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BACTERICIDAL ACTIVITY OF THE MEMBRANE FRACTION ISOLATED FROM PHAGOCYTES OF MICE AND ITS STIMULATION BY MELITTIN

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SUMMARY: The membrane fraction prepared from mouse peritoneal exudate cells was incubated with mycobacteria, staphylococci, or *E. coli* in acetate buffer of pH 5.6 to follow the fate of viable bacilli. The membrane fraction exhibited bactericidal effect on mycobacteria and staphylococci, but not on *E. coli*. The activity to kill mycobacteria, as well as the endogenous phospholipase A₂ activity, of the membrane fraction was markedly enhanced by melittin, a basic peptide from bee venom, and inhibited by indomethacin and EDTA. The role of the enzyme activity in the bactericidal activity was discussed.

INTRODUCTION

As one of the candidates for the non-oxidative mechanism of the bacterial killing by phagocytes, we have been suspecting degradation products of cell membrane phospholipids in lesions, especially long-chain fatty acids and lysophospholipids (1-3). This idea was based on our discrete findings concerning tissue lipid analysis in mycobacterial infection (4) and also on the observations in a model experiment in which mycobacteria were killed in an incubation mixture with vesicles of artificial lipid membrane (liposomes) in the presence of phospholipase A₂ added (5). Furthermore, we have been encouraged in this idea by ultrastructural findings that mycobacteria ingested by phagocytes were in close contact with the phagosomal membrane, and that such areas of contact very often produced membrane degradation accompanying the damage to the inner structure of the bacterial cell (6). The present paper is an extension along this line of study to observe the events in an incubation mixture with an isolated membrane fraction of phagocytes and mycobacteria or staphylococci, with particular reference to the stimulating effect of a membrane-active agent, melittin.

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This attempt was undertaken taking the recent information into consideration that endogenous phospholipase A₂ in cultured cells is highly activated by melittin (7), and that such activation is observed even when isolated plasma membranes of neutrophils are used (8).

MATERIALS AND METHODS

Microorganisms: *M. tuberculosis* (H37Rv R-KM) and *M. bovis* (BCG) were employed. H37RvR-KM has a marker of kanamycin-resistance to 100 µg per ml. The strain has been maintained by subculture on Sauton synthetic liquid medium. If not otherwise stated, aqueous bacterial suspensions were prepared from the growth of 10 days or so by routine procedures (9). *Staphylococcus aureus* (209P) and *Escherichia coli* (K-12) are also kanamycin-resistant strains to 5 µg per ml. Before use, these strains were subcultured overnight in tryptose broth (Eiken), and a 1:4 saline dilution of the former and a 1:10 dilution of the latter were employed as test inocula.

Experimental animals: Conventional male mice of ddY strain weighing around 30 g were mainly employed to harvest peritoneal exudate cells.

Harvest of peritoneal exudate cells: Mice were injected intraperitoneally with 3 ml of a 9% solution of Hammersten casein (Nutritional Biochemicals Corporation, Cleveland, OH). Fifteen hours or 3 days later, the animals were sacrificed by cervical dislocation or by air embolism, and the peritoneal cavity was opened to be washed with chilled Hanks' salt solution containing heparine in 5 units per ml. The lavaged fluid was pooled and let pass through a stainless steel sieve of 100 mesh. The filtrate was centrifuged at 1,000 rpm for 5 min. The resulting cell pellet was washed twice with Hanks' solution and subjected to cell counting with a hemocytometer and to differential cell counting by Giemsa staining (mononuclear or polymorphonuclear cell). Finally, the cells were suspended in a known volume of 0.2 M KCl containing kanamycin in 50 µg per ml so that the cell concentration was to be 5×10^7 to 1×10^8 cells per ml and kept at 4 C. In each experiment, 50 to 100 mice were employed for cell harvest.

Preparation of "membrane fraction": The cells, 5×10^8 to 1×10^9 , were suspended in 10 ml of 0.2 M KCl and disrupted by sonication at 50 w for one minute with an ultrasonic apparatus (Model LP-200P) of Tomy Seiko Co., Tokyo. The sonicated suspension was centrifuged sequentially at 1,000 rpm for 10 min and at 3,000 rpm for 20 min to remove cell debris and nuclear components. The resulting supernatant was centrifuged at 26,500 xg for one hour with a refrigerated centrifuge. The pellet was resuspended in 10 ml of 0.2 M KCl and the suspension was centrifuged again as above. The washed sediment was resuspended in 2 ml of deionized water and dispersed by sonication for a few seconds. Further purification was not carried out in view of the decreasing yield of material. To prevent bacterial contamination, kanamycin and penicillin were added to this suspension at concentrations of 50 µg per ml and 100 units per ml, respectively. When *S. aureus* or *E. coli* was to be used as test microorganisms, penicillin was omitted and kanamycin was added at the concentration of 5 µg per ml. Thus prepared materials had phospholipids in an amount of 100 to 200 µg (estimated by King method) and proteins in an amount of 200 to 300 µg (Folin test with BSA as standard) per 100 µl. This post-nuclear fraction enriched in membrane fragments

(designated as MF) was presented to bactericidal tests.

A corresponding fraction of red blood cells was prepared in a similar fashion from the blood obtained at the time of animal sacrifice for cell harvest.

Bactericidal test: The system for mycobacteria was an incubation mixture consisting of 100 μ l of 0.05 M acetate buffer (pH 5.6), 10 μ l of 100 mM CaCl_2 , 100 μ l of bacterial suspension (1 mg/ml), and 100 μ l of MF as stated above. After a given period of incubation at 37 C, the mixture was added with 0.7 ml of the acetate buffer to make the total volume 1 ml and it was incubated for additional 30 min. Enumeration of viable units therein was made with Kirchner agar slants containing bovine serum in 5% and kanamycin in 100 μ g per ml.

As modifiers of the bactericidal effect, melittin, indomethacin and EDTA were employed. Indomethacin purchased from Sigma (St. Louis, MO) was used in an amount of 20 μ l of a 0.5 mg per ml (1.4×10^{-3} M) solution. The solution was prepared immediately before use by dissolving first the drug in 0.5 ml of 95% ethyl alcohol and then adding 9.5 ml of PBS (10). Melittin (Sigma) was used in an amount of 10 μ l of a 250 μ g/ml solution which did not show antimycobacterial activity at all. EDTA was used in an amount of 20 μ l of 60 mM.

The system for examining bactericidal effect on *S. aureus* and *E. coli* consisted of 100 μ l of 0.01 M tris buffered saline (pH 7.0), 100 μ l of a bacterial suspension, 100 μ l of MF and 10 μ l of 100 mM CaCl_2 . After a given period of incubation, 0.7 ml of 0.005 M acetate buffer (pH 5.6) was added to the mixture and it was left to stand at 37 C for additional 15 min. Enumeration of viable units was made with triptosoy broth agar plates containing kanamycin in 5 μ g per ml.

Phospholipase A₂ assay: As labeled lecithin, L- α -phosphatidylcholine, β -[1- 14 C] arachidonyl, α' -stearoyl (The Radio-chemical Centre Amersham, England) was used: 0.2 μ Ci of this was put into a test tube of 10-ml capacity and dried in vacuo. Then, 100 μ l of 0.05 M acetate buffer (pH 5.6), 10 μ l of 100 mM CaCl_2 , and 100 μ l of MF were added to the tube and stirred. After a given period of incubation at 37 C, the reaction was stopped by addition of 1.2 ml of chloroform-methanol (2:1) and the mixture left standing overnight. The lower part of the extract was then employed for lipid analysis. The procedure to determine percent degradation of labeled lecithin was the same as that described previously (11). Addition of the reaction modifiers to the mixture was made as described in "bactericidal test".

RESULTS

Melittin-stimulated Bactericidal Activity of MF on H37Rv Tubercle Bacilli and Its Inhibition by Indomethacin

A sample of MF was prepared from BCG-vaccinated mouse peritoneal cells consisting of mononuclear cells (81%) and polymorphonuclear cells (19%). It was used in an amount of 560 μ g as protein and 168 μ g as phospholipid per incubation mixture of 330 μ l and against 100 μ g of target bacteria. When melittin and indomethacin were incorporated into the incubation system, their

Fig. 1. Melittin-stimulated bactericidal activity on H37Rv tubercle bacilli of a membrane fraction (MF) and its inhibition by indomethacin. The amount of MF was 560 μ g as protein and 168 μ g as phospholipid per incubation mixture (330 μ l).

final concentrations were adjusted to 0.6 μ g and 3 μ g per 100 μ l, respectively. Time-course enumeration of viable units was conducted according to the standard method as stated before. The results are given in Fig. 1. MF was active in killing the bacilli as to reduce viable count down to less than one-tenth of the starting count in 16 hr. This activity was further enhanced in the presence of melittin. The viable count in 16 hr was nearly one-thousandth of the initial. Indomethacin manifested definite inhibition toward this bactericidal phenomenon. MF was preincubated with indomethacin for 30 min before addition of melittin.

Bactericidal Activity on H37Rv Tubercle Bacilli and Staphylococci of MF Separated from Different Cell Populations

Three samples of normal mouse MF were prepared from a mononuclear cell-predominant (78%) cell population, polymorphonuclear cell-predominant (90%)

cell population, or red blood cells. The bactericidal activities of these membrane preparations and the inhibition by indomethacin were compared in the experimental design as usual. Test microbial strains were H37Rv and *S. aureus*. The results are shown in Figs. 2 and 3. The preparations from peritoneal cells were highly active in killing both tubercle bacilli and staphylococci regardless of the cell population in the starting material. Staphylococci were killed more rapidly than tubercle bacilli. However, the corresponding fraction obtained from red blood cells was much less active in this respect, though a much larger amount was employed in the incubation system. In these experiments, the stimulation effect of melittin and the suppressing effect of indomethacin were also demonstrated.

Fig. 2. Bactericidal activity on H37Rv tubercle bacilli of membrane fractions (MF) separated from three different cell populations. In A, MF was obtained from a cell population consisting of mononuclear cells (78%) and polymorphonuclear leukocytes (22%). In B, MF was from a cell population of mononuclear cells (10%) and polymorphonuclear cells (90%). In C, MF was obtained from red blood cells. The amounts of MF per incubation mixture (330 μ l) were 232 μ g as protein and 60 μ g as phospholipid in A, 300 μ g as protein and 95 μ g as phospholipid in B, and 360 μ g as protein and 260 μ g as phospholipid in C.

Fig. 3. Bactericidal activity on *S. aureus* of membrane fractions separated from three different cell populations. The experimental conditions were the same as those in Fig. 2 except for the test microorganisms.

Fig. 4. Comparison of the sensitivity to the bactericidal activity of a membrane fraction between *S. aureus* and *E. coli*. The amount of MF was 300 μ g as protein and 65 μ g as phospholipid per μ l of the incubation mixture. Heat-treatment of MF was made at 100 C for 5 min.

Absence of Bactericidal Activity on E. coli in the Incubation System

With MF (240 μ g as protein and 113 μ g as phospholipid) obtained from mouse polymorphonuclear cells, the bactericidal tests on *E. coli* and staphylococci were conducted by the routine procedure. MF heated at 100 C for 5 min was also used. The results are shown in Fig. 4. MF was highly active in killing staphylococci as usual, but it was quite inactive on *E. coli*. Heat-treatment at 100 C for 5 min reduced the staphylocidal activity.

Bactericidal Activity on H37Rv Tubercle Bacilli of MF and Its Inhibition by EDTA

An experiment similar to the preceding one was undertaken with MF prepared from a mononuclear-predominant (71%) cell population of normal mouse origin and EDTA in place of indomethacin. The final concentration of EDTA was 4 mM, and it was incorporated into the incubation system simultaneously with other components. The results are shown in Fig. 5. The activities of MF were reversed by EDTA.

Fig. 5. Bactericidal activity on H37Rv tubercle bacilli of a membrane fraction (MF) and its inhibition by EDTA. The amount of MF was 300 μ g as protein and 65 μ g as phospholipid per 100 μ l of the incubation mixture.

Phospholipase A₂ Activity of MF Stimulated by Melittin and Inhibited by Indomethacin and EDTA

With labeled L- α -phosphatidylcholine, β -[1-¹⁴C]arachidonyl, α' -stearoyl, as substrate, phospholipase A₂ activity of a sample of MF was examined by the method already described. MF of mouse origin was obtained from a cell population consisting of mononuclear cells (85%) and polymorphonuclear leukocytes (15%). The amount used for the test was 280 μ g as protein and 168 μ g as phospholipid per 100 μ l of the incubation mixture. The enzymatic activity was expressed in percent degradation of labeled lecithin as calculated from the scanning chart of radioactivity on thin-layer chromatography. The results are shown in Fig. 6. The activity of MF alone was 4% degradation in 6 hr, but it increased up to 21.3% in 3 hr and to 36.5% in 6 hr in the presence of melittin. These activities were partially inhibited by indomethacin

Fig. 6. Phospholipase A₂ activity of the membrane fraction (MF) as activated by melittin and inhibited by indomethacin. The amount of MF was 280 μ g as protein and 165 μ g as phospholipid per 100 μ l of the incubation mixture.

in such a way as from 20% to 10% and from 36.5% to 22%. Also in another experiment with the mouse MF shown in Fig. 5, melittin-stimulated degradation of labeled lecithin was 88% in 8 hr and inhibited almost completely by EDTA. In this experiment, the activity reduced by indomethacin was 59.7%. The activity of MF alone was 3.3%.

DISCUSSION

In the previous paper (2), we reported that the degradation products of lecithin namely lysolecithin and fatty acid, can kill mycobacteria and staphylococci in vitro. An assumption was presented that, if this could also be the case in phagocytic cells as a protective mechanism, the site of event would be the closely contacting areas between the phagosomal membrane and parasite. Because, disruption and degradation of the membrane are prominent features in such areas (6), and we speculated that phospholipids there might be degraded into toxic fatty acids and lysolecithins possibly on the basis of hyper-activation of endogenous phospholipase A (7). Those degradation products may have good chances to act on the contacting bacteria before they are diffused into environmental tissues or neutralized by tissue fluid protein (12). The present study was undertaken to examine the feasibility of the above idea in an in vitro incubation model with an isolated membrane fraction of phagocytes and target microorganisms. As a condition to make the membrane-bacillus contact as close as possible, the incubation mixture was set up in a very limited space. Besides, melittin, a basic peptide of bee venom, was employed as an activator of membrane phospholipase A₂. It is known that melittin can stimulate various biochemical reactions in plasma membrane through activation of endogenous phospholipase A₂ (7,13). More recently, phospholipase A₂ in isolated plasma membranes of rabbit peritoneal neutrophils was found to be activated through receptor-mediated binding of various chemotactic factors (8).

In the present incubation system, MF exhibited constantly the bactericidal effect on mycobacteria and staphylococci but not on *E. coli*. The effect was inhibited more or less by indomethacin, EDTA, and heat-treatment of the fraction but enhanced by melittin. All these observations suggest that the underlying mechanism may be activation of endogenous phospholipase A of MF and subsequent degradation of phospholipids to release toxic fatty

acids and lysolecithin on a pathological level. The activation was actually demonstrated by an experiment with ^{14}C -labeled lecithin.

MF, may it be prepared from macrophages or polymorphonuclear leukocytes, was effective on mycobacteria. This may appear controversial against the general view that macrophages are the principal phagocytes responsible for protection against mycobacterial infection (14). It is difficult to answer this question at this moment. However, one explanation may be that, though polymorphonuclear leukocytes are essentially active in mycobacterial killing (15), mycobacteria are feeble in inducing acute inflammatory lesions and the cellular response soon turns into macrophage infiltration before polymorphonuclear leukocytes accumulate in an amount enough to play a main role of protection.

Dye and Kapral (16) suggested that the bactericidal material in staphylococcal abscesses might be lipid as degradation products of accumulated polymorphonuclear leukocytes. Noda et al. (17) reported that staphylococcal leucocidin stimulates the phospholipase A activity of the leukocytes membrane through binding to its receptors and brings about degradation of phospholipids. These two papers are favorable for our concept that phagocyte destruction as the result of host-parasite interaction can be interpreted as a protective mechanism by "self-sacrifice". It is generally known that viable cells of *S. aureus* are sometimes few in abscess (18) and viable tubercle bacilli are rather rare in caseous lesions (19).

Melittin was bactericidal to staphylococci at least in the concentration employed for mycobacteria. Therefore, we did not use it as an MF-stimulating agent when staphylococci were target bacteria. Staphylococci are known to be sensitive to basic peptide (21).

E. coli was not sensitive to the bactericidal activity of the present incubation system. This may be due to the fact that this species of bacteria is not sensitive to free fatty acids or lysolecithins (2) because of the presence of polysaccharide chains on the outer membrane as a barrier to them.

The basic and surface-active characteristics of the melittin molecule (20) may give it unique biological properties. Perturbation of the membrane structure caused by such physicochemical properties of melittin may result in activation of endogenous phospholipase A in the membrane (Fig. 6).

A study similar to the present paper has been made by Elsbach and his

associates (22-24). They isolated from rabbit polymorphonuclear leukocytes a highly bactericidal fraction, which was a basic peptide associated with phospholipase A₂ activities but devoid of lysozyme, myeloperoxidase and protease activities. Unlike our results, however, their fraction kills *E. coli* through the effects on the envelope as the permeability barrier. Their data suggest that the accumulation of lytic products of lipids within the bacterial envelope, rather than the loss of phospholipids per se, causes increased permeability. Here again, free fatty acids and lysolecithins come into the scope of discussion.

Since the purpose of this study was to see direct interaction between some host cell component and the parasite, we employed such an experimental approach that may appear tricky and artificial. When intact peritoneal cells were used in that system, viable counts of target bacteria did not decrease. The present author's concern is, however, not the early event within infected phagocytes but the event in a much later stage of infection or in the lesions consisting of degenerated phagocytes. In any case, the question to what extent the experimental model employed here reflects the real in vivo situation requires further study.

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