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Inhibition of Murine Macrophage Protein Kinase C Activity by Nonviable *Histoplasma capsulatum*

JUDITH E. WOLF,* STEVEN E. MASSOF, JOSEPH R. SHERWIN, AND ROBERT V. CONSIDINE

Departments of Medicine and Physiology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

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Ingestion of *Histoplasma capsulatum* yeast cells inhibits the oxidative burst response of murine macrophages (Mϕ's). Since protein kinase C (PKC) is believed to be an integral part of the signal transduction pathway involved in the production of reactive oxygen intermediates, we investigated the relationship between PKC activity and oxidative burst inhibition in *H. capsulatum*-containing murine peritoneal Mϕ's. An inhibitory effect on both basal and phorbol myristate acetate-mobilized membrane PKC activities was observed in Mϕ's which had ingested *H. capsulatum* but not latex spheres. These results suggest that one way in which *H. capsulatum* may disrupt the oxidative burst is through a PKC-dependent mechanism.

The oxidative burst of phagocytes represents an important host defense mechanism against invading microorganisms (1, 25). Initiated by the interaction between external stimuli and specific receptors on the macrophage cell membrane, the burst ultimately culminates in the activation of a plasma membrane-bound NAD(P)H oxidase and the generation of toxic oxygen species (2). Pathogens which survive within macrophages must therefore evolve strategies to circumvent the lethal effects of these reactive oxidants.

As an intracellular fungal parasite, *Histoplasma capsulatum* has been shown to inhibit the oxidative burst in murine peritoneal macrophages (37). However, the biochemical basis for the changes in oxidative burst activity observed in *H. capsulatum*-containing macrophages is unknown. One possibility is that the yeast cells directly inhibit the membrane oxidase. Alternatively, the yeast cells may disrupt the link between receptor and oxidase by altering the action of second messengers such as protein kinase C (PKC), a calcium- and phospholipid-dependent kinase speculated to be a critical component of the signal transduction network of phagocytic cells (19, 21, 34).

In this article, we characterize the activity of murine macrophage membrane PKC after ingestion of *H. capsulatum*. We also examine the effect of prior ingestion of the yeast cells on secondary stimulus-induced PKC mobilization.

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MATERIALS AND METHODS

Animals. Female 8-week-old AKR/J mice were obtained from Jackson Laboratory, Bar Harbor, Maine.

***H. capsulatum* G217B.** Heat-killed lyophilized yeast-phase cells of *H. capsulatum* G217B (37) were obtained from G. Kobayashi, Washington University, St. Louis, Mo., and stored at 4°C until used.

Reagents. Latex spheres (1.06-μm diameter), phorbol myristate acetate (PMA), superoxide dismutase from *Aspergillus niger*, staurosporine, and digitonin were purchased from Sigma Chemical Co., St. Louis, Mo.; proteose peptone was from Difco Laboratories, Detroit, Mich.; ferricytochrome *c* was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.; RPMI 1640 and Hanks buffered saline solution were from Fisher Scientific, Pittsburgh, Pa.; [γ-^{32}P]ATP was from New England Nuclear, Boston, Mass.; and P-81 ion-exchange paper was from Whatman, Maidstone, England.

Preparation of macrophage monolayers. Murine peritoneal macrophages were elicited by intraperitoneal injection of 1.0 ml of 10% proteose peptone, harvested by peritoneal lavage 72 h later, and plated onto 24-well tissue culture dishes in RPMI 1640 at a concentration of 3.5×10^6 cells per well as previously described (37). After 2 h of incubation at 37°C, in 5% CO_2, the resulting adherent monolayers (>98% macrophages by nonspecific esterase staining) were rinsed twice in RPMI 1640 before use. Giemsa-stained Mϕ monolayers were examined by light microscopy to confirm uniform density of intact adherent Mϕ's. Selected monolayers were exposed to particles at designated concentrations for various periods of time at 37°C and rinsed twice with RPMI 1640, and phagocytosis was quantified by differential counting of 200 macrophages at a magnification of 100×. Ingestion of two to eight particles per cell by at least 80% of the Mϕ's was observed in particle-containing preparations. Mϕ viability was assessed by trypan blue exclusion and by the ability of the Mϕ's to ingest latex spheres 24 h after ingesting *H. capsulatum*.

Superoxide anion (O_2^-) release. By using a modification of the ferricytochrome *c* reduction assay (20), macrophage monolayers were exposed to 10^8 *H. capsulatum* yeast cells for 1 h or to 0.01 μmol of staurosporine per ml for 15 min, washed twice with RPMI 1640, and then incubated for 90 min at 37°C in 1.0 ml of Hanks buffered saline solution (without phenol red) containing 0.1 μmol of PMA and 360 μmol of ferricytochrome *c* with or without 40 μg of superoxide dismutase per ml. O_2^- release was quantified by measuring the absorbance of culture supernatants in a spectrophotometer. The change in extinction coefficient at 550 nm for ferricytochrome *c* reduction was 21,000 M^{-1} cm^{-1}, which was applied to the difference in absorbance of culture supernatants in the presence or absence of superoxide dismutase.

Measurement of membrane-associated PKC activity. A permeabilized cell-synthetic peptide assay described by

* Corresponding author.

TABLE 1. Effect of increasing concentrations of *H. capsulatum* on Mϕ membrane PKC activity[a]

Group	No. of expts	Membrane PKC activity (pmol/mg/10 min)		% Inhibition of basal PKC activity[b]	% Inhibition of PMA-stimulated PKC activity	% Phagocytosis	Mean no. of particles per Mϕ
		−PMA	+PMA[c]				
Control	5	53.1 ± 13.6	143.4 ± 23.0				
H. capsulatum (3:1)[d]	5	38.5 ± 10.2[e]	119.2 ± 20.2	25.1 ± 10.4	12.0 ± 4.0	68.3 ± 18.3	5.4 ± 1.6
Control	11	90.6 ± 16.5	274.9 ± 49.6				
H. capsulatum (10:1)[d]	11	42.4 ± 6.3[e]	180.3 ± 31.5	48.1 ± 5.9	20.0 ± 8.5[f]	72.0 ± 8.4	7.3 ± 2.9
Control	4	63.6 ± 12.1	168.2 ± 16.7				
H. capsulatum (30:1)[d]	4	34.5 ± 9.4[e]	94.2 ± 13.1	47.8 ± 6.1	43.1 ± 6.4[f]	96.0 ± 5.2	10.7 ± 6.0

[a] Mϕ monolayers were incubated for 1 h in the presence or absence of increasing concentrations of *H. capsulatum*, washed, and then exposed to 0.1 μM PMA in fresh medium for 15 min. Values represent the mean ± standard error of the mean of the indicated number of experiments.
[b] $P < 0.05$; Sheffes' test comparison with percent inhibition in the absence of *H. capsulatum*.
[c] $P < 0.05$; Student's paired *t* test comparison with cells not exposed to PMA.
[d] Value in parentheses indicates particle-to-cell ratio.
[e] $P < 0.05$; Student's paired *t* test comparison with cells not exposed to *H. capsulatum*.
[f] $P < 0.05$; Sheffes' test comparison with percent inhibition in the absence of *H. capsulatum*.

Heasley and Johnson (16) was used to measure membrane-associated PKC activity. In this procedure, cells that have been pretreated with hormones or phorbol ester are permeabilized with digitonin in the presence of the synthetic peptide substrate, KRTLRR, and radioactive ATP. The sequence of the synthetic peptide is based on the major PKC phosphorylation site on the epidermal growth factor receptor and is specific for PKC in vitro. In vivo, the peptide is phosphorylated exclusively by membrane PKC (17). In our hands, the phosphorylation reaction is linear with permeabilization time up to 20 min.

The KRTLRR peptide was synthesized by using Fmoc chemistry and a solid-phase SAM TWO 9500 automated peptide synthesizer (Biosearch, San Rafael, Calif.). The purity of the preparation was determined by high-performance liquid chromatography analysis to be greater than 95%. The peptide sequence was confirmed by amino acid analysis.

Briefly, macrophage monolayers were exposed to PMA (100 nmol), heat-killed lyophilized *H. capsulatum* (2×10^8 to 4×10^8 particles per 100 μl), or latex spheres (10^9 spheres per 100 μl) in RPMI 1640 for the times indicated. The supernatants were removed, the monolayers were washed free of particles, and 200 μl of a salt solution (containing 137 mmol of NaCl, 5.4 mmol of KCl, 0.3 mmol of NaH_2PO_4, 0.4 mmol of KH_2PO_4, 5.6 mmol of dextrose, and 20 mmol of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) [pH 7.2] supplemented with 40 μmol of digitonin, 10 mmol of $MgCl_2$, 25 mmol of β-glycerophosphate, 100 μmol of [γ-^{32}P]ATP [~1,000 cpm/pmol], 5 mmol of EGTA [ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], 2.5 mmol of $CaCl_2$, and 600 μmol of KRTLRR peptide) was added to the Mϕ preparations. After 10 min of incubation at 30°C, the reaction was terminated by the addition of 40 μl of 25% trichloroacetic acid. The phosphorylated peptide was blotted onto P-81 ion-exchange paper, and the paper was washed with 75 mmol of phosphoric acid (three times) and 75 mmol of NaH_2PO_4 (pH 7.5) (once). The phosphocellulose sheets were then placed in scintillation fluid, and the radioactivity incorporated into the peptide was quantified by scintillation spectroscopy. Background phosphorylation, defined as the phosphorylation of substrates other than KRTLRR which bind to the filter paper, was subtracted from all values in each experiment. Monolayer protein content was determined by the method of Lowry et al. (22).

To assess the effect of prior *H. capsulatum* ingestion on secondary stimulus-induced PKC mobilization, the cells were pretreated with *H. capsulatum* or latex particles at various concentrations and times in 1 ml of RPMI 1640, washed twice with RPMI 1640, and then exposed to PMA (final concentration, 0.1 μmol) for 15 min before measurement of PKC activity. Percent inhibition of basal PKC activity was calculated as the difference in membrane PKC activity in the presence and absence of *H. capsulatum* divided by the activity in the absence of *H. capsulatum* multiplied by 100. The PMA-induced increase in membrane PKC (ΔPMA) was calculated as the difference in activity in the presence and absence of PMA treatment. Percent inhibition of PMA-stimulated PKC activity was then calculated as the difference between ΔPMA in the presence and absence of *H. capsulatum* divided by ΔPMA multiplied by 100.

RESULTS

Effect of *H. capsulatum* on membrane PKC activity. Basal membrane PKC activity was measured in Mϕ preparations incubated with increasing concentrations of heat-killed *H. capsulatum* and compared with that of untreated controls. As shown in Table 1, basal membrane PKC activity in the absence of *H. capsulatum* ranged from 53.1 ± 13.6 to 90.6 ± 16.5 pmol/mg/10 min. A one-way analysis of variance failed to demonstrate a significant difference among the three experimental groups; therefore, all basal data were grouped together ($n = 20$) for statistical analysis. Mϕ's exposed to increasing concentrations of *H. capsulatum* yeast cells exhibited a reduction in basal PKC activity which reached a plateau at a particle-to-cell ratio of approximately 10:1. Examination of Giemsa-stained Mϕ monolayers failed to reveal any significant differences in phagocytic index among the different preparations.

PMA stimulation resulted in a threefold increase in membrane PKC activity in all experimental groups. Inhibition of PMA-stimulated membrane PKC activity was observed in *H. capsulatum*-containing Mϕ's. However, except at the 30:1 particle-to-cell ratio, the inhibition of PMA-stimulated PKC activity was less than that observed for basal activity.

Specificity of PKC inhibition. To ensure that the above

TABLE 2. Lack of effect of latex particles on PKC activity[a]

Group	Membrane PKC activity (pmol/mg/10 min)		PMA-stimulated PKC activity (pmol/mg/10 min)
	−PMA	+PMA[b]	
Control	73.3 ±16.1	233.7 ±37.4	160.4 ±27.0
Latex	78.2 ±19.5	221.5 ±35.0	143.3 ±24.1

[a] Mϕ monolayers were incubated for 1 h in the presence or absence of 10^7 latex beads and then stimulated with 0.1 μM PMA for 15 min. PMA-mobilized PKC activity was calculated for each experiment as described in Materials and Methods. Values represent the mean ± standard error of the mean of three experiments.

[b] $P < 0.05$; Student's paired t test comparison with cells not exposed to PMA.

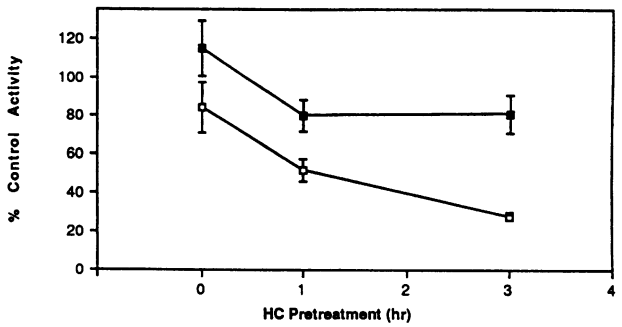

FIG. 1. Time course of inhibition of basal (open squares) and PMA-stimulated (closed squares) PKC activity. Results are expressed as percent PKC activity compared with that of particle-free Mϕ preparations (control).

observations were directly related to ingestion of *H. capsulatum* rather than a nonspecific effect of yeast cells, KRTLRR was incubated with 2×10^8 to 4×10^8 heat-killed *H. capsulatum* yeast cells for 1 h. The pretreated peptide was then used to measure PKC activity in non-yeast-cell-containing Mϕ's. The extent of phosphorylation of the *H. capsulatum*-treated peptide in the absence and presence of 0.1 μmol of PMA (27.8 and 100.6 pmol/mg/10 min, respectively) was comparable to that of untreated peptide under the same conditions (24.3 and 107.3 pmol/mg/10 min, respectively). In other experiments, 10^9 heat-killed *H. capsulatum* yeast cells were incubated in 1 ml of RPMI 1640 for 1 h, the supernatants from pelleted samples were applied to Mϕ monolayers for 15 min, and PKC activity was measured. No differences in either basal or PMA-stimulated PKC activity were found between Mϕ preparations which had been incubated with *H. capsulatum* supernatants (73.7 ± 14.4 and 226.9 ± 36.1 pmol/mg/10 min, respectively; $n = 3$) versus medium alone (79.4 ± 5.4 and 182.4 ± 16.6 pmol/mg/10 min, respectively, $n = 3$).

To assess whether the alteration in PKC activity was particle specific, membrane PKC activity was measured in Mϕ's which had ingested latex spheres. As shown in Table 2, phagocytosis of latex had no effect on basal or PMA-stimulated PKC activity.

Time course of PKC inhibition. To define the relationship between length of exposure to *H. capsulatum* and the degree of PKC inhibition, Mϕ's were incubated with heat-killed *H. capsulatum* at a 10:1 particle-to-Mϕ ratio and PKC activity was measured at various times over a 3-h period. The results indicate that inhibition of basal PKC activity was directly related to phagocytosis of yeast cell particles and was progressive over time (Fig. 1). At time zero, at which time no significant inhibition of PKC activity was observed, microscopic examination of Mϕ monolayers revealed contact between approximately 50% of Mϕ's and at least one *H. capsulatum* yeast cell but few internalized yeast cells. At 1 h, 72.0% ± 8.4% of Mϕ's had ingested 7.3 ± 2.9 yeast cells per Mϕ. This result did not differ significantly from that at the 3-h time point (64.3% ± 11.3% of Mϕ's containing 8.0 ± 3.2 yeast cells per Mϕ). In PMA-stimulated Mϕ's, there was no inhibition of PKC activity at time zero; however, at 1 and 3 h, PKC was inhibited by approximately 20%.

To determine whether *H. capsulatum* exposure could completely eliminate PKC activity, monolayers were incubated with *H. capsulatum* at a 10:1 particle-to-Mϕ ratio for 24 h before basal and PMA-stimulated PKC activities were measured. Results show that inhibition of basal PKC activity (56.9% ± 13.4%; $n = 4$) was not significantly different from that observed at 3 h (72.3% ± 2.2%; $n = 3$; one-way analysis of variance). In contrast, there was no longer any inhibitory effect on PMA-stimulated PKC activity. No difference in Mϕ viability was observed among the 1-, 3-, and 24-h preparations.

Relationship between reduction in PKC activity and generation of O_2^-. To determine whether the level of reduction in basal PKC activity in *H. capsulatum*-containing Mϕ's correlated with inhibition of the oxidative burst, we measured both membrane PKC activity and PMA-stimulated superoxide release from Mϕ's which had been incubated with *H. capsulatum* under the same conditions. As a control, we also measured the same parameters in Mϕ's which had been exposed to the PKC inhibitor staurosporine. The reduction in basal and PMA-stimulated Mϕ membrane PKC activities observed at a 10:1 *H. capsulatum* particle-to-Mϕ ratio (Table 1) was accompanied by a decrease in PMA-stimulated Mϕ O_2^- release from 4.4 ± 1.4 nmol in untreated Mϕ's to 0.9 ± 1.5 nmol in *H. capsulatum*-containing Mϕ's ($P \leq 0.05$; $n = 2$). Treating Mϕ's for 15 min with 0.01 μmol of staurosporine inhibited basal membrane PKC activity by 51% ± 15% ($n = 3$). The ability of PMA to increase PKC activity was inhibited by 59% ± 26% ($n = 2$). PMA-stimulated O_2^- release was reduced from 3.9 ± 0.2 nmol of O_2^- in untreated Mϕ's to 0.8 ± 0.9 nmol in staurosporine-treated monolayers ($P \leq 0.05$; $n = 3$). No O_2^- release was observed in Mϕ's treated with *H. capsulatum* or staurosporine alone.

DISCUSSION

Macrophage activation has been shown to result in enhanced cytotoxicity for intracellular microorganisms (1, 25). In general, there is a good correlation between murine Mϕ antimicrobial activity and the secretion of toxic oxygen metabolites during the oxidative burst, although nonoxidative mechanisms also contribute to Mϕ effector function. The oxidative burst is initiated by a series of events that culminates in the activation of a plasma membrane-bound NAD(P)H oxidase, proposed to be a multicomponent electron transfer chain composed of a flavoprotein and cytochrome b_{559} (2, 34). However, the biochemical mechanisms linking external stimuli with activation of the oxidase are still largely unknown.

Recent studies have suggested that the calcium- and

phospholipid-dependent kinase, PKC, may play a critical role in signal transduction in phagocytic cells (19, 21, 34). Potent stimuli of the oxidative burst such as PMA induce changes in the subcellular localization of PKC in phagocytic cells. Increases in membrane PKC activity in these cells appear to precede activation of the NAD(P)H oxidase and release of O_2^- (7, 10, 24, 26).

Previously, we showed that *H. capsulatum* yeast cells, both viable and heat killed, fail to stimulate an oxidative burst in unprimed murine peritoneal macrophages. Furthermore, the ingestion of *H. capsulatum* by murine macrophages inhibits subsequent responses to potent stimuli such as PMA or zymosan particles (36, 37). Similar findings have been described with a number of other intracellular parasites which evade phagocyte effector function (5, 9, 32). The ability of these pathogens to deactivate the oxidative burst may involve modulation of one or more of its biochemical components. Possibilities include changes in the number or function of cell surface receptors, in the NAD(P)H oxidase, or in the PKC-regulated signal transduction pathway from receptor to oxidase. Although other reports in the literature provide evidence for *H. capsulatum*-mediated production of intracellular reactive oxidants by human monocytes (6) and neutrophils (29), some controversy exists as to the reliability of nitroblue tetrazolium reduction when used to assay responses to *H. capsulatum* (11, 28). The observation that human peripheral blood monocyte-derived macrophages can be stimulated by *H. capsulatum* to release H_2O_2 (6) may reflect differences in assay conditions and in the properties of distinct mononuclear phagocyte populations.

In the present study, we focused on the signal transduction network of murine peritoneal Mϕ's by examining the activity of PKC after ingestion of heat-killed *H. capsulatum*. PKC activity was measured at various time intervals after phagocytosis of yeast cells by using a permeabilized cell-synthetic peptide method (16). This method has several advantages over the traditional homogenization or fractionation technique which measures the incorporation of ^{32}P from radiolabelled ATP into histone (7). First, the sequence of the synthetic peptide (KRTLRR) is based on a major PKC phosphorylation site on the epidermal growth factor receptor. Therefore, in contrast to histone protein which is a suitable substrate for several kinases, KRTLRR peptide has been shown to be phosphorylated specifically by PKC (17). Second, homogenization or fractionation of the cells is not required, reducing the possibility of PKC partitioning into the cytosol or membrane fraction because of the isolation procedure (27). Finally, since there is no Triton solubilization of the membrane fraction, the measurement of membrane PKC activity is more specific. One concern with the use of digitonin to permeabilize cells is the relatively large holes that are produced. However, experiments performed in our laboratory indicate that phosphorylation of KRTLRR peptide in digitonin-treated nonstimulated Mϕ monolayers is linear with permeabilization time up to 20 min, suggesting that activated PKC does not diffuse from the cells. In addition, no gross morphological alterations or cell losses from the culture dish have been observed.

The results reported here demonstrate inhibition of both basal and PMA-stimulated membrane PKC activities in *H. capsulatum*-containing Mϕ's. Inhibition of PKC activity was both time (Fig. 1) and concentration (Table 1) dependent, although complete inhibition was not attained. One explanation for the latter finding is that complete inhibition of PKC activity may not be necessary to eliminate the oxidative burst. Alternatively, *H. capsulatum* may simultaneously affect other sites in the signal transduction pathway or directly inhibit the NAD(P)H oxidase. Since the effect of *H. capsulatum* occurred earlier and to a greater extent on basal activity than on PMA-stimulated PKC activity, we speculate that *H. capsulatum* may act primarily by reducing the level of basal PKC activity within the Mϕ cell membrane. This reduction in membrane PKC activity may in turn affect the phosphorylation state of the NAD(P)H oxidase responsible for the Mϕ oxidative burst. Because measurements of PMA-stimulated PKC reflect not only increases in kinase activity but also translocation of the kinase from the cytosol to the membrane, the observation that *H. capsulatum* is only able to reduce PMA-stimulated PKC activity by 20% implies that kinase translocation is less effectively inhibited by *H. capsulatum* than is PKC membrane activity.

The relationship between PKC activity and the oxidative burst was evaluated by measuring both membrane PKC and PMA-stimulated superoxide release in *H. capsulatum*-containing Mϕ's. Our data confirm that a reduction in basal (and PMA-stimulated) Mϕ membrane PKC was associated with a significant inhibition of superoxide release. Further evidence that a moderate (30 to 50%) decrement in PKC activity correlated with a significant inhibition of oxidant release was provided by experiments using staurosporine, a known inhibitor of both PKC and oxidative burst activities (18, 33).

In previous work, we compared the effects of *H. capsulatum* with those of latex challenge on Mϕ oxidative metabolism, since latex has not been shown to either stimulate or inhibit the oxidative burst in murine macrophages (12, 30, 37). While neither particle was able to stimulate the oxidative burst (37), ingestion of *H. capsulatum* (but not latex) was capable of inhibiting the capacity of Mϕ's to respond to other stimuli. In corroboration of these findings, the results reported here show that unlike *H. capsulatum*, latex spheres failed to alter either basal or PMA-stimulated PKC activity (Table 2). Although the reasons underlying these differences in particle-mediated effects is not well understood, they likely represent stimulation of distinct metabolic pathways initiated by different mechanisms of phagocytosis. Nonspecific adsorption of the peptide to *H. capsulatum* yeast cells was effectively excluded by documenting that phosphorylation of KRTLRR peptide was unaffected by preincubation with *H. capsulatum*.

Although the morphologic and biochemical characteristics of the yeast form of *H. capsulatum* responsible for inhibition of murine Mϕ effector function are poorly understood, several observations are worthy of comment. The fact that active fungal metabolism was not required for Mϕ deactivation to occur suggests that a structural component of the yeast may be active in disease pathogenesis. Other studies have shown that *H. capsulatum* yeast cell wall and cell membrane antigens are capable of modulating cellular immune function (13). Moreover, since supernatants of RPMI 1640 incubated with *H. capsulatum* had no effect on PKC activity, it was unlikely that *H. capsulatum*-mediated PKC inhibition could be ascribed to a soluble component of the yeast particles which diffused into the culture medium.

Recently, cell wall-associated glycolipids have been hypothesized to play a role in the pathogenesis of infection with *Leishmania donovani* (23), *Mycobacterium tuberculosis* (8), and *Mycobacterium leprae* (31) via their ability to inhibit PKC activity and triggering of the oxidative burst. Sphingolipids and lysosphingolipids have also been shown to act as potent, reversible inhibitors of PKC in vitro (14, 15), in platelets (14, 15) and in neutrophils (35). Since novel antigenic phosphoinositol-containing sphingolipids have been

isolated from the pathogenic yeast phase but not the mycelial phase of *H. capsulatum* (3, 4), their potential role in the down-regulation of Mϕ oxidative metabolism may represent an intriguing area for future investigation.

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