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INHIBITORY ACTIVITY OF LIPID FRACTIONS OF *MYCOBACTERIUM AVIUM* COMPLEX AGAINST MACROPHAGE RESPIRATORY BURST

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To explore possible mechanisms of the resistance of *Mycobacterium avium* complex (MAC) intracellular parasites to the antimicrobial activity of macrophages (MΦs), effects of the lipid components of these parasites on the MΦ respiratory burst were investigated. In this study, the MΦ respiratory burst was measured by luminol-dependent chemiluminescence generated through the peroxidase-mediated halogenation reaction in murine peritoneal MΦs in response to phorbol myristate acetate (PMA) triggering. Some lipid fractions of MAC organisms including phospholipid (PL), polar mycoside, and apolar mycoside irreversibly suppressed PMA-induced chemiluminescence of MΦs. Their inhibitory activity was dose-dependent, and their potency of inhibition was in the order listed above. PL potently suppressed MΦ chemiluminescence when administered to MΦs 10 s before PMA triggering. However, PL showed less efficacy in reducing MΦ chemiluminescence when added at 30 s or 80 s after PMA triggering. Moreover, when PL was administered to MΦs producing chemiluminescence in response to PMA triggering 30 s before PL addition, an approximately 1-min time lapse was observed before apparent suppression of MΦ chemiluminescence. These findings suggest that the PL-mediated suppression of MΦ chemiluminescence was due in part to inhibitory effects of PL on the PMA triggering process and process of photoemission by target MΦs.

Key words: *Mycobacterium avium* complex/lipid, mycoside, phospholipid / macrophage / respiratory burst / chemiluminescence

Mycobacterium avium complex (MAC) infections are frequently encountered in patients with AIDS and in other types of compromised hosts (1,2). MAC organisms can grow primarily in host macrophages (MΦs), because of their high resistance to MΦ microbicidal mechanisms (3,4,5). Certain mycobacterial components including the polysaccharide outer layer (6), inhibitors of phagosome-lysosome fusion such as sulfatide (6,7), inhibitors of MΦ activation such as lipoarabinomannan (LAM) (8), and endogenous scavengers of reactive oxygen intermediates (ROI) such as LAM and phenolicglycolipid-I (PGL-I) (9,10) are thought to be responsible for the resistance of mycobacterial organisms to intracellular killing by MΦs. LAM, PGL-I, and sulfatide also suppress MΦ production of ROI by inhibiting the respiratory burst (6,9,11,12) . Here, we studied the effect of certain lipid fractions of MAC, such as phospholipid (PL) and mycosides, on respiratory burst of peritoneal MΦs measured in terms of chemiluminescence, in response to phorbol myristate acetate (PMA) triggering.

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MATERIALS AND METHODS

Organisms

M.intracellulare N-260 strain, identified by using Gen-Probe Rapid Diagnostic System for the MAC (Gen-Probe, Inc., San Diego, CA.) was used.

Mice

Unless otherwise noted, female, eight to 12-week-old CBA/JN mice purchased from Charles River Co., Kanagawa, Japan were used throughout the study.

Preparation of MAC lipid fractions

Apolar and polar mycosides and phospholipids were partially purified from *M.intracellulare* N-260 using the method of Dhariwal *et al.* (13) with slight modifications (14). Briefly, the organisms were cultured on 7H11 agar plates, harvested by centrifugation, washed twice with distilled water, and lyophilized. The dried cells were extracted with acetone, and the acetone layer was concentrated by evaporation, yielding the "apolar mycoside-enriched fraction". The residue was then extracted with hot (60℃) methanol, evaporated and subsequently extracted with hexane. The resultant supernatant was concentrated by evaporation, yielding the "phospholipid-enriched fraction". The residue was then extracted with acetone and concentrated by evaporation, yielding the "polar mycoside-enriched fraction". All three lipid fractions were dissolved in dimethylsulfoxide (DMSO). A whole lipid fraction was prepared from MAC organisms by chloroform-methanol (2:1) extraction and subsequent concentration by evaporation.

MΦ chemiluminescence

MΦ chemiluminescence was measured as previously reported (15). Briefly, peritoneal exudate cells of mice (2×10^6 cells), harvested 4 days after ip injection of Zymosan A (1 mg), were incubated at 37℃ for up to 3 min in the following reaction mixture (1 ml in a 13 ×32-mm vial) : 1 ml of phenol red-free Hanks' balanced salt solution (HBSS) containing 10 mM N-2-hydroxyethyl-piperazine-N'-2-ethansulfonic acid (HEPES buffer), pH 7.4, and 0.1mM luminol with or without the addition of 0.1 μg/ml of PMA (Sigma Chmeical Co., St. Louis, Mo., U.S.A.). Lipid fractions of *M.intracellulare* were dissolved in 10 μl of DMSO and added immediately before the addition of PMA, unless otherwise specified. Photoemission was measured using a Lumicounter ATP-237 ATP lumiphotometer (Toyo Kagaku Ind., Tokyo, Japan) at 37 ℃ for 3 min. MΦ chemiluminescence measurement was performed in duplicate. The average deviation between the values was 8.6% of the mean value.

RESULTS

Fig. 1 shows effects of crude lipid (whole lipid), apolar mycoside, polar mycoside, and PL fractions on PMA-triggered MΦ chemiluminescence, when added immediately before PMA addition. All lipid fractions inhibited MΦ chemiluminescence in a dose-dependent manner. The potency of inhibitory effect of the three lipid fractions was in the order of PL, polar mycoside, and apolar mycoside. It is unlikely that the observed phenomenon was due to absorption or inactivation of PMA by these lipid fractions, since ten-fold increase of PMA concentration in the reaction mixture caused no decrease in the efficacy of the MAC lipid in inhibiting MΦ chemiluminescence (unpublished observation).

MΦs were preincubated with MAC crude lipid fraction (1,000 μg/ml) for 10 min, washed with HBSS, and then measured for PMA-triggered chemiluminescence in the absence of MAC lipid. Significant reduction was observed for the MAC lipid-treated MΦs, compared with untreated control MΦs (Fig. 2A). In this case, the decrease in the intensity of chemiluminescence observed for MAC lipid-treated MΦs (Fig. 2A) was similar in level to the reduction in chemiluminescence of control MΦs due to simultaneous addition of MAC lipid (Fig. 2B). This finding indicates that MAC lipid acted directly on MΦs to irreversibly reduce their ability to produce ROI. It thus appears that MAC lipid-mediated suppression of MΦ chemiluminescence is not due to quenching of ROI-derived light by MAC lipid.

Further detailed study was performed using the PL fraction, since it displayed strong inhibitory activity against MΦ chemiluminescence and was the most abundant lipid fraction obtained from MAC organisms (14). Fig. 3 shows the effect of the PL fraction added at different stages on PMA-triggered MΦ chemiluminescence. When the PL fraction was added 10 s before the addition of PMA, initiation of photoemission was delayed and photoemission intensity was strongly reduced. In this case, 59% inhibition of the peak chemiluminescence was observed (Fig. 3A). When the PL fraction was added at 30 s after PMA triggering, MΦ photoemission increased at the same rate as in the control incubation (PL-non added) until 60 s, and thereafter weak inhibition emerged, resulting in 22% reduction of peak chemiluminescence (Fig. 3B). PL thus appears to cause only incomplete suppression of photoemission of MΦs in cases in which the PMA triggering process has already been completed. When the PL fraction was added 80 s after PMA triggering, the intensity of MΦ photoemission was rapidly decreased within 30 s, and about 36% reduction in chemiluminescence was observed at 3 min after PMA-triggering (Fig. 3C). The reduction of MΦ photoemission observed immediately after addition of PL was probably not due to direct quenching of MΦ-derived light by PL, since there was an approximately 1-min time lapse between PL addition and the onset of PL-mediated reduction of MΦ chemiluminescence.

These findings indicate that MAC PL affected the MΦ chemiluminescence generating system itself, and that MAC PL-mediated suppression of chemiluminescence by PMA-triggered MΦs was due not only to hindrance of PMA triggering process of MΦs but also to inhibition of MΦ photoemission itself. Similar findings were also obtained for the crude lipid fraction of MAC (unpublished observation).

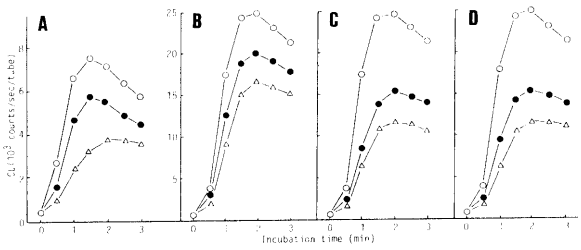

Fig. 1. MΦ chemiluminescence (CL)-inhibitory activity of crude lipid (A), apolar mycoside (B), polar mycoside (C), and PL (D) fractions of MAC. Each lipid fraction was added at concentrations of 0 (○), 250 (●), and 500 (△) μg/ml immediately before PMA-triggering at time 0. In the experiment shown in Fig. A, PECs were obtained from male mice whose MΦs generally generate lower chemiluminescence than that of MΦs of female mice used in Figs. B to D.

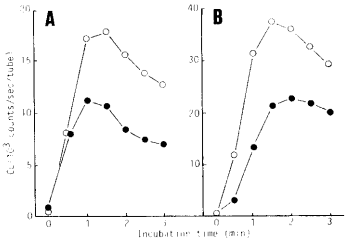

Fig. 2. Pretreatment of MΦs with MAC lipid fraction irreversibly decreased their ability to produce chemiluminescence (CL) in response to PMA-triggering. (A) ●: MΦs were incubated in HBSS containing 1,000 μg/ml of MAC lipid at 37°C for 10 min, washed with HBSS, and then measured for PMA-triggered chemiluminescence in the absence of MAC lipid. PMA-triggering was administered at time 0. ○: Control MΦs preincubated in the same fashion in the absence of MAC lipid were measured for PMA-triggered chemiluminescence in the same way. (B) Untreated (intact) MΦs were measured for PMA-triggered chemiluminescence in the presence (●) or absence (○) of MAC lipid (1,000 μg/ml). MAC lipid was added immediately before PMA-triggering at time 0.

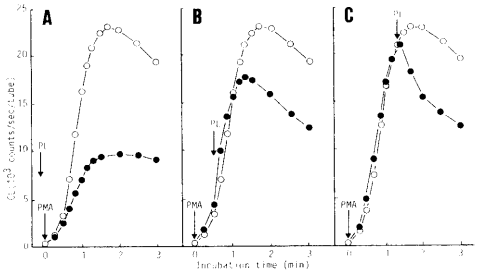

Fig. 3. Phase-dependency of chemiluminescence (CL)-inhibitory effects of the MAC PL fraction added before or after PMA-triggering at time 0. ○: MΦ chemiluminescence in the absence of PL. ●: PL was added at 500 μg/ml either 10 s before (A), 30 s after (B), or 80 s after (C) PMA-triggering.

DISCUSSION

This study indicates that MAC organisms possess lipid components with inhibitory activity against the PMA-triggered respiratory burst of MΦs. Among these compounds, PL and polar mycoside displayed relatively potent activity. Luminol-dependent chemiluminescence originates largely from HOCl molecules, which are generated in H_2O_2-dependent halogenation reaction, in addition to photoemission from other ROI including O_2^-, H_2O_2, •OH, and $^1O_2$ (16). These MAC lipids thus appear to affect the production of O_2^- by NADPH oxidase, dismutation of O_2^-, and/or H_2O_2- and halogen-dependent formation of HOCl molecules (17). In addition, it appears that MAC PL and whole lipid also inhibit the PMA-triggering process of the respiratory burst of MΦs, as described above.

The significance of ROI in the expression of antimicrobial activity of MΦs against mycobacteria including MAC and *M.tuberculosis* remains unclear (18). Although some investigators have reported that active oxygen species produced by lymphokine-activated MΦs killed *M.tuberculosis* (19), other studies have provided evidence that oxygen radicals may not be sufficient to inhibit and/or kill mycobacteria (20,21,22). Indeed, increase in MΦ active oxygen producing ability is not always accompanied by enhancement of the antimycobacterial activity of the same MΦs (23,24). However, we previously found that various mycobacterial species including MAC and *M.tuberculosis* were effectively killed by an H_2O_2-Fe^{2+}-mediated halogenation system but not by direct toxic effects of ROI (25,26). Hence, the oxygen-dependent bacterial killing mechanisms, in particular, halogenation reaction, may participate in expression of the antimycobacterial ability of MΦs, provided that sufficient MΦ respiratory burst is elicited by the interaction of MΦs with mycobacterial organisms. Indeeed, we found that virulent strains of MAC induced a much weaker respiratory burst in MΦs than did avirulent MAC strains (27), providing evidence that virulent MAC organisms evade active oxygen-mediated microbicidal mechanisms by parasitizing MΦs via binding to MΦ receptors whose interactions with bacterial ligands do not trigger ROI production.

The present findings suggest another important mechanism for the escape of MAC organisms from the toxicity of ROI produced by MΦs. All three lipid fractions tested, particularly PL, efficiently inhibited the respiratory burst of MΦs as measured by PMA-triggered chemiluminescence. These lipid components may thus serve as virulence factors of MAC organisms. However, it is still unclear whether the MAC lipids play an important role in the establishment and progression of MAC infection in vivo. In our recent study, PL and polar mycoside but not apolar mycoside inhibited mitogen-induced T cell blastogenesis (14). Analysis using thin-layer chromatography showed that the PL fraction of MAC consisted of four components with phosphate groups and T cell mitogenesis-inhibitory activity was found in the two PL components with high hydrophobicity. It is of interest to determine whether the MΦ chemiluminescence-inhibiting activity is shared by such PL components. Large-scale purification of these PL components and more detailed studies of their functions and chemical structures are required to elucidate the functional properties of PL as a virulence factor.

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