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# INHIBITION OF MACROPHAGE MIGRATION BY SYNTHETIC MURAMYL DIPEPTIDE

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Summary: N-acetylmuramyl-L-alanyl-D-isoglutamine, a synthetic compound which is known to have a minimal effective structure for an adjuvant activity of cell wall peptidoglycans, was found to inhibit the migration of normal macrophages. It was shown that the inhibition was neither due to cytotoxic or agglutinating effect of the muramyl dipeptide on macrophages nor due to lymphokine production uopn stimulation of lymphocytes by the muramyl dipeptide.

## INTRODUCTION

Most microorganisms and bacterial products which are potent immunological adjuvants enhance functions of macrophages.

Recently, a simple synthetic compound, N-acetylmuramyl-L-alanyl-D-isoglutamine, was found to have a minimal effective structure for adjuvant activity of cell wall peptidoglycans (1-3). Tanaka et al. showed recently that injection of this muramyl dipeptide (MDP) increased the rate of carbon clearance, while its adjuvant-inactive diasteroisomer, N-acetylmuramyl-L-alanyl-L-isoglutamine (MDP L-L) did not (4). This suggested a possible relation between adjuvant activity and the activation of the reticuloendothelial system.

In order to understand the mechanism of the activation of the reticuloendothelial system by MDP, we have searched for a suitable in vitro test and found that MDP inhibited the migration of macrophages in vitro.

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## MATERIALS AND METHODS

Muramyl dipeptide (MDP). The synthetic MDP was obtained through the courtesy of Drs. Shozo Kotani and Atsuro Inoue from Daiichi Seiyaku Co. (Tokyo, Japan). The synthesis of this compound has been reported by Kusumoto et al. (5).

Animals. Nonsensitized outbred Hartley female guinea pigs, weighing 400-600 g, were used throughout this study.

Culture medium. Hank's balanced salt solution (HBSS) was purchased from Nissui Pharmaceutial Co. Ltd., Japan. Tissue culture medium 199 (Chiba-ken Serum Institute, Japan) was supplemented with 10 % heat-inactivated fetal calf serum (FCS), 7mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Doujin Yakukagaku Institute, Japan) and was added with 50 units of penicillin and 50 μg streptomycin/ml. This culture medium is referred to as 199-FCS medium.

Collection of peritoneal exudate cells. Peritoneal exudate cells (PEC) were collected from nonsensitized guinea pigs 4 days after intraperitoneal injection of sterilized liquid paraffin as described previously (6). These cells consisted of about 70-75 % macrophages, 10-15 % lymphocytes and 10-15 % polymorphonuclear cells as determined by morphological examination.

Purification of adherent cells. PEC were incubated in 199-FCS medium at 37°C for 30 min in glass dishes and the cells adhered on the glass surface were collected as described previously (6). The purified adherent cells consisted of more than 99 % of macrophages, as judged by morphological criteria, phagocytic activity and rosette-forming capacity with sheep erythrocytes (SRBC) coated with rabbit anti-SRBC IgG antibody (7). T lymphocytes were not detected by the method of rosette formation with rabbit erythrocytes (8).

Test for cell migration. Cell migration was measured by capillary method (9) conventionally used for the assay of migration inhibitory factor (10). Briefly, migration of PEC or adherent cells packed in capillary tubes were measured in duplicate in 199-FCS media containing various concentrations of MDP. The migration area was determined after 24 hr in case of PEC and 36 hr in case of purified adherent cells. The migration index was calculated as follows:

$$\text{Migration Index} = \frac{\text{mean of migration area in the test sample}}{\text{mean of migration area in 199-FCS medium}}$$

Macrophage agglutination test. PEC ($1 \times 10^7$) were suspended in 0.5 ml HBSS containing phytohemagglutinin P (Difco, Detroit, Mich.) or MDP in each tube. After 15 min and 4 hr, agglutination of these cells were observed microscopically. The degree of agglutination was arbitrarily graded from + to +++.

## RESULTS

Inhibition of the migration of peritoneal exudate cells (PEC) by MDP (MurNAc-L-Ala-D-isoGln). As shown in Table 1, MDP caused a dose-related inhibition of the migration of PEC of normal guinea pigs. A marked inhibition was observed at a concentration of as low as 0.1 μg/ml of MDP.

Table 1. Effect of MDP on migration of peritoneal exudate cells

| MDP* | Experiment No. | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| µg/ml | Migration index (%) | | | | | |
| 0.001 | 90 | 89 | 92 | - | - | - |
| 0.01 | 87 | 43 | 80 | - | - | - |
| 0.1 | 71 | 29 | 50 | 50 | 47 | - |
| 1.0 | 59 | 14 | 48 | 44 | 40 | 27 |
| 10 | 51 | 11 | 40 | 47 | 33 | 26 |
| 100 | 41 | 12 | 34 | - | - | - |

* N-acetylmuramyl-L-alanyl-D-isoglutamine

Inhibition of the migration of peritoneal adherent cells by MDP. Next experiments were performed using adherent cells which consisted of more than 99 % macrophages as indicator cells. As shown in Table 2, MDP caused also definite inhibition of the migration. Removal of lymphocytes and polymorphonuclear cells did not reduce the extent of inhibition caused by MDP, which was shown by an experiment in which PEC and adherent cells purified from the same cell source were used (compare Exp. 3 of Table 1 and Exp. 3 of Table 2). Interestingly, MDP L-L isomer at 10 µg/ml did not show any inhibition. Data obtained with this and several other analogs will be reported separately.

The inhibition of migration with MDP was not due to an agglutinating effect of the material on PEC, because MDP did not agglutinate PEC (Table 3). In contrast, phytohemagglutinin used as a positive control showed a good agglutinating activity.

Table 2. Effect of MDP on migration of adherent cells

| MDP* | Experiment No. | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| μg/ml | Migration index (%) | | | | | |
| 0.001 | 79 | 94 | 92 | - | - | - |
| 0.01 | 75 | 80 | 74 | - | - | - |
| 0.1 | 33 | 39 | 42 | 35 | - | - |
| 1.0 | 25 | 35 | 30 | 35 | 45 | 52 |
| 10 | 25 | 30 | 28 | 34 | 43 | 50 |
| 100 | 25 | 30 | 27 | - | - | - |

* N-acetylmuramyl-L-alanyl-D-isoglutamine

Furthermore, the phenomenon was not due to cytotoxic effect of MDP, since addition of MDP (100 μg/ml) to the culture medium caused no decrease in the cell viability during 24 hr of culture (viable cells > 90 %).

## DISCUSSION

The present study revealed that the presence of MDP in the medium inhibited the migration of oil-induced peritoneal adherent cells (macrophages) of normal guinea pigs. The inhibition was dose-dependent, the minimal effective dose being less than 0.1 μg/ml. MDP neither exerted cytotoxicity to PEC nor agglutinated them, which showed that the inhibition phenomenon should not be due to such effects. Further, a possible involvement of lymphokine-induced migration inhibition in this phenomenon would be excluded because (a) no lymphocyte was detected in the adherent cell population used and (b) we observed no skin reaction to MDP in normal guinea pigs (unpublished data).

Table 3. Lack of agglutinating activity of MDP*

| Incubation Periods | Control Medium | PHA-P** | | MDP*** | |
|---|---|---|---|---|---|
| | | 10 μg/ml | 100 μg/ml | 10 μg/ml | 100 μg/ml |
| 15 min | - | + | ++ | - | - |
| 4 hr | - | + | +++ | - | - |

* Peritoneal exudate cells were used for agglutination test.

** Phytohemagglutinin P

*** N-acetylmuramyl-L-alanyl-D-isoglutamine

Probably, macrophage is the target cell of MDP in the present assay system, though an involvement of other types of cells or mediator(s), undetected for their scarcity in the cell preparation, is not completely excluded.

Heymer et al. (11) observed that the migration of PEC from normal rats and guinea pigs was inhibited by streptococcal cell wall mucopeptides under conditions similar to the present study. The phenomenon seems to be similar to that observed here. However, they found the inhibitory activity only in mucopeptide fractions of large molecular weight; those of low molecular weight (less than 5 - 10 x $10^3$ daltons) was not inhibitory even at a concentration of 200 μg/ml. Now we demonstrated that MDP, a simple synthetic compound of defined structure, has a definite effect at a concentration of as low as 0.1 μg/ml.

Concerning the target cell involved in the immunoadjuvant effect of MDP, Löwy et al. reported that the adjuvant effect of MDP was exerted through T cells (12). Using a peptidoglycan-containing cell wall fraction ("water-soluble adjuvant"),

Modollel et al. reported that macrophages seemed to be primarily required for the adjuvant effect (13). Tanaka et al. found that the phagocytic function of the reticuloendothelial system of mice was enhanced by MDP but not by its adjuvant-inactive diastereoisomer, MDP L-L (4).

In a study which will be reported separately, we also found that all the four adjuvant-active analogs of MDP tested inhibited the migration of adherent cells, while all the other five adjuvant-inactive analogs did not. The close parallelism observed between these activities suggests a possible relation between adjuvant activity and macrophage-stimulating activity, since migration inhibition phenomenon may be a reflect of macrophage stimulation. This point requires further investigation.

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