Abstract

Inflammation is a key process of many diseases. This may occur as a result of gram-negative bacteria sepsis including Escherichia coli infection that gives rise to excessive production of inflammatory mediators and causes severe tissue injuries. We have reported earlier that the lipid of attenuated Leishmania donovani suppresses the inflammatory responses in arthritis patients. Using heat killed E. coli and LPS stimulated macrophages, we have now investigated the effect of leishmanial total lipid (LTL) isolated from Leishmania donovani (MHO/IN/1978/UR6) for amelioration of the inflammatory mediators and transcriptional factor. To evaluate the in vivo effect, E. coli induced murine sepsis model was used focusing on the changes in different inflammatory mediator levels and pathophysiology. Significant improvement was observed in the mortality of endotoxemic mice. The carrageeban and formalin-induced paw edema thicknesses were found to be reduced significantly with the treatment of LTL in time dependent manner. These findings indicate that LTL may prove to be a potential anti-inflammatory agent and provide protection against gram-negative bacterial toxin.

Key Words: Leishmania, Lipid, Bacteria, Toxin, Macrophage

Introduction

Endotoxins are constituents of the outer membrane of Gram-negative bacteria. Bacterial endotoxin is a major candidate for the inflammatory reactions. Endotoxins are agents
of pathogenicity of Gram-negative bacteria, implicated in the development of Gram-negative shock. Endotoxins are lipopolysaccharides (LPS). In many cases of Gram-negative bacteria, LPS are found to consist of three covalently-linked regions, the lipid A, the core oligosaccharide and the O-specific polysaccharide. All three parts of the LPS molecule are immunogenic, eliciting the formation of antibodies interacting specifically with distinct epitopes in the respective region. There are a large spectrum of toxic biological activities that are found to be expressed by purified LPS or isolated free lipid A. These activities are not direct effects of the LPS molecule but are induced indirectly by endogenous mediators that are produced following interaction of endotoxin with LPS-sensitive cells. Macrophages are cells mediating the toxic activities of LPS and tumour necrosis factor alpha (TNF-α) is a primary mediator of the lethal action of endotoxin.

It has been well-established that endotoxins are mitogenic for B-cells and function as polyclonal B-cell activators. They also mediate cell activation of macrophages and activate the complement cascade. They act as a physiological stimulus for the synthesis of pro-inflammatory cytokines such as TNFα, IL1, IL6, IL8 and non-protein mediators, which in turn, are responsible for most pathophysiological consequences of a bacterial infection¹. Endotoxic shock is a complex phenomenon resulting from systemic release of inflammatory mediators. Endotoxin interacts with inflammatory cells, platelets, and vascular endothelium. Cytokines, such as tumor necrosis factor and interleukins, and lipid mediators (platelet activating factor, thromboxane, prostacyclin, leukotrienes) are released. These primary mediators act synergistically to cause many of the harmful effects associated with endotoxemia. Multiple secondary mediators are released in response to the primary mediators, compounding the damage. The end result is the species-specific clinical syndrome recognized as endotoxemia². Treatment of endotoxemia is difficult because of the numerous mediators involved in the body's response to endotoxin. There are three possible approaches in treating endotoxemia. The interaction of endotoxin with target cells can be blocked by inducing tolerance, decreasing plasma endotoxin concentrations, or interfering with endotoxin binding. Once endotoxin has interacted with target cells, endogenous mediators can be blocked with a huge variety of drugs. The effects of corticosteroids, cyclooxygenase blockers, leukotriene blockers, platelet activating factor blockers, tumor necrosis factor blockers, oxygen radical scavengers, opiate antagonists, antihistamines and calcium channel blockers are detailed. Supportive care of the endotoxemic patient continues to be a critical aspect of treatment³. There are reports that immune response of host cells is significantly modified by leishmanial lipids. They impair
inflammatory cytokines and NO production by stimulated macrophages. L. donovani-induced immunosuppression and alteration of host cell signaling is mediated by ceramide, a pleiotropic second messenger playing an important role in regulation of several kinases, including mitogen-activated protein kinase and phosphatases. Recently, we have shown that a sphingolipid-rich lipid fraction isolated from an attenuated Leishmania donovani promastigote (MHO/IN/1978/UR6) induces apoptosis when administered exogenously in mouse melanoma (B16F10) and human melanoma (A375) cells. In 2008, our findings shown that leishmanial lipid has strong anti-inflammatory and apoptosis-inducing effects on SFMCs from patients with RA and that apoptosis occurs via the mitochondrial pathway. Leishmanial lipid is a strong immunosuppressor of host cells. Inhibition of the inflammatory responses of synovial cells through induction of apoptosis is one of the main targets of therapeutic intervention in rheumatoid arthritis (RA). This study was undertaken to examine the anti-inflammatory and apoptosis-inducing effects of leishmanial lipid on adherent synovial fluid mononuclear cells (SFMCs) in patients with RA. Leishmanial lipid inhibited the release of tumor necrosis factor, interleukin-1, and NO in the culture, decreased their cytosolic protein levels, and decreased NF-κB p65 levels in SFMCs, in a dose-dependent manner. It had the reverse effect on interleukin-10 levels. Leishmanial lipid-induced apoptosis involved the activation of caspase 3, caspase 9, and Bax, the release of cytochrome c, the alteration of mitochondrial membrane potential, and the down-regulation of Bcl-2.

These results suggest that leishmanial lipid has strong anti-inflammatory and apoptosis-inducing effects on SFMCs from patients with RA, and that apoptosis occurs via the mitochondrial pathway. Septic shock remains one of the leading causes of death in hospital patients. Barely more than 50% of the patients with severe sepsis survive their hospital admission. The incidence of sepsis is increasing year by year. Septic shock is a serious medical condition caused by decreased tissue perfusion and oxygen delivery as a result of infection and sepsis, though the microbe may be systemic or localized to a particular site. It can cause multiple organ dysfunction syndrome (formerly known as multiple organ failure) and death. Its most common victims are children, immunocompromised individuals, and the elderly, as their immune systems cannot deal with the infection as effectively as those of healthy adults. The mortality rate from septic shock is approximately 50 percent.

According to the US CDC, septic shock is the 13th leading cause of death in the United States, and the #1 cause of deaths in intensive care units. There has been an increase in the rate of septic shock deaths in recent decades, which is attributed to an increase in invasive medical devices and procedures, increases in immunocompromised patients, and an overall increase in
elderly patients. Tertiary care centers (such as hospice care facilities) have 2-4 times the rate of bacteremia than primary care centers, 75% of which are nosocomial infections. The mortality rate from sepsis is approximately 40% in adults, and 25% in children\textsuperscript{12}. Treatment primarily consists of a) Oxygen administration and airway support, b) Volume resuscitation, c) Early antibiotic administration, d) Rapid source identification and control, e) Support of major organ dysfunction.

All medications may cause side effects. Antibiotics can cause allergic reactions, stomach upset, and other side effects. Other effects depend on the medicines used. A ventilator can rarely cause a new infection or lung damage. Surgery can be complicated by bleeding, infection, an allergic reaction to the anaesthetic or even death. Vasopressin and terlipressin are thus last resort therapies in septic shock states that are refractory to fluid expansion and catecholamines. However, current data in humans remain modest, and properly powered, randomized controlled trials with survival as the primary endpoint are required before these drugs can be recommended for more widespread use. So the fact which can be accentuated unhesitatingly that there is a genuine demand for an effective preparation with therapeutic potentiality in treating sepsis. As mentioned earlier, the outcomes of our previous investigations has prompted us to enthusiastically speculate that the findings of our present study as proposed will definitely be an effective march ahead to satisfy that serious ongoing demand.

Our present study is designed to explore the anti-inflammatory role of leishmanial lipid in LPS stimulated macrophage responses. Monocytes-macrophages are the first line of defence against infections and varieties of immunomodulatory diseases.

Lipids from Leishmania spp. are known to have immunosuppressive role on immune systems. Monocyte-macrophages are the host for Leishmania where it grows and multiply. Leishmanial lipid impairs the signalling system and the immune responses of macrophages. So the lipids from leishmania are expected to be immunosuppressive against inflammation.

**Materials and Methods**

**Isolation of Lipid from Leishmania donovani Promastigote Cells:**

Leishmania strain UR6 (MHO/IN/1978/UR6) will be grown in Ray’s modified medium and subcultured at 72-hour intervals. Cells will be collected and washed in phosphate buffered saline (PBS). For extraction of lipids from *Leishmania* promastigote cells standard methods like Bligh and Dyer method will be followed. The total lipid thus obtained from the lower organic phase after evaporation to dryness at -40°C will be stored at 4°C in vacuum desiccators.
until used. Since lipids are usually not soluble in aqueous medium, leishmanial lipid will then be dissolved in solvents like DMSO, the required amount of leishmanial lipid will be emulsified by sonication in RPMI 1640 containing 10% FBS. The emulsion and will then be used for subsequent experiments\textsuperscript{13}.

**Thin-Layer Chromatography (TLC):**

The leishmanial lipid was dissolved in 2:1 (v/v) chloroform-methanol. TLC was performed in chloroform-methanol-water (90:10:1, v/v) and lipid spots were visualized using iodine spray\textsuperscript{14}.

**Isolation of mouse peritoneal macrophages:**

Macrophages will be obtained from peritoneal cavities of BALB/c mice by washing with 5 ml of Hanks balanced salt solution (HBSS). The pooled cells will be washed and resuspended in HBSS. Approximately 2.5 x 10\textsuperscript{6} cells will be cultivated in sterile cell culture plate in HBSS supplemented with 10% calf serum. After 2 to 4 h of incubation at 37°C in a moist atmosphere of 5% CO\textsubscript{2}, non-adhering cells on each plate will be removed by intensive rinsing with phosphate-buffered saline (PBS). The number of adherent cells on each plate will be determined by counting 10 to 15 microscopic fields and multiplying the arithmetic means of the counts by the ratio of plate area to the area of the microscopic field\textsuperscript{15}.

**Cytokine assays in vitro:**

To determine the effect of LTL on the production of cytokines from LPS-stimulated cells, RAW 264.7 or macrophages cells were plated onto 24-well plates (1x10\textsuperscript{6} cells/well), pretreated in the presence or absence of LTL for 1 h, and then stimulated with LPS for time dependent manner at 37°C in a 5% CO\textsubscript{2} incubator. At each time point, cell-free supernatants were collected and the concentrations of cytokines TNF-\textalpha, IL-1\beta and IL-6 were measured by sandwich ELISA, using commercially available assay kit from GE Healthcare Bio-Sciences (NJ,USA) according to the manufacturer's instructions.

**Extraction of nuclear proteins and assay of NF-kB p65:**

RAW 264.7 cells were plated onto 24-well plates (1x10\textsuperscript{6} cells/well), pretreated in the presence or absence of LTL for 1 h, and stimulated with LPS (1\mu g/mL) for 12 h. After centrifugation, it was resuspended in 400 \mu L of ice cold hypotonic buffer for 10 min, vortexed, and centrifuged at 15,000 g at 4°C to get the supernatant containing nuclear protein. Aliquots of this were added to incubation wells precoated with the NF-kB p65 DNA-binding consensus sequence, and the translocated p65 subunits present in the nuclear lysate were assayed according to the recommendations of the manufacturer of the NF-kB assay kit (Cayman, Michigan, USA).
NO assay:

RAW 264.7 cells (1×10^6 cells/well) were plated onto 24-well plates, pretreated with the indicated concentrations of LTL for 1 h, and subjected to stimulation with of LPS for time dependent manner. The sample supernatants were mixed with equal volumes of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride) and then incubated at room temperature for 10 min. The absorbance was measured at 540 nm on a microplate reader. Nitrite concentration was determined using a dilution of sodium nitrite as a standard. Concentration values were determined for two wells of each sample and the experiment was performed in triplicate.

Carrageenan-induced paw edema:

Animals were divided into four groups (n=10). In all groups, inflammation was induced by single sub-plantar injection of 0.02 mL of freshly prepared 1% carrageenan in normal saline. The group treated with carrageenan alone served as control. Three groups received LTL, i.p. at different time point, 30 min before the carrageenan injection. The paw thickness was measured using vernier calipers and the difference between paw volumes was calculated every hour after carrageenan injection.

Formalin-induced paw edema:

The experiment was the same as described before for carrageenan induced paw edema except that a single dose of 0.02 mL of formalin (2%) was used as the inflammation inducer.

Results

Morphological changes of macrophage cells Fig.1 presents the cell morphology of the macrophage RAW 264.7 cells under Leishmanial total lipid (LTL) treatment in the presence or absence of LPS (1μg/ml). The cells were monitored under optical microscopy (200X) and pictures were captured after 24h of treatment. In the LPS-unstimulated cells, the cell morphology generally showed round in form whereas LPS-activated RAW 264.7 cells had changed to an irregular form with accelerated spreading and forming pseudopodia. Leishmanial total lipid (LTL) reduced the level of cell spreading and pseudopodia formation by suppressing cell differentiation.
Effects of Leishmanial total lipid (LTL) on the expressions of inflammatory mediators in LPS stimulated macrophage cells:

To determine whether Leishmanial total lipid (LTL) modulates the production of pro-inflammatory cytokines, the productions of TNF-α, IL-1β, IL-6, IL-10 and NO were examined using ELISA methods in the LPS stimulated RAW 264.7 macrophage supernatants. These compounds alone had no significant effect on the secretions of those inflammatory mediators in the normal RAW264.7 cells (not stimulated with LPS) (data not shown). The treatment with different concentrations of LTL resulted in an inhibition of the LPS-induced (1µg/µl) IL-1β (Fig. 2A) TNF-α (Fig. 2B), IL-6 (Fig. 2C) and NO (Fig. 2F) secretions. With 30µg/ml of LTL a remarkable >50% inhibition compared to control set were noticed in each cases. Simultaneous release of another cytokines, IL-10 was also observed expectedly with more than 50% increase in production with 30µg/ml of LTL (compared to control) (Fig. 2D). The result of Western Blot analysis convincingly corroborated the above findings as shown in Figure 2E. These observed effects were not due to the cytotoxicity of LTL, showed no impairment of the cell viability in concentration ranges used in this study, compared with the cells treated with LPS alone.
Figure 2: Effects of Leishmanial total lipid (LTL) on the expressions of inflammatory mediators in LPS stimulated macrophage cells

Effects of Leishmanial total lipid (LTL) on the expressions of inflammatory mediators in E. coli K-13 stimulated macrophage cells:

To determine whether Leishmanial total lipid (LTL) modulates the production of pro-inflammatory cytokines, the productions of TNF-α, IL-1β, IL-6, IL-10 and NO were examined using ELISA methods in the E. coli K-13 stimulated RAW 264.7 macrophage supernatants just in the similar manner as above. The findings are equally impressive as are delineated in Fig. 3A-3E below. Here also, our LTL responded in a remarkably effective manner as the figures described.
Figure 3: Effects of Leishmanial total lipid (LTL) on the expressions of inflammatory mediators in E. coli K-13 stimulated RAW 264.7 macrophage cells.
Effects of Leishmanial total lipid (LTL) on the expressions of inflammatory mediators in LPS stimulated serum and peritoneum macrophages of mice:

Next we have conducted our experiments on in vivo mouse model to see whether our LTL is equally effective in in vivo system. To determine whether Leishmanial total lipid (LTL) modulates the production of pro-inflammatory cytokines, the productions of TNF-α, IL-1β, IL-6, IL-10 and NO were examined using ELISA methods in both serum and peritoneum macrophages of mice pre-treated with LTL and then with sublethal doses of LPS. Here also very much encouraging results were found as can be clearly observed from Fig. 4A-4E. The animals were separately pre-treated with 3mg/kg wt and 6mg/kg wt of LTL followed by a sublethal dose of LPS (2.5mg/kg wt). After first 24 hours of study inhibitory effect of LTL on the production of inflammatory mediators, like TNF-α, IL-1β, IL-6 and NO, were distinctly noticed and were recorded in a remarkable fashion after 36 hours of study (compared to only LPS-treated control). For IL-6 production, the inhibitory effect of LTL even after 24 hours was significant (Fig. 4A-4D). Also, in complete agreement of our expectation the production of anti-inflammatory cytokine IL-10 was conspicuously manifested after first 24 hour of study and after 36 hours the increase in production was really noteworthy (Fig. 4E). Fig. 4F significantly delineates the efficacy of LTL on survival rate of mouse treated with LPS compared to treatment with LPS alone as control.

Effects of Leishmanial total lipid (LTL) on the expressions of inflammatory mediators in E. coli K-13 stimulated serum and peritoneum macrophages of mice:

Next, we have conducted similar experiments with on in vivo mouse model by using sublethal doses of E. coli K-13 strain to determine whether Leishmanial total lipid (LTL) modulates the production of pro-inflammatory cytokines, the productions of TNF-α, IL-1β, IL-6, IL-10 and NO were examined using ELISA methods in both serum and peritoneum macrophages of mice. The findings are equally impressive as are delineated in Fig. 5A-5E below. Here also, our LTL responded in a remarkably effective manner as the figures described. Fig. 5F significantly delineates the efficacy of LTL on survival rate of mouse treated with E. coli K-13 compared to treatment with E. coli K-13 alone as control.
Figure 4: Level of cytokines in serum and peritoneum macrophages of mice pre-treated with lipid and then with sub-lethal doses of LPS for 24 hrs.
Figure 5: Effects of Leishmanial total lipid (LTL) on the expressions of inflammatory mediators in E. coli K-13 stimulated serum and peritoneum macrophages of mice
Figure 5 (contd.): Effects of Leishmanial total lipid (LTL) on the expressions of inflammatory mediators in E. coli K-13 stimulated serum and peritoneum macrophages of mice

LTL inhibits NF-κB expression:

LPS induced inflammatory stress is known to cause activation of the transcriptional factor NF-κB and the subsequent release of inflammatory mediators with signalling cascade. Thus, we examined if LTL inhibited the levels of NF-κB p65 expression in 100μg/ml manner. Immunocytochemistry studies provided evidence that the expression level of NF-κBp65 subunit is lowered in LPS stimulated macrophage cells in the presence of LTL. NF-κB activation represents a paradigm for controlling the function of different regulatory proteins via phosphorylation based on the cascade series including proteolytic degradation of IκB followed by TLR4-CD14 signalling pathway.
Carageenan induced paw edema

Leishmanial total lipid (LTL) was administered intra-peritoneally at the doses of 200µg, 500µg and 2mg/kg of body weight. These LTL at different doses inhibited carageenan-induced rat paw edema up to 4hrs observations. Moreover, the anti-inflammatory effect was more significant at the dose of 2mg/kg than dose of the 200µg, 500µg/kg LTL as compared with control (carageenan induced group) (Figure 7).

Figure 6: Immunofluorescence expression measured by confocal laser scanning microscopy (magnification 600x)

Figure 7: Effect of Leishmanial total lipid (LTL) at different doses on local effect of carrageenan induced rat paw edema (mean±SEM; n=6)
Formalin induced paw edema

Leishmanial total lipid (LTL) was administered intraperitoneally at the concentration of 200µg, 500µg and 2mg/kg of body weight. These LTL at different doses inhibited formalin-induced rat paw edema up to 4 hrs observation. Moreover the anti-inflammatory effect was more significant at the dose of 2mg/kg than dose of the 200µg, 500µg/kg LTL as compared with control (formalin induced group) (Figure 8).

Figure 8: Effect of Leishmanial total lipid (LTL) at different doses on local effect of formalin-induced rat paw edema (mean±SEM; n=6)

Discussion

Our previous study has found that a novel compound, lipid isolated from Leishmanial donovani inhibitory effects on endotoxin induced TNF-α and IL-6 release in mouse macrophages. In the present study, we demonstrated anti-inflammatory activities of this compound both in vitro and in vivo, using LPS-stimulated Raw 264.7 cells and several mouse models of topical inflammation respectively.

Macrophages play important roles in a host’s immune defense system during infection as well as in the processes of disease development\(^{18}\). Activation of macrophages by stimuli, such as LPS, increases the production of numerous inflammatory mediators, including various cytokines and nitric oxide (NO)\(^{10}\). Excessive production of NO has been widely reported to be associated with inflammatory response\(^{19,20}\). First, we showed that LTL has inhibitory activity upon morphological features Raw 264.7 cells (Figure 1B). Our next experiments provided the evidence that LTL significantly inhibits TNF-α, IL-1β, IL-6, IL-10 and NO in macrophages cells. (Figure 2). Strong inhibition of inflammatory cytokine release has been ascribed to down-regulation of NF-kB dependent gene transcription\(^{21,22}\). The validation of experiment was
done with E.coli also and we found the similar result. The significant reduction was found in inflammatory mediators and cytokines level with the LTL in stimulated macrophages. Next, we designed to study the effect of LTL in murine serum and macrophages also in presence of LSP or E.coli. LTL subdued the level of TNF-α, IL-1β, IL-6, IL-10 in vivo system also in time dependent manner with simultaneous improvement of the survival rate of the endotoxin or E.coli challenged mice.

Besides the endotoxin and etiologic agents, some chemicals also induce the production of inflammation. During tissue inflammation, there is normally vasodilatation and transient increases in capillary permeability producing extravasation of plasma proteins and tissue oedema. Generally, these reactions are linked to painful perception or hyperalgesic sensitization\textsuperscript{23-25}. Our results shown in Figure 6 indicate that pre-treatment with LTL reduced the inflammatory pain, and also reduced the paw oedema induced by carrageenan and formalin model. These inflammatory models are mainly related to local production of bradykinin and prostaglandins, such as PGE2 and leukotrienes, both derivatives of arachidonic acid. These prostanoids bind the prostaglandin sub-type receptors, triggering the inflammatory and hyperalgesic pathway in tissues. Bradykinin receptor B1 is associated with a metabotropic signaling pathway producing vasodilatation, an increase of vascular permeability, and an increase of eicosanoids production, such as PGE2 and NO produced by PGES, COX-2 and NO. On the other hand, B1 receptors promote pain stimuli and inflammation by the NF-κβ pathway\textsuperscript{25}. In another inflammatory model of formalin-caused pain and oedema, the pain in the first phase is induced through formalin directly stimulating C-fibers, whereas in the second phase, the pain is generated by the release of several inflammatory mediators\textsuperscript{26,27}. The higher inhibitory rate of LTL in phase II than phase I also partly indicated that the main mechanism of LTL activity is to inhibit the production of inflammatory mediators.

This study represents that leishmanial total lipid (LTL) exerts anti-inflammatory responses via regulating the inflammatory factors \textit{in vitro} and \textit{in vivo}. Thus, LTL reduces mortality rate of gram-negative bacteria induced septic mice.

\textbf{Acknowledgments}

The authors convey their sincere thanks to Dr. Subires Bhattacharyya, Principal, Fakir Chand College, Diamond Harbour, and to Prof. (Dr.) Siddhartha Roy, Director, CSIR-Indian Institute of Chemical Biology, Jadavpur, Kolkata, for their relentless support to carry out the
present work. Financial assistance from University Grants Commission, Govt. of India, to the first author is also duly acknowledged.

References


9. Majumdar, K. N., Banerjee, A., Ratha, J., Mandal, M., Sarkar, R. N., Das Saha, K. Leishmanial lipid suppresses TNFα, IL1β, and nitric oxide production by adherent synovial fluid mononuclear cells in rheumatoid arthritis patients and induces apoptosis


