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Complement-mediated Killing of *Leishmania martiniquensis* Promastigotes

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Authors' contributions

This work was carried out in collaboration among all authors. Authors WS designed the work and contributed to the drafting of the manuscript. Authors WS,AP conducted laboratory work and interpretation of the results, authors AP,WS Performed the data analysis. All authors have approved the final manuscript and agree to be held accountable for all aspects of the work.

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ABSTRACT

Background: Leishmaniasis caused by various species is now an emerging autochthonous disease. *L. martiniquensis was* described first from a human case in Martinique island and to date is frequently causing human infections in many countries. To eliminate the invading pathogen, complement activation of the innate system plays an effective role in the host's defense. This study aims to test the parasiticidal activity of the complement activation against *L. martiniquensis* promastigotes.
Methods: An in vitro examination of parasite mortality rate incubated in fresh normal human serum (NHS) was performed compared to heat-inactivated, and ion chelated sera.
Results: We observed a lethal effect on the parasite when exposed to fresh NHS. Our parasites when tested with heat-inactivated at 56 ^{0}C for 30 min and ethylene diamine tetra acetic acid (EDTA) chelated sera indicated complement dependent parasiticidal activity. Ethyleneglycol tetraacetic acid (EGTA) chelated, and EDTA+$MgCl_2$ treated sera demonstrated that *L. martiniquensis* promastigotes activated the complement via alternative pathway. This pathway was also revealed when the parasite was subjected to zymosan treated sera and that of the heat inactivated (50^{0}C for 15 min). Interestingly, positive mannose-binding lectin (MBL) of the parasite surfaces demonstrated by using anti- MBL antibody immunofluorescent test.

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Conclusion: The parasitical activity is antibody independent mediated via the alternative pathway. The involvement of MBL could not exclude. These preliminary data might provide a further study for the explanation of the immune evasion strategy of the *Leishmania* species.

Keywords: Complement; leishmania martiniquensis; promastigote; alternative pathway; mannose-binding lectin.

1. INTRODUCTION

Leishmaniasis is caused by parasites belonging to the genus *Leishmania* [1]. More than 20 species have been determined as human pathogens which endemic in many countries, including, the Middle East, Central and North America, Indian subcontinent, and Mediterranean basins [1]. The parasites have a digenetic life cycle, composed of the vertebrate host (amastigote stage) and an insect vector (promastigote stage) [2]. The disease is transmitted to vertebrate hosts by the bite (intradermal inoculation of the promastigote) of an infected female sandfly [3]. *L. martiniquensis*, firstly discovered on Martinique Island, is now the cause of autochthonous disease in the majority of cases of many countries [4,5]. Clinical presentation of this species infection includes diffuse cutaneous, visceral, and overlapping diffuse cutaneous and visceral forms [6,7]. The infected cases are mostly found in immuno-compromised patients [8]. Innate response principally plays an important first line against pathogens. A number of studies showed the lethal effect on various pathogens which depend upon the complement activation by forming the membrane attack complex (MAC) and promoting an inflammatory reaction [9,10]. There are three major pathways of the complement system: classical, alternative, and lectin. The binding of antibodies to the parasites will initiate the classical pathway, while the binding of mannose-binding lectin (MBL) can activate the lectin pathway. An alternative pathway can be directly activated by the pathogen's molecule [11]. *Leishmania* stays inside the host cell in form of amastigote might be the manner for escaping complement attack, as a survival strategy and propagation are not well understood [12]. The humoral factor of the complement system showed the lethal effect on many species includes *L. major*, *L. mexican*, *L. chagasi*, *L. infantum*, and others [13]. In vitro of those studies, more than 80% of the promastigotes exhibited lysis within minutes after being cultured with fresh normal human sera (NHS). The process was mediated by the alternative pathway of complement which is antibody independent [13].

The present study examines the lethal effect on *L. martiniquensis* promastigotes when incubated with fresh NHS, and various conditions of sera treatment. To destroy or inactivate all complement systems and have only the alternative pathway done, sera were heated at 56^0C, 30 min, and 50^0C, 15 min. Serum treatment with divalent chelate ions of EDTA (calcium and magnesium chelating) to inactivation both classical and alternative pathways were used. In addition, those treated with EGTA (calcium chelating) to destroy only the classical pathway have also been studied. More testing is undertaken in zymosan treated serum because this polysaccharide composed primarily of glucan and mannan residues can predominantly activate the complement system through the alternative complement pathway. The suspected MBL on our *L. martiniquensis* promastigotes surface was revealed by an anti-MBL immunofluorescence antibody test. Results obtained from this study may increase understanding of the host innate defense of the complement system, the parasite evasion strategies, and immuno-pathogenicity of the infection.

2. MATERIALS AND METHODS

2.1 Parasite, Sera, and Chelating Agents

L. martiniquensis promastigotes used were that from a leishmaniasis patient of southern Thailand (CU1 isolate) [14]. The parasites were cultured in Schneider's Insect Medium (Sigma-Aldrich, USA) at a pH of 6.7 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were maintained at 25 ± 2^0C and inspected for viability every 24 h under an inverted microscope (Olympus, Japan). In this experiment, stationary phase promastigotes were harvested by

centrifugation (1,100xg at 20^0C for 10 min) and washed twice in 1x phosphate buffer saline (PBS) (Sigma-Aldrich, USA).

Five sera donors (male, age 21-22 years old) from the 3rd-year-student of the faculty of Allied Health Sciences, Chulalongkorn University, who have no history of leishmaniasis, and are negative for Leishmanial antibody test were consent to use. The blood was taken, allowed to clot at 4^0C for 2 h, and subjected to a refrigerated centrifuge (400xg for 20 min). The decanted NHS was aliquoted and stored at -80^0C until further use. The experiments were carried out with sera that were frozen and thawed only once in order to minimize the inactivation of complement.

Different chelating agents, EDTA (Sigma-Aldrich, USA) and EGTA (Sigma-Aldrich, USA) were prepared as a stock solution of 12 mM. Mg++ ion in form of $MgCl_2$ (Sigma-Aldrich, USA) was provided at a concentration of 8 mM in order to add in the chelated sera for accession the requirement ion for alternative pathway process. Zymosan suspension (Sigma-Aldrich, USA) in PBS and boiled to sterilize was prepared. The concentration of the chelating agents and zymosan suspension used as described [15] were tested for viability prior to treatment because high concentrations of the agents may directly affect the parasite.

Fresh NHS were mixed with a chelating agent and zymosan (ratio 1:1), and incubated at 37 C for 30 min. The zymosan was removed from the treated sera by centrifugation at 2,000 rpm for 30 min at 4 C. The pre-treated sera were all then centrifuged at 3,200 rpm for 20 min prior to use.

2.2 *In vitro* Test for Parasiticidal Activity

The lethal effect on *L. martiniquensis* promastigotes (10^6 parasites/ml) was determined by reacting the with various conditions/ treatment of the sera and incubated at 25±2^0C in a plastic tissue culture plate (Corning, USA) at a ratio of 1:1 (volume by volume) for 15, 30, and 60 min.

Analysis of *L. martiniquensis* promastigotes killing was performed by gently mixing of 20 µl reacted promastigotes with 20 µl of trypan blue solution stock (Sigma-Aldrich, USA), and kept at room temperature for 5 min. Then, 10 µl of the cells were counted on a hemocytometer for the number of viable (unstained with trypan blue) and dead (stained with trypan blue) cells. The mortality rate in percentage was calculated.

2.3 Immunofluorescence Analysis

An immunofluorescence test was performed to detect MBL on the parasite surface. Our cultured *L. martiniquensis promastigotes* with fresh NHS and 56^0C 30 min heated sera were smeared onto microscope slides, air-dried, and fixed in 4% paraformaldehyde (Sigma-Aldrich, USA). The cells were permeabilized with 1xPBS containing 1% BSA and 0.1% Triton for 10 min. Slides were washed and reacted with rabbit monoclonal anti-MBL antibody (Abcam, Cambridge, UK) in PBS and tris-buffer containing 10% normal horse serum at concentration 1:500 for 2 h at room temperature. The slides were then incubated with a secondary antibody (goat anti-rabbit IgG fluorescence (Alexa fluor®488) **(**Abcam, Cambridge, UK**)** at a concentration of 1:1000 and left for 30 min before examination under a fluorescence microscope at 494 nm (Olympus, Japan).

3. RESULTS

Incubation of *L. martiniquensis* promastigotes with fresh NHS exhibited the maximum killing of the parasite 74.0, 94.6 and 90.6%) at 15, 30, and 60 mins, respectively (Fig. 1). This lethal effect of sera became undetectable by diluting the serum beyond 1:16. (Table 1). The effects were all significantly abrogated (5.6-5.8%) in heated serum (at 56^0C for 30 min) which indicated that the activity was heat-labile, suggesting the involvement of complement in the process. Based on the variance, among the different incubation periods (15, 30, and 60 mins) they are not significantly different which indicated that the complement action can complete within a short time period (15 mins). Factor B of the alternative pathway appeared to be more labile than other complement components. The mortality rate of our parasite in 50^0C for 15 min heated sera showed a significant decrease from 90.6% to 27.8% (at 60 mins) which was associated with the alternative pathway activation (Fig. 1).

To obtain further evidence for supporting the above suggestion, our fresh NHS was then

treated with a chelating agent. All divalent cations including Ca++ and Mg++ would be chelated in EDTA treated sera, thus all the three pathways of complement activation are blocked. When EGTA was added to the serum, only Ca++ would be chelated and the alternative pathway which requires Mg++ could still be activated. In the experiment, alternative pathway activation was optimized by adding an extraneous source of Mg++ to the EDTA treated serum for essential ions providing.

In our study concentration of 6 mM of neither the EDTA nor EGTA had any effect when incubated with our promastigote. Four mM $MgCl_2$ added to the parasite culture medium also did not affect our parasite survival. The fresh NHS was treated with EGTA at a final concentration of 6 mM to selectively chelate Ca^{2+}, thus stopping both the classical and MBL pathways activation, but not the alternative pathway which requires Mg ions. This did not subsequently decrease the parasiticidal activity, since the parasite was mostly killed in EGTA treated serum (78.2% mortality rate, (range 65-82% Table 1). The results indicate that the parasiticidal activity was inhibited in EDTA-treated sera whereas most promastigotes were killed in EGTA treated sera, suggesting the involvement of alternative pathways in this process. The addition of $MgCl_2$ (4 mM) in EDTA chelated sera showed that *L. martiniquensis* promastigotes can activate the alternative complement pathway. (86.4% mortality rate, (range 78-95%) Table 1).

However, we confirmed the pathway by pretreatment of fresh NHS with zymosan. Zymosan is known to activate or consume complement predominantly via the alternative pathway. Incubating the promastigotes with this agent (40 mg/ml) exhibited no effect on the parasite. In the experiment, the alternative depleted serum reduced the percentage of the mortality (from 90.6 ± 2.3 to 47.8 ± 5.6%, Table1).

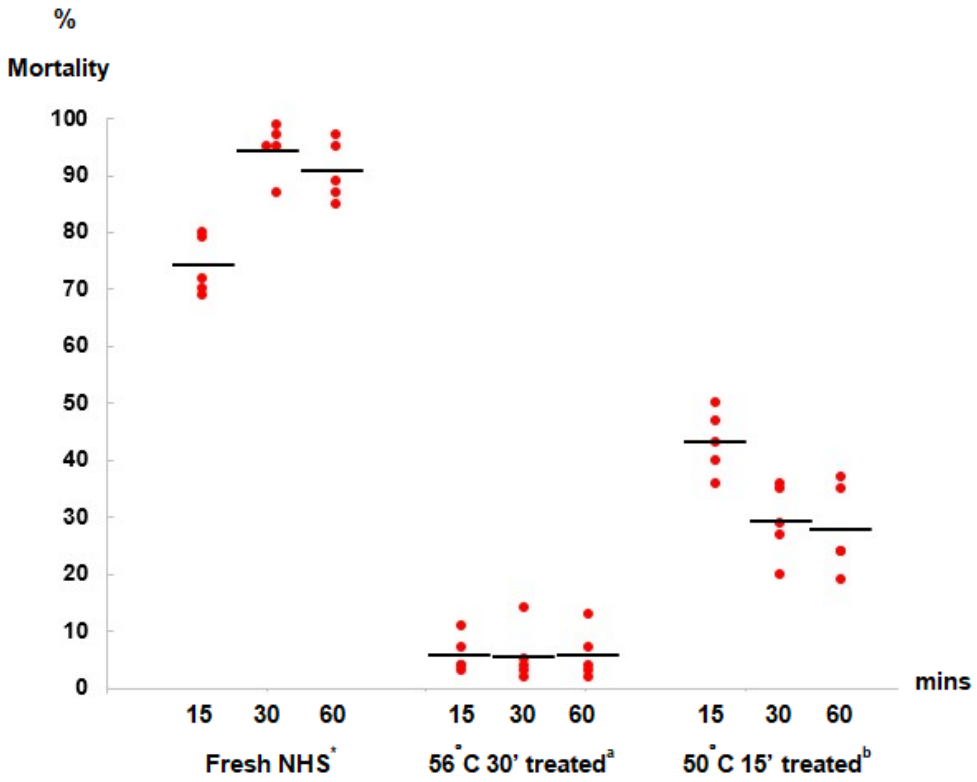

Fig. 1. Percentage of parasite mortality rate incubated with undiluted fresh normal human sera, heat-treated sera (56 ^{0}C 30 min) and (50^{0}C 15 min) against *L. martiniquensis* promastigotes at the different time intervals of 15, 30, and 60 mins

**, Fresh normal human serum is the untreated sera of control used in the system.*

[a b], Values within a row with different treatment are significantly different ($P < 0.01$; T-test)

Table 1. Effect of chelating agents on parasiticidal activity of fresh normal human sera against *L. martiniquensis* promastigotes

Treatment of Fresh normal human sera	% Mortality at 60 min incubation	
	range	mean ± SE
Untreated**	85-97	96.6 ± 2.3
1:4 diluted	70-80	73.2 ± 1.9
1:8 diluted	4-36	16.2 ± 5.4
1:16 diluted	4-15	8.8 ± 2.0
1:32 diluted	2-10	4.8 ± 1.4
6 mM EDTA	28-40	32.6 ± 2.2[c]
6 mM EGTA	65-82	78.2 ± 3.6
6 mM EDTA + 4 mM $MgCl_2$	78-95	86.4 ± 3.1
40 mg/ml Zymosan	33-62	47.8 ± 5.6[c]

** *Untreated is the control used in the system*

[c] *Values within a column with different treatment are significantly different* (*P <0.01; T- test*)

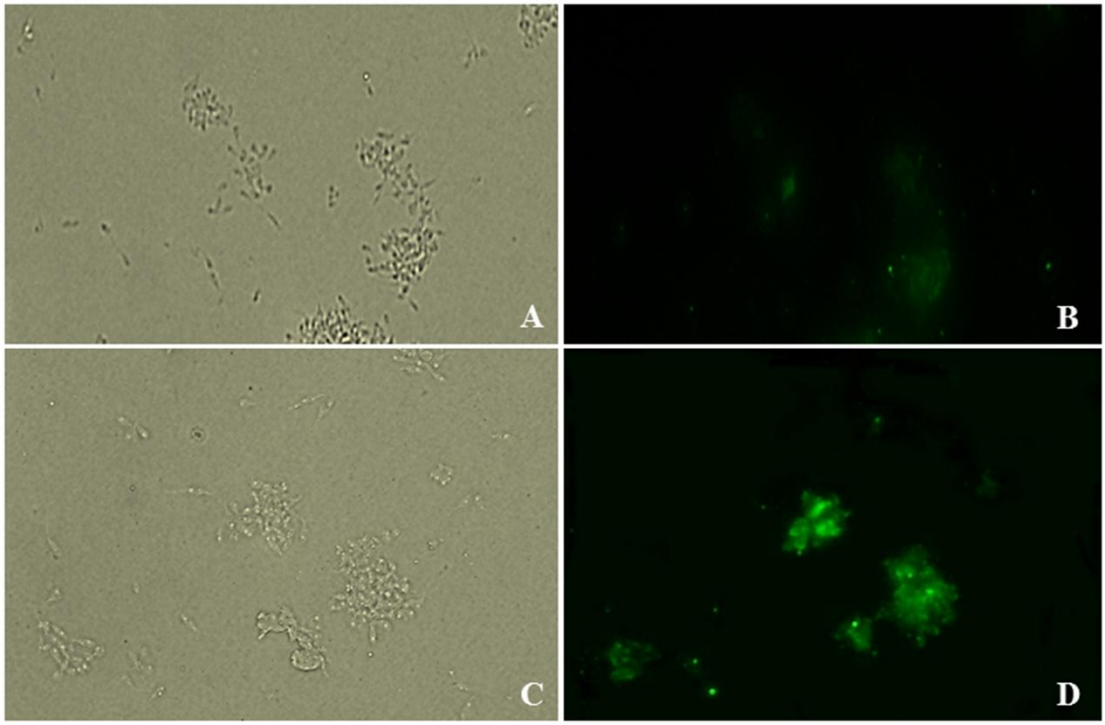

Fig. 2. Bright-field microscopic images of immunofluorescence staining using an anti-MBL antibody (magnification 40x) for *L. martiniquensis* promastigotes incubated in heated NHS at 56^{0}C for 30 min (A, B) and fresh NHS (C, D)

We observed the presence of MBL on our promastigotes surface incubated with fresh NHS by immunofluorescence test using a monoclonal anti-MBL antibody. Surface MBL-negative binding was detected when the parasite was cultured in heat-activated NHS (at 56 ^{0}C for 30 min) (Fig. 2 A, B). In contrast, the anti-MBL antibody showed positive staining around the surface of the cell which was exposed to fresh NHS (Fig. 2 C, D).

4. DISCUSSION

In this study, the data presented suggest that *L. martiniquensis* promastigotes can be readily killed when exposed to fresh NHS. The lethal effect of the serum is complement-dependent, since the activity was inhibited by heat inactivation (56 ^{0}C for 30 min) and by EDTA treatment. It is generally accepted that EDTA would chelate all divalent cations including Ca^{2+}

and Mg^{2+}, thus all pathways of complement activation are blocked [11]. Only Ca^{2+} ions would be chelated by EGTA, thus the alternative pathways requiring Mg^{2+} ions would still be activated [11]. Parasite mortality is diminished by pre-treatment of the serum with either zymosan or anti-human factor B which indicated a proceeding activation of the alternative pathway. The selective factor B can be destroyed by heating the serum at 50^0C for 15 min. The activity can be restored by the addition of purified human factor B (data not shown). The involvement of the alternative pathway in these parasiticidal processes is strongly suggested.

Investigation for MBL of the surface demonstrated positive using the immunofluorescence test. MBL is a well-known complement activator and an efficient opsonin. Binding and promoting lysis of live promastigotes of *L. braziliensis* by MBL were reported [16]. Results of our study show that the biological circumstance of the parasiticidal action in normal serum has not been unexpected. The clinical outcome and severity reported of this disease greatly depend upon the species of *Leishmania* and the host immune situation [17,18]. Our results are the first to demonstrate the complement-mediated killing of the species *L. martiniquensis* promastigotes in vitro study. The results were compared to the treating/ chelating NHS which revealed that the parasiticidal activity depends upon the alternative pathway; an antibody-independent process. This parasite killing process requires Mg^{2+} but not Ca^{2+}since the process was inhibited by zymosan treated sera which suggested an activation proceeds via an alternative pathway. However, the involvement of the MBL pathway might not exclude.

There is a question of which complement pathways are critical in resistance or clearance of *Leishmania* in the infected vertebrate host. An alternative complement pathway is seen as the major essential pathway that can eliminate various species of the *Leishmania* pathogen [13]. *L. donovani* (the Sudan strain), *L. major* (strains 1-S and 252), *L. braziliensis guyanensis, L.* dependent on human serum and would activate the alternative complement pathway [13,19]. Complement mediated clearance of *L. tropica* and *L. enriettii* promastigotes by NHS was shown to be antibodies or C4 independent, but mediated by the alternative pathway following the formation of C5b-9 MAC [20,21]. The IgM antibody in *Leishmania* activated the complement by the classical pathway leading to promastigote agglutination and death as in *L. donovani* was reported [22-24]. *Leishmania* promastigotes that are covered by a surface protein such as the gp63 metalloproteinase, proteophosphoglycans (PPGs), abundant lipophosphoglycan (LPG), and the MBL have been shown [18,25].

Serum MBL binding of *L. braziliensis* promastigotes also suggested the possible contribution of the lectin pathway in the pathogen killing [16]. MBL is a calcium-dependent plasma lectin that can bind to various microorganisms. It has shown a strong association with the severity of microbial infections [12,26]. The binding of MBL by the pathogen could subsequently spur complement activation by increasing uptake of PMN (opsonophagocytosis) [11]. This process needs further research and observation for the species *L. martiniquensis*. It may explain the increased risk of these microorganism infections in MBL-deficient individuals [27]. Interestingly, there are reports demonstrating that the level of MBL is directly associated with pathogenesis and the likelihood of developing visceral leishmaniasis upon *L. chagasi* infection [28,29].

Nevertheless, to promote parasite clearance many mechanisms and/or pathways have been elucidated during the recent decades. Interaction between parasites and macrophages, different species are recognized by a variety of host cell receptors not only complement receptors (CRs), and mannose receptors but also Fc receptors (FcRs), fibronectin, and others [30]. These may impact the fate of intracellular parasite conditions as well as the course of infection. In addition, in this protozoa, infection autophagy has been currently shown to adaptively interact with the host cells in controlling leishmaniasis [31].

5. CONCLUSIONS

Data presented in this experiment suggested that *L. martiniquensis* promastigotes are readily killed following exposure to fresh NHS. To the best of our knowledge, this is the first study examining alternative pathways and the presenting of surface MBL of this species of *L. martiniquensis* promastigotes. Future studies should focus attention on the important role of the complement

system and interaction of the host cell (macrophage) receptor and the parasite *L. martiniquensis* to help deepen our understanding of parasite evasion strategies, host immunity, and pathogenicity.

CONSENT AND ETHICAL APPROVAL

The experimental procedures used in this study were approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (COA No. 768/2012). Sera donors were explained for the purpose with written informed consent.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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