Effect of *Leishmania major* on human polymorphonuclear leucocyte function *in vitro*

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Summary. The effect of various antigens of *Leishmania major* promastigotes on the function of human polymorphonuclear leucocytes (PMNLs) was examined. Different concentrations of *L. major* antigens were incubated with isolated PMNLs for various periods and the respiratory burst was assessed by Luminol-dependent chemiluminescence. All the *Leishmania* antigens employed inhibited the PMNL respiratory burst by 35–64%. PMNL viability was not affected either by the concentrations or type of the parasite antigen. Oxygen free radical scavengers enhanced the action of the antigens on the PMNL respiratory burst.

Introduction

Cutaneous leishmaniasis caused by *Leishmania major* is endemic in Saudi Arabia.^{1–3} Recently, we have shown that *Leishmania* promastigotes inhibit human whole blood phagocytic activity *in vitro*.⁴ The role of polymorphonuclear leucocytes (PMNLs) in host defence against a wide range of micro-organisms is well established. The microbicidal activity of PMNLs depends partly upon the generation of large quantities of oxygen free radicals (OFRs) such as superoxide anion, hydrogen peroxide, hydroxyl radicals and singlet oxygen.^{5–6} As the role of PMNLs in the pathogenicity of leishmaniasis is ill defined, the present study was designed to examine the effect of *Leishmania* and its antigens on the OFR-mediated microbicidal activity of PMNLs. Various forms of *Leishmania* antigens (viable, non-viable, lysate and denatured promastigotes) were incubated with human PMNLs and the effect on the oxygen-dependent respiratory burst was assessed with chemiluminescence (CL). Phorbol myristate acetate (soluble agent) and opsonised (particulate antigen) yeast were used to produce a high CL response. Combined exposure of PMNL to *Leishmania* and OFR scavengers such as superoxide dismutase (SOD) dimethyl sulphoxide (DMSO), sodium azide (SA) and allopurinol was also studied.

Materials and methods

Parasite and antigen preparation

L. major strain HOM/SA/1983/Sayed was isolated and

maintained as described by Mahmoud *et al.*³ Promastigotes were collected and centrifuged, the pellet was washed twice with phosphate-buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 12 mM Na₂HPO₄, 0.9 mM CaCl₂ in distilled water), and the promastigotes were counted as described previously.⁴ The supernate was filtered through a 22- μ m membrane filter (Millipore) to obtain promastigote-free lysate. Non-viable promastigotes were prepared by freezing and thawing several times. Another preparation of promastigotes was heated for 1 h at 90°C to produce denatured antigen. The protein concentration of the various antigens was determined by the method of Lowry *et al.*⁷

Polymorphonuclear leucocytes

Blood was collected by venepuncture from apparently healthy donors in sterile containers with heparin (10 IU/ml; Fisher Scientific Co., NJ, USA). Ten ml of heparinised blood was mixed with 10 ml of Dextran T 500 6% w/v in saline 0.9% w/v in a 25-ml tube and kept at room temperature for 30 min before the leucocyte-rich plasma layer was removed. Erythrocytes were lysed by adding an equal volume of distilled water and the cells were then suspended in PBS. About 5–6 ml of the leucocyte rich suspension was layered over 3 ml of Nycodenz Solution (Nyegaard and Co. AS, Torshov, Norway) in a 12-ml tube and centrifuged at 1800 rpm for 15 min to harvest PMNLs. The PMNLs were then resuspended in PBS, counted and adjusted to the desired concentrations. PMNL viability was assessed by trypan blue (0.1%) exclusion and only PMNL suspensions with a viability > 90% were used.

Measurement of Luminol-enhanced chemiluminescence

An LKB-Wallac 1251 luminometer with a constant temperature (37°C) controller (Wallac Oy, 20101 Turku

10, Finland) connected to an Apple IIe computer, as described by Tono-Oka *et al.*,⁸ was used. Light emission was recorded in mV. Two methods were used to quantify CL emission—the peak height in mV and the total light emission during the reaction period (area under the curve). The readings were recorded at 2-min intervals for 30 min. The reaction mixture consisted of 100 μ l of PMNL suspension and 900 μ l medium containing 10^{-4} M Luminol and 10^{-4} M phorbol myristate acetate (PMA) (Sigma).

Oxygen free radical scavengers

Superoxide dismutase (SOD) (Sigma) 0.02 mg/ml and 6.6 mM dimethyl sulphoxide (DMSO; Fluka AG, Chemische Fabrik, Buchs, Switzerland), sodium azide (B.D.H. Chemicals Ltd, Poole) 0.02 mg/ml and 2 mM allpurinol (Al Hikma, Jordan) were used as oxygen scavengers.

PMNL viability

The effect of *Leishmania* antigens on PMNL viability was determined by trypan blue exclusion examined after incubation for 0, 30, 60 and 120 min at 37°C with the antigen preparations.

Yeast opsonisation

Bakers yeast was washed twice and resuspended in PBS and the concentration was adjusted to 2×10^8 particles/ml and killed by boiling for 30 min. The yeast was opsonised by incubating 100 μ l of autologous human serum with 900 μ l of yeast suspension at 37°C for 30 min, washing with PBS, and was resuspended in PBS to a final concentration of 2×10^5 particles/ml.

Statistical analysis

The results of CL were computed by an LKB computer program that gave the CL maximum peak response in mV and the integral area under the curve of each graph. Differences between experiments with *Leishmania* preparations and controls were compared with the two-tailed Student's *t*-test and considered significant when $p < 0.05$.

Results

All the *Leishmania* promastigote antigens (viable, non-viable, denatured and lysate) inhibited PMNL CL. Viable and denatured promastigotes showed a significant CL inhibition of 64% and 68%, respectively ($p < 0.001$; table I and fig. 1). The non-viable (frozen and thawed) and lysate antigens caused less inhibition. The background activity of unstimulated PMNL CL was always < 0.8 mV and was not affected by addition of *Leishmania* antigen preparations. Also, there was no effect of *Leishmania*

Table I. Effect of various forms of *L. major* antigens on the chemiluminescence of PMNL stimulated with PMA

Antigen preparation	CL Measurement (mV) with		Percentage inhibition	p value
	PBS	<i>L. major</i>		
AP	1215.6 $\pm$ 31.1	442.1 $\pm$ 24.1	64	0.001
NP	1038.0 $\pm$ 36.2	664.9 $\pm$ 22.1	36	0.01
DP	1460.0 $\pm$ 77.2	460.0 $\pm$ 79.9	68	0.001
LP	1337.9 $\pm$ 97.2	870.0 $\pm$ 37.8	35	0.01

AP, Viable promastigotes; NP, non-viable promastigotes; DP denatured promastigotes; LP, promastigote lysate.

All values were expressed as the mean of six observations $\pm$ SE. Number of promastigotes (viable or nonviable) was 1×10^5 /test (equivalent to 20 mg of protein/test). PBS was used as a control. PMNLs count 5×10^5 .

Incubation time 30 min at 37°C.

Denatured promastigotes were heated at 90°C for 1 h.

Percentage of CL inhibition was calculated as follows:

$$\text{Percentage CL inhibition} = \frac{(\text{Control} - \text{Leishmania promastigotes})}{\text{Control}} \times 100$$

antigen on PMNL-free media. The antigens, in the concentration used, did not induce CL when added to unstimulated PMNLs.

A dose-dependent reduction in the PMNL CL response was observed with denatured *Leishmania* antigen (fig. 2). The maximum peak PMNL CL response was significantly reduced when PBS was replaced with different doses of denatured *Leishmania* antigen. When compared with non-viable promastigotes, equivalent concentrations of the

Fig. 1. Percentage inhibition of PMA-induced CL of PMNLs (5×10^4) by denatured (▲—▲) and non-viable (●—●) promastigotes.

denatured antigen preparation caused greater inhibition at all the concentrations tested (table II and fig. 3).

The maximum light output with Luminol-enhanced CL from PMNLs induced by opsonised yeast was recorded when PBS was used alone. Addition of *Leishmania* antigens caused a significant inhibition of CL. The inhibitory effect of *Leishmania* antigens was dose-dependent (table II).

OFR scavengers—SOD, DMSO, sodium azide and allopurinol—were incubated with PMNLs in the presence or absence of *Leishmania* antigens. Simultaneous incubation of non-viable promastigotes and SOD significantly ($p < 0.05$) inhibited PMNL CL compared with either non-viable promastigotes, or OFR scavengers alone (table III).

Incubation of *Leishmania* antigen with PMNLs for different lengths of time did not have any significant effect on PMNL viability. The effect of *Leishmania* non-viable promastigotes at the highest dose used (1×10^7) was reversible by washing the antigen from the incubation medium (table IV) with PBS.

Discussion

Earlier studies in this laboratory have shown that *Leishmania major* significantly inhibited phagocytosis in laboratory animals. The intravascular clearance of carbon colloid or 99 MTC sulphur colloid was markedly depressed and correlated with parasite proliferation in the liver and spleen. Furthermore, serum lysozyme, an index of macrophage activity, was reduced in infected mice.^{4,9}

The results of the present study show that all types of *Leishmania* antigens (viable, non-viable, lysate and denatured) inhibit PMNL CL. More-

Table II. The effect of *L. major* antigens on CL of PMNL (1×10^5) stimulated with PMA (100 $\mu\text{g}/\text{ml}$) or opsonised yeast cells ($2 \times 10^5/\text{ml}$) after incubation for 30 min

Antigen preparation (promastigote concentration)	CL peak response (mV) with	
	PMA	Opsonised yeast
Control	292.4 $\pm$ 12.6	484.4 $\pm$ 16.7
Non-viable promastigotes		
$1 \times 10^4/\text{ml}$	240.9 $\pm$ 7.3	324.4 $\pm$ 14.3
$1 \times 10^5/\text{ml}$	80.6 $\pm$ 5.1	316.3 $\pm$ 10.7
$1 \times 10^7/\text{ml}$	58.03 $\pm$ 2.7	290.7 $\pm$ 9.5
Denatured promastigotes		
1×10^4	92.8 $\pm$ 8.4	300.9 $\pm$ 6.7
1×10^5	44.9 $\pm$ 5.3	296.9 $\pm$ 11.3
1×10^7	9.8 $\pm$ 14.7	271.5 $\pm$ 8.0

Fig. 2. Inhibition of PMA-induced CL of PMNLs (5×10^4) by various concentrations of denatured promastigotes: 1×10^3 (S—S), 1×10^5 (O—O), 1×10^6 (X—X), 1×10^7 (Z—Z) and control (V—V).

over, this inhibitory effect is dose-dependent and is directly proportional to antigen concentration expressed either as the number of promastigotes or the protein concentration. The inhibition of PMNL respiratory burst by denatured *Leishmania* antigen was shown to be reversible after the antigen was removed from the incubation medium. Promastigotes denatured by heat exhibited a greater inhibitory effect than either the viable or non-viable promastigotes. It is suggested that the heating enhanced the release of factor(s) that induced the inhibitory effect on the PMNLs. When the antigen was boiled for 1 h, it was still potent enough to exert a marked inhibitory effect on PMNLs, indicating that the inhibitory factor(s) of the parasite are heat stable.

As well as macrophages, PMNLs are considered to be important in generating OFRs as a part of their microbicidal activities.^{5,6} Co-culturing of PMNLs with any one of the OFR scavengers (SOD, DMSO, azide, or allopurinol) plus non-viable promastigotes as used in the present study, significantly enhanced the inhibitory effect on PMNL CL in comparison with either OFR scavengers or the

Table III. Effect of OFR scavengers on inhibition of PMNL CL by *Leishmania* non-viable promastigotes

OFR scavenger	Control (PBS) preparation		Preparations with non-viable promastigotes†	
	Mean (SE) CL peak response (mV)	CL inhibition (%)	Mean (SE) CL peak response (mV)	CL inhibition (%)
PBS (control)	1393 (141)	...	646.6 (18.2)	54
SOD (0.02 mg/ml)	488.42 (180)	62	323.47 (97.8)‡	77
DMSO (6.6 mM)	948.3 (130)	32	521 (12.7)§	63
Sodium azide (0.02 mg/ml)	87.8 (31)	94	63.2 (17.9)	95
Allopurinol (2 mM)	977.7 (40)	30	691.6 (1.2)§	50

* Percentage CL inhibition = $\frac{\text{Control (PBS) CL} - \text{Leishmania promastigote CL}}{\text{control CL}} \times 100$.

† Non-viable promastigote protein 20 mg/test; PMNL count 5×10^5 /ml; incubation time, 30 min; temperature, 37°C.

‡ $p < 0.01$.

§ $p < 0.001$.

parasite antigen alone. This potentiation between the antigen and OFRs on the inhibition of PMNL CL responses suggests that either *Leishmania* antigen does not interfere with OFR scavengers or that a certain component of the parasitic antigen may possess a factor that acts in a fashion similar to OFR scavengers. This phenomenon was observed with malaria in which the survival of the

parasite intracellularly is partly attributed to the indigenous OFR scavenger content of the plasmodia.^{10,11} Furthermore, the toxic effect of the *Leishmania* parasite on PMNLs could be a direct action on the cell membrane or at the level of the intracellular organelles. Generation of OFRs by PMNLs stimulated with PMA is reported to be through specific receptors, e.g., phospholipid-dependent kinase, protein kinase C.^{12,13} In the light of this, it could be stated that the inhibitory action of *L. major* antigens on the PMA-induced respiratory burst observed in this study is probably due to interference with PMA binding to protein kinase receptors. On the other hand, the source of oxygen reactive metabolites produced by PMNLs following stimulation with opsonised particles (yeast or Zymosan) is believed to be due to the activation of receptors of the alternative complement pathways.¹⁴

In conclusion, we suggest that PMNLs may play some role in the pathogenicity of *Leishmania* parasites.

Table IV. The effect on the Luminol-dependent CL PMNLs stimulated with PMA of washing *L. major* non-viable promastigote antigen from the incubation medium with PBS

CL reaction mixture	Mean (SE) CL peak response (mV)*
Control (PMNL + PBS), not washed	1120.1 (2.2)
PMNL + <i>Leishmania</i> antigen (not washed)	537.2 (10.7)
Control (PMNL + PBS), washed	1235.2 (7.4)
PMNL + <i>Leishmania</i> antigen (washed)	1197.3 (20.0)

Non-viable promastigotes and PMNL counts were 1×10^5 /test; incubation time 30 min.

* Data are expressed as mean (SE) of four observations.

Fig. 3. Inhibition of PMA-induced CL of PMNLs (5×10^4) by various concentrations of non-viable promastigotes: 1×10^3 (S—S), 1×10^5 (O—O), 1×10^6 (X—X), 1×10^7 (Z—Z) and control (V—V).

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