), which was capable of splitting the cross-links between the neighboring stem peptides of staphylococcal cell walls. Details of the preparation methods of SALE, isolation procedures, chemical analyses, and proposed chemical structures of SEPS and SEPS-M were described previously (11).

MDP and 6-*O*-acyl derivatives. MDP, 6-*O*-octadecanoyl-MDP, 6-*O*-(2-tetradecylhexadecanoyl)-MDP, and 6-*O*-(3-hydroxy-2-docosylhexadecanoyl)-*N*-acetylmuramyl-L-seryl-D-isoglutamine were synthesized as described in previous papers (21–23, 30). These synthetic preparations, referred to as MDP, L18-MDP, B30-MDP, and BH48-MDP(L-Ser), respectively, were kindly supplied by A. Inoue, Daiichi Seiyaku Co., Ltd., Tokyo, Japan.

Chemotactic agents used as reference standards. One reference standard, *N*-formylmethionyl-leucyl-phenylalanine (FMLP), was purchased from Sigma Chemical Co., St. Louis, Mo., and was dissolved in ethanol at a concentration of 10^{-2} M. The other was a lipopolysaccharide (LPS)-activated serum which was prepared by incubation of 1 ml of a fresh human serum specimen with 200 μg of LPS from *Escherichia coli* O55:B5 (Difco Laboratories, Detroit, Mich.) in 0.1 ml of physiological saline at 37°C for 60 min with shaking (Eyelashaker SS-8, Tokyo Rikakikai Co., Tokyo, Japan). The LPS-activated serum was centrifuged at 20,000 × *g* (4°C, 15 min) to remove little debris and was heated at 56°C for 30 min. These two reference preparations were stored at −20°C for 3 to 5 days in 0.5-ml portions. Portions were thawed before use, diluted to indicated concentrations with the medium described below, and used as positive controls for chemotaxis assays (12, 35).

Media for test specimens. Test materials and reference chemotactic agents were diluted with a mixture of seven parts of Gey balanced salt solution containing 2% bovine serum albumin (Sigma) and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (Sigma) (Gey BSA, pH 7.0) and five parts of gelatin-Veronal buffer with 2 M $MgCl_2$ and 1 M $CaCl_2$ (GVB^{2+}). This mixture will be referred to as Gey BSA-GVB^{2+} (7, 33). It should be noted that neither sera nor serum products as possible sources of complement (except BSA) were added to the medium.

Human monocytes. Heparinized venous blood drawn from healthy donors was subjected to fractionation by a modification of the Ficoll-Hypaque method (5) to obtain a cell fraction in which monocytes were enriched. Cells were washed with phosphate-buffered saline (pH 7.0) and suspended at a cell density of 5 × 10^6 cells per ml of Gey BSA. The cells consisted of approximately 20% monocytes and 80% lymphocytes.

Assay for migration of monocytes. Assays were performed by use of a multiwell chemotaxis assembly (Neuro Probe, Cabin John, Md.) (4, 7, 12, 33). Each of the wells on a bottom plate was filled with 25 μl of test or reference specimens dissolved or suspended in Gey BSA-GVB^{2+}. A polycarbonate filter sheet (Neuro Probe) of 5-μm pore size and about 10-μm thickness was then placed on the bottom plate. A gasket and a top plate were fixed in place. Fifty microliters of leukocyte suspension (enriched with monocytes) was then added to each well on the top plate (see Table 1). The whole assembly was incubated at 37°C for 90 min in humidified air containing 5% (vol/vol) CO_2. After incubation, the filter was removed, fixed, and stained with Diff-Quick (International Reagents Co., Kobe, Japan). Migration of monocytes from the upper well toward the bottom well containing the test or reference specimen was estimated by counting the number of monocytes which had completely passed through the filter; 20 microscopic fields on the filter surface adjacent to the bottom wells were randomly selected for counting at ×1,000 magnification (by combination of a ×100 oil immersion objective lens and a ×10 ocular lens equipped with a microgrid [5 by 5 mm; Olympus Optical Co., Tokyo, Japan]). The assay for each specimen and each dose was carried out with triplicate filters. Data are expressed as the mean ± standard error of the number of monocytes per oil immersion field, obtained in a representative of at least three repeated experiments.

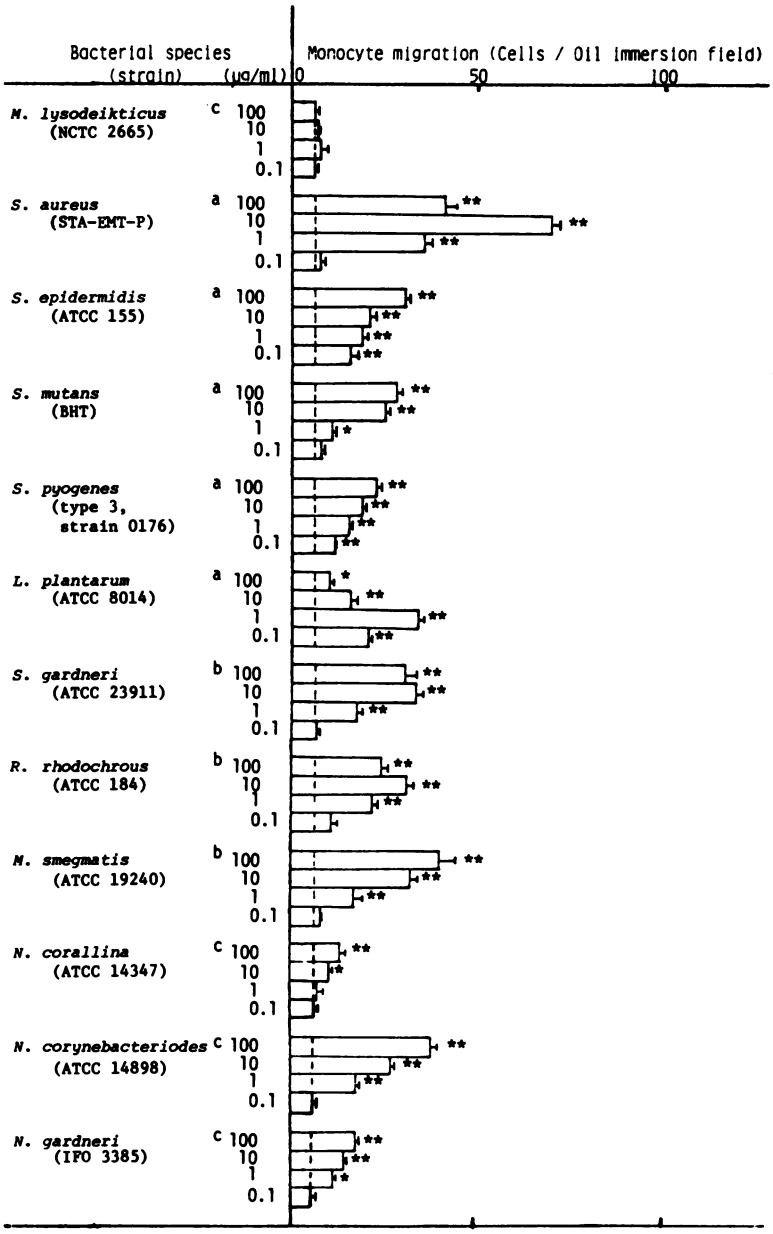

FIG. 2. Monocyte migration in response to various bacterial cell walls. Details are as for Fig. 1 except that the migration values of positive controls were: 99 ± 2 (a), 59 ± 3 (b), and 47 ± 1 (c) for FMLP (10^{-8} M) and 96 ± 3 (a), 64 ± 4 (b), and 55 ± 2 (c) for LPS-activated serum (1:10); that of all negative controls was 6 ± 1. The difference between the test sample and negative control was significant at a level of 5% (*) or 1% (**) by Student's *t*-test.

RESULTS

Dose response of human monocytes to FMLP and LPS-activated serum. Monocyte chemotaxis by two well-known chemoattractants was assayed to evaluate the validity of the experimental conditions adopted in the present study. The dose-response data for FMLP are shown in Fig. 1A. The maximum chemotactic response occurred at an FMLP concentration of 10^{-8} M. The response of monocytes to various dilutions of LPS-activated serum is shown in Fig. 1B. An optimal chemotactic response was obtained between dilutions of 1:16 and 1:8. LPS specimens

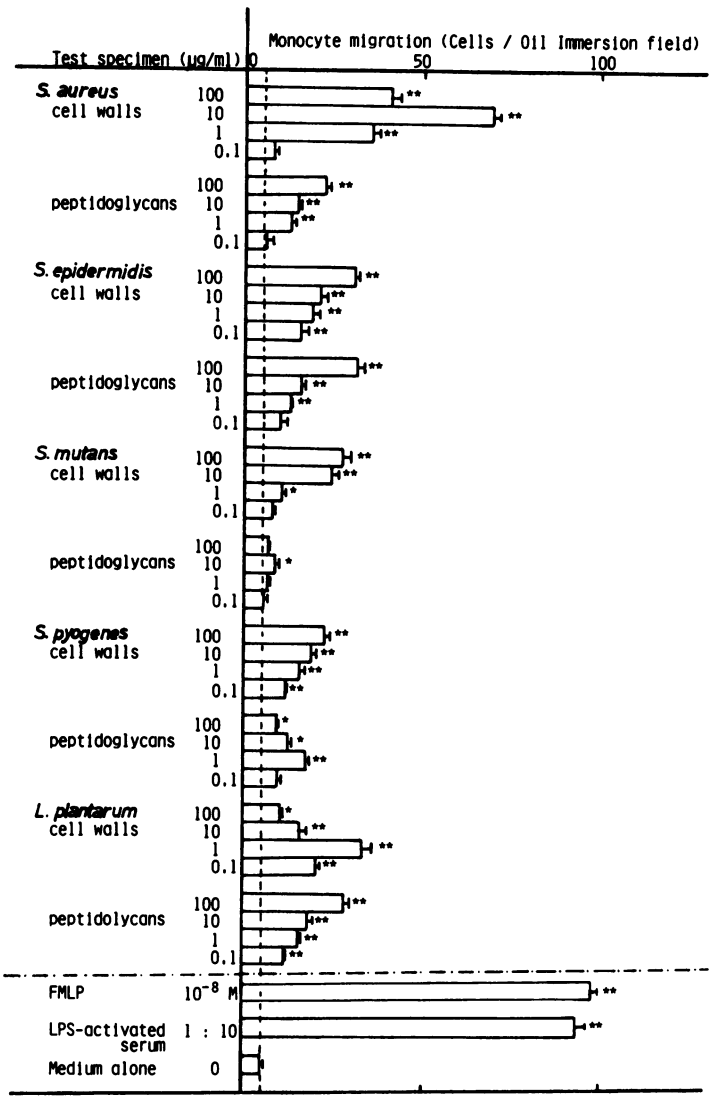

FIG. 3. Comparison of monocyte migration in response to cell walls and peptidoglycans from various bacterial species. Details are as for Fig. 1 except that the migration values of positive controls were 99 ± 2 for FMLP (10^{-8} M) and 96 ± 3 for LPS-activated serum (1:10); that of the negative control was 6 ± 1. The difference between the test sample and negative control was significant at a level of 5% (*) or 1% (**) by Student's *t*-test.

(Difco Laboratories) of *E. coli* and *Salmonella enteritidis* by themselves were inactive in the enhancement of monocyte migration (data not shown).

Stimulation of monocyte migration by bacterial cell walls and peptidoglycans. Figure 2 summarizes the results of the assay made with cell walls of 12 bacterial species. All of the test cell walls except those of *M. lysodeikticus* significantly enhanced the migration of human monocytes through a polycarbonate filter membrane at concentrations ranging from 0.1 to 100 μg/ml. The walls of *M. lysodeikticus* completely lacked the enhancing activity. The cell walls of *N. corallina* showed only a weak activity, and the walls of *S. aureus* exerted the strongest stimulating effect. Existence of the optimum dosage was noticed with some cell walls such as those of *S. aureus*, *L. plantarum*, and *R. rhodochrous*. Figure 3 shows the monocyte migration-enhancing activities of cell walls and peptidoglyans of *S. aureus*, *S. epidermidis*, *S. pyogenes*, and *L. plantarum*. Stronger activity was obtained with cell walls than with peptidoglycans, except for *S. mutans*, wherein the wall was scarcely active for an unexplainable reason.

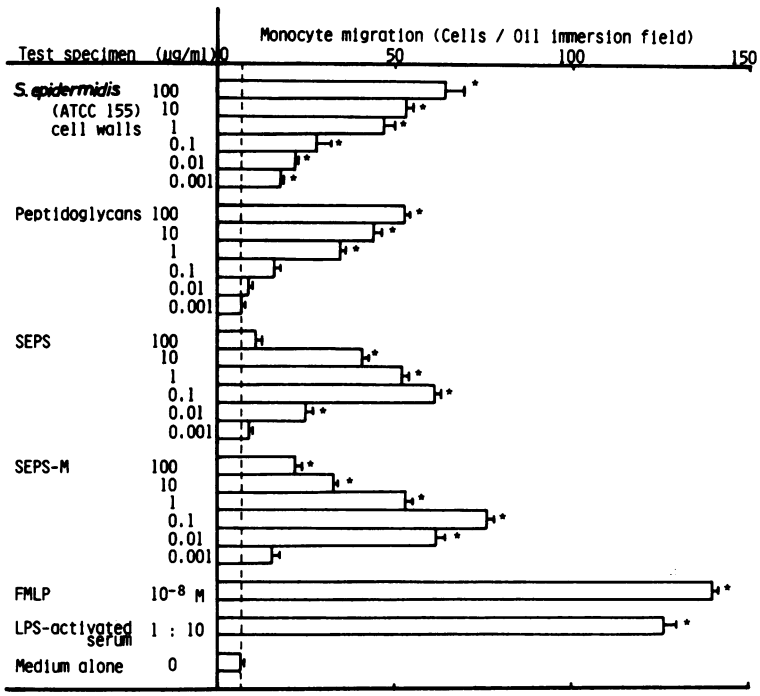

FIG. 4. Monocyte migration in response to *S. epidermidis* cell walls, peptidoglycans, and their enzymatic digests SEPS and SEPS-M. Details are as for Fig. 1. The migration values of positive controls were 140 ± 2 for FMLP (10^{-8} M) and 126 ± 4 for LPS-activated serum (1:10); that of negative controls was 7 ± 1. The difference between the test sample and negative control was significant at a level of 1% (*) by Student's *t*-test.

Migration-stimulating activity of a water-soluble enzymatic lysate of *S. epidermidis* cell wall peptidoglycan. Figure 4 indicates that SEPS-M, a monomer of disaccharide peptide, as well as SEPS, a polymer, significantly increased the migration of monocytes through the filter toward them, and that the wall and peptidoglycan required higher dosages than did SEPS and SEPS-M to exhibit a similar level of enhanced migration (100 μg/ml with the wall and peptidoglycan and 0.1 μg/ml with SEPS and SEPS-M). The extent of enhancement of monocyte migration by SEPS and SEPS-M, however, was significantly less than that caused by two positive controls (FMLP and LPS-activated serum).

Stimulating activity of synthetic MDP and 6-*O*-acyl derivatives. Since SEPS-M, a monomeric peptidoglycan subunit, was shown to stimulate the migration of monocytes significantly, synthetic MDP and its 6-*O*-acyl derivatives were submitted to the assay for possible enhancement of the monocyte migration. All of the test synthetic specimens significantly enhanced the monocyte migration in the range of doses from 0.001 to 1 μg/ml, though the extent of enhancement was generally less than that of cell walls, peptidoglycans, and their enzymatic digests (Fig. 5). It seems that synthetic MDP exhibited stronger activity than did 6-*O*-acyl derivatives, and the introduction of 6-*O*-acyl groups to MDP weakened rather than raised the migration-stimulating activity of the molecule.

Checkerboard assay with *S. epidermidis* cell walls. To determine whether the observed increase of monocyte migration caused by *S. epidermidis* cell walls was merely due to increased chemokinesis or directed migration toward a positive stimulus (chemotaxis), various concentrations of cell walls were added to either the upper well, the lower well, or both of the wells in a checkerboard pattern (Table 1), and the monocyte migrations in these wells were compared. The assay result clearly indicates that the observed increase of monocyte migration was mainly directed by the concentration gradient of cell wall preparations, although increased chemokinesis might intervene.

DISCUSSION

The present study has demonstrated that all of 12 test cell walls, except those of *M. lysodeikticus*, and peptidoglycan specimens from five walls, except those of *S. mutans*, caused a

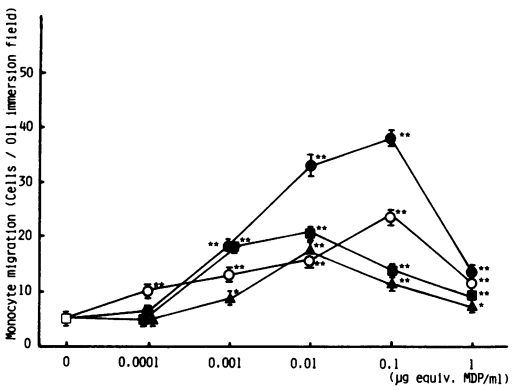

FIG. 5. Enhanced monocyte migration induced by MDP (●) and its 6-*O*-acyl derivatives L18-MDP (○), B30-MDP (■), and BH48-MDP (L-Ser) (▲). Details are as for Fig. 1 except that the migration values of positive controls were 52 ± 3 for FMLP (10^{-8} M) and 70 ± 4 for LPS-activated serum (1:10); that of the negative control (□) was 5 ± 1. The difference between the test sample and negative control was significant at a level of 5% (*) or 1% (**) by Student's *t*-test.

definite increase of monocyte migration directly, i.e., in the absence of fresh serum as a source of complement. The optimum dose of bacterial cell walls that caused the maximum stimulation of monocyte migration and extent of enhanced migration significantly differed from walls to walls. The reason for the inability of *M. lysodeikticus* walls to enhance monocyte migration is not known at present since the cell wall of this organism holds MDP as a part of its peptidoglycan structure as in other active cell walls.

Studies with water-soluble preparations enzymatically prepared from *S. epidermidis* peptidoglycan showed that the monocyte migration-enhancing activity was retained by enzymatic degradation of the peptidoglycan into a monomeric subunit. Further study revealed that, like many of other immunomodulating activities, the monocyte migration-enhancing activity of the cell walls was held by synthetic MDP. By dose-response analyses, the concentration of MDP inducing the maximum enhancement of monocyte migration was shown to coincide with that of SEPS and SEPS-M (0.1 μg/ml). The minimum effective concentration of MDP was estimated to be as low as 0.001 μg/ml (2×10^{-9} M). In view of previous reports that some macrophage- and monocyte-stimulating activities of MDP could be significantly increased by substitution of a hydroxy group at position C-6 of the muramic acid residue with appropriate acyl groups such as the 2-tetradecylhexadecanoyl group (27, 39), attempts were made to increase the migration-enhancing activity of MDP by 6-*O*-acylation, but were not rewarded by positive results (Fig. 5).

There have so far been no reports on the enhancement of leukocyte migration by either bacterial cell walls or MDPs without the involvement of serum complement. Regarding microbial products other than cell walls, Adamu et al. (2) reported that an outer membrane fraction obtained from *Bacteroides fragilis* ATCC 23475 caused an enhanced migration of rabbit polymorphonuclear leukocytes under assay conditions similar to those of the present study. They showed that the effective dosage ranged from 50 to 250 μg/ml, so that the enhancing activity of the outer membrane was in terms of the effective dose. There are a few reports on the chemotactic activity of bacterial endotoxins on leukocytes (19, 34, 36). The activity of LPS, however, is dependent on factors in fresh serum, particularly on complement system. It has been shown that bacterial cell walls and SEPS, but neither SEPS-M nor MDP, can activate serum complement through the alternative pathway irrespective of participation of antibody, as reported by Kawasaki (18) and others (9, 44, 48). It may be emphasized that LPS specimens hardly exerted stimulating activity on monocyte migration in the present assay conditions: this finding clearly indicates that the activity of cell walls and related compounds reported here was not due to contaminated LPS. Regarding the production of chemotactic principles by bacterial peptidoglycans through the activation of complement system, Heymer and Rietschel (13) reported that peptidoglycans isolated from *S. aureus* and *Bordetella pertussis* exerted the chemotactic activity for human granulocytes in the presence of normal human serum. However, none of the

TABLE 1. Dependence of enhanced monocyte migration on the concentration gradient of *S. epidermidis* cell walls[a]

Concn (μg/ml) of cell walls in lower well	Cells/field ± SE[b] at following concn (μg/ml) of cell walls in upper well:			
	0	1	10	100
0	**5 ± 2**[c]	8 ± 1	8 ± 1	6 ± 1
1	19 ± 1	**8 ± 1**	9 ± 1	11 ± 1
10	22 ± 2	19 ± 2	**12 ± 2**	9 ± 1
100	32 ± 1	29 ± 0	27 ± 2	**8 ± 1**

[a] *S. epidermidis* cell walls were added to both wells or either of the lower and upper wells of the multiwell chemotaxis chamber at indicated concentrations. Monocytes were added to the upper well.

[b] The extent of monocyte migration was determined as described in the text. The number of monocytes (per oil immersion field) on the lower surface of a polycarbonate filter was triplicately counted on 20 microscopic fields to get the mean ± standard error.

[c] Boldface numbers indicate those values obtained when the concentrations of chemoattractant on both sides of the filter were the same.

activity was found in heat-inactivated serum or in balanced salt solution (13). No data are available on the activity of peptidoglycans toward monocytes. In this connection, our preliminary experiment showed that *S. epidermidis* cell wall peptidoglycan did not stimulate the migration of human polymorphonuclear leukocytes under the present assay conditions. Thus, bacterial wall peptidoglycan seems to be not chemotaxin but chemotaxigen for human granulocytes or polymorphonuclear neutrophils.

Finally, with regard to the question of whether the monocyte migration-enhancing effect of bacterial cell walls reported here is actually chemotactic, i.e., a directed migration in response to a concentration gradient of active principles, or is simply because of increased random migration of monocytes (chemokinesis), we demonstrated by the checkerboard assay described by Zigmond and Hirsch (51) that *S. epidermidis* cell walls caused an enhanced migration of human monocytes directed by a concentration gradient and that *S. epidermidis* cell walls acted as a chemotaxin. Analyses for synthetic MDPs by the checkerboard technique are in progress (manuscript in preparation).

ACKNOWLEDGMENT

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