Plasma lipoproteins in visceral leishmaniasis and their effect on Leishmania-infected macrophages


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SUMMARY

This work aimed at investigating the lipid profile of zoonotic visceral leishmaniasis (VL) patients’ sera and the effect of lipoproteins on the in vitro production of tumour necrosis factor-α (TNF-α), interleukin (IL)-6, IL-10 and IL-12 by Leishmania infantum-infected and uninfected macrophages. Lipids were quantified in 26 VL patients’ sera and 26 healthy controls from a VL endemic area. The patients’ sera had higher triglyceride and very low density lipoprotein (VLDL) levels, and much lower apolipoprotein A1, total cholesterol, low density lipoprotein (LDL) and high density lipoprotein (HDL) levels than the control sera. Lipoprotein fractions were obtained by ultracentrifugation of sera. The addition of LDL and HDL to Leishmania-infected and uninfected macrophages, in physiological concentrations, enhanced the production of IL-6 and IL-10, but not of IL-12. LDL stimulated the production of TNF-α only in infected macrophages, whereas HDL stimulated the production of lower amounts of TNF-α in both infected and uninfected macrophages. VLDL stimulated only the production of IL-10. It is proposed herein that LDL may influence the development of VL by promoting the production of TNF-α by infected macrophages. A decrease in plasma LDL in some VL patients (to 20 mg/mL or less); however, would tend to reduce the production of TNF-α and therefore to limit the development of immune-mediated pathology, notwithstanding the fact that it would perhaps increase the permissiveness of macrophages to Leishmania growth.

Keywords cytokines, Leishmania, lipoproteins

INTRODUCTION

Zoonotic visceral leishmaniasis (VL) is a systemic infectious disease caused by the protozoa of the Leishmania genus, which infect mononuclear phagocytes in the spleen, the liver and the bone marrow of human beings and other mammals (1). When left untreated, most patients die of opportunistic bacterial infections (2) because of a disease-associated nonspecific immunodeficient state (3–5). Zoonotic VL is caused by Leishmania infantum Nicolle, 1908, or Leishmania chagasi Cunha & Chagas, 1937, which are believed to be the same parasite (6). In the American continent, it is transmitted by an insect vector of the genus Lutzomyia (7,8).

Some studies on both zoonotic VL and anthropoctic (caused by Leishmania donovani) VL have demonstrated that the control of the disease is associated with Th1-type cellular immune responses, with production of interleukin (IL)-12 and interferon-γ (IFN-γ) (9–12). IL-12 stimulates the production of IFN-γ in VL patients who respond well to treatment (9) and its plasma levels are lower in L. donovani-infected individuals with overt VL than in L. donovani-infected individuals who develop skin delayed-type hypersensitivity reactions to Leishmania antigens without developing a disease (11). Moreover, peripheral blood mononuclear cells (PBMC) from individuals with asymptomatic or self-healing oligosymptomatic L. infantum infection, and not from individuals that eventually develop progressive VL, respond in vitro to Leishmania antigen with the production of IFN-γ and IL-2 (9). When the disease is already established; however, increased levels of the pro-inflammatory cytokines IFN-γ, tumour necrosis factor-α (TNF-α), IL-6 and IL-8 can be found in VL patients’ plasma (13–16). Despite the increase in IFN-γ and TNF-α plasma levels during active disease, the numbers of TNF-α-containing monocyes (16) and the production of IFN-γ by antigen-stimulated PBMC (15) are reduced when compared with that in
asymptomatic and cured individuals respectively. Moreover, in *L. infantum* and *L. donovani*-infected mice and human beings, the development of disease occurs in the presence of increased amounts of IL-10, which is a potent immunosuppressive and anti-inflammatory cytokine (15–19).

Lipoproteins or lipoprotein particles are capable of modulating cellular immune responses. Among these are chylomicrons, very low density lipoproteins (VLDL) and low density lipoproteins (LDL), which inhibit the proliferation of lymphocytes *in vitro* (20,21). Macrophages, which are the cellular hosts of *Leishmania* parasites, have natural or modified lipoprotein receptors (22) and are therefore possible targets for the immunomodulatory activities of lipoproteins.

Plasma lipid alterations have been demonstrated in some infectious diseases. In acquired immune deficiency syndrome (AIDS), levels of serum triglycerides are elevated and those of high density lipoprotein (HDL) are decreased (23,24). On mansonic schistosomiasis, the plasmatic concentration of total cholesterol and triglyceride levels are found to be decreased (25). In canine VL, high levels of triglycerides and low levels of HDL have been described (26). Alterations in circulating lipids in a low number of VL human patients have also been reported (27–30). Interestingly, several recent reports have described that depleting cholesterol from host-cell membranes impairs the penetration of intracellular pathogens, such as *Mycobacteria*, *Plasmodium falciparum*, *Chlamydia trachomatis*, *Listeria monocytogenes*, *L. donovani* and *L. infantum* (31–36). These findings have led to the proposition that cholesterol-depleting medications could play a therapeutic role in VL (37). On the other hand, the reduction in cholesterol concentrations in antigen-presenting cell membranes has been shown to impair antigen presentation to lymphocytes (38), a phenomenon that could theoretically lead to sub-optimal immune responses, and, therefore, favour parasitism. Studies on plasma lipid abnormalities in VL patients, and on the possible effect of the addition of these plasma lipoproteins to *Leishmania*-infected macrophages, therefore, may provide important clues on the role of these lipoproteins in the development of the infection.

In this report, the nature of the lipidic abnormalities in 26 VL patients’ sera is described. In addition, the effects of lipoproteins on the *in vitro* production of pro-inflammatory and regulatory cytokines by *L. infantum*-infected and uninfected macrophages are reported, and a parallel between the lipoprotein concentrations found in the patients, and how these concentrations affect the cytokine production by infected and uninfected macrophages *in vitro*, is made.

## MATERIALS AND METHOD

### Serum samples

Samples of sera from 26 parasitologically diagnosed patients with acute VL were obtained at the Unity of Endemic Diseases Pirajá da Silva in Jequié (PIEJ), Bahia, Brazil. Blood samples for serum preparation were collected before chemotherapy. Control sera from 26 apparently healthy, age- and gender-matched individuals, who did not have detectable anti-*Leishmania* antibody levels in an ELISA (39), were obtained at the same region. All patients and volunteers were previously informed of the nature of the research and they agreed to participate in the study. The project was approved by the Committee of Ethics in Research of the Gonçalo Moniz Research Centre.

### Parasite culture

*Leishmania infantum* was characterized by a panel of species-specific monoclonal antibodies and isoenzymatic patterns, by Dr. G. Grimaldi, at the Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. Promastigotes were cultured in Schneider’s medium supplemented with 10% foetal bovine serum (Microbiology, Rio de Janeiro, Brazil).

### Quantification of plasma lipids

The serum amounts of triglycerides, cholesterol and fractions and apolipoproteins were determined with the use of commercially available kits (Labtest Diagnostica S.A., Belo Horizonte, Brazil), using enzymatic methodologies or Trinder’s final (quinoneimine formation) reaction.

### Lipoprotein separation

Lipoprotein fractions were obtained from a pool of 10 sera of healthy individuals with hypertriglyceridaemia, by ultracentrifugation on a NaCl gradient (40). Lipoproteins were not prepared from VL patients sera because of clinical limitations in the volume of blood that can be collected from them. These fractions were dialysed against RPMI 1640 (GIBCO, Grand Island, NY, USA), pH 7.2, at 4°C, for 24 h, in sterile conditions, and stored at −20°C.

### Infection of macrophages with *Leishmania infantum* and their incubation with lipoproteins

Healthy donors’ PBMC were separated using Ficoll-Paque (Sigma Chemical Co., St. Louis, MO, USA) and cultured in 24-well plates in RPMI medium supplemented with...
amino acids and 10% normal AB human serum (complete RPMI), at 37°C and 5% of CO₂. After 48 h of culture, the wells were washed with RPMI at 37°C for removal of non-adherent cells and incubated in complete RPMI for 7 days, with the same culture conditions. On the eighth day of culture, 2 x 10⁶ L. infantum promastigotes, in stationary phase of growth, were added to some wells in the proportion of 10 promastigotes per macrophage. The cultures were incubated again for 4 h and washed with RPMI for removal of Leishmania from the supernatants. LDL, HDL and VLDL fractions were then added, leading to the concentrations given in the Figure 1, both to infected and uninfected macrophages. At least 70% of the macrophages were infected in any of the cultures to which Leishmania was added. After a 24-h incubation, the culture supernatants were collected and stored at −20°C.

Quantification of cytokines

Commercially available detection kits were used to determine the concentrations of IL-6, IL-12p40, TNF-α (Duo-set-Kit; R&D Systems, Inc., Minneapolis, MN, USA, http://www.rndsystems.com), and IL-10 (human ELISA Set kit, BD Biosciences, Franklin Lakes, NJ, USA, http://www.bdbiosciences.com), following the manufacturer’s instructions.

![Figure 1](image-url)  
*Figure 1* Concentration-response curves of the effects of LDL, HDL or VLDL on the production of TNF-α (a, b, c), IL-6 (d, e, f), IL-10 (g, h, i) and IL-12 (j, k, l) by human monocyte-derived macrophages *in vitro*. The macrophages were either infected with Leishmania infantum (■) or uninfected (▲), as explained in the Materials and Method. Cytokine concentrations, in culture supernatants collected after 24 h after the addition of lipoproteins, were determined by ELISA. The data represent the mean of three experiments with cells from different donors. Vertical bars represent the standard deviations of the means. Within each experiment, each concentration of lipoprotein was assessed in triplicate. The values shown on the x-axis correspond to the total concentrations of each lipoprotein present in the cultures (i.e. the sums of the concentrations of the added purified lipoproteins with those of the lipoproteins that were present in the 10% normal human AB serum-containing culture medium; the first point on the left represents the result that was obtained when no purified lipoprotein was added).
Statistical analyses

Analyses of the statistical significance of differences in lipidic fractions found in VL patients’ sera and in normal individual sera were carried out using the paired Student’s t-test. The statistical significance of differences in the production of cytokines by infected or uninfected macrophages, either stimulated or not stimulated with lipoproteins, was determined by using the Friedman’s test and the repeated measures ANOVA.

RESULTS

Analysis of lipidic profile in VL patients’ sera

High levels of triglycerides and VLDL, and low levels of apolipoprotein A1, apolipoprotein B, total cholesterol, LDL and especially HDL, were found in sera from patients with VL (Table 1). Values of HDL in these patients’ sera were about six times lower than in normal individuals’ sera ($P < 0.05$).

Effect of lipoproteins on cytokine production by *Leishmania infantum*-infected macrophages

Low density lipoprotein stimulated the production of TNF-$\alpha$ by infected PBMC-derived macrophages, and not by uninfected macrophages, starting at the concentration of 30 mg/dL, and reaching a plateau with the concentration of 50 mg/dL ($P < 0.05$; Figure 1b). Although a small increase in TNF-$\alpha$ production was observed in cultures of both infected and uninfected macrophages at HDL concentrations above 13 mg/dL, such difference was not statistically significant ($P > 0.05$; Figure 1a). VLDL did not stimulate the production of TNF-$\alpha$ in either infected or uninfected macrophages ($P > 0.05$; Figure 1c).

The incubation with HDL and LDL, starting at the concentrations of 13 and 30 mg/dL respectively, increased IL-6 concentrations in both infected and uninfected macrophage cultures (Figure 1d, e). Addition of HDL to the cultures at concentrations higher than 20 mg/dL did not lead to increased IL-6 production. Although there was about three times more IL-6 in uninfected than in infected macrophage cultures that contained 20 mg/dL of HDL (Figure 1d), this difference was not statistically significant ($P > 0.05$). The addition of VLDL did not affect the concentration of IL-6 (Figure 1f).

The incubation of both infected and uninfected macrophages with concentrations equal to or above 13 mg/dL of HDL, 30 mg/dL of LDL or 47 mg/dL of VLDL led to an increase in IL-10 production ($P < 0.05$; Figure 1g, h, i). This increase was not seen when 65 mg/dL of HDL was added to the cultures. No statistically significant differences in IL-10 concentrations between infected and uninfected macrophage cultures were observed ($P > 0.05$).

Table 1 Lipid fractions in 26 zoonotic visceral leishmaniasis (VL) patients’ and in 26 healthy individuals’ sera

<table>
<thead>
<tr>
<th>Lipid fractions</th>
<th>Age (years)</th>
<th>Number of individuals</th>
<th>Concentrations of lipid fractions in sera from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>VL patients</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>1–12</td>
<td>12</td>
<td>91 ± 33$^b$</td>
</tr>
<tr>
<td></td>
<td>&gt;18</td>
<td>14</td>
<td>104 ± 37</td>
</tr>
<tr>
<td>LDL</td>
<td>1–12</td>
<td>12</td>
<td>30 ± 13</td>
</tr>
<tr>
<td></td>
<td>&gt;18</td>
<td>14</td>
<td>53 ± 23</td>
</tr>
<tr>
<td>HDL</td>
<td>1–12</td>
<td>12</td>
<td>8 ± 5</td>
</tr>
<tr>
<td></td>
<td>&gt;18</td>
<td>14</td>
<td>8 ± 6</td>
</tr>
<tr>
<td>VLDL</td>
<td>1–12</td>
<td>12</td>
<td>53 ± 32</td>
</tr>
<tr>
<td></td>
<td>&gt;18</td>
<td>14</td>
<td>43 ± 14</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1–12</td>
<td>12</td>
<td>266 ± 158</td>
</tr>
<tr>
<td></td>
<td>&gt;18</td>
<td>14</td>
<td>216 ± 71</td>
</tr>
<tr>
<td>APO A1</td>
<td>1–12</td>
<td>12</td>
<td>79 ± 38</td>
</tr>
<tr>
<td></td>
<td>&gt;18</td>
<td>14</td>
<td>80 ± 31</td>
</tr>
<tr>
<td>APO B</td>
<td>1–12</td>
<td>12</td>
<td>80 ± 25</td>
</tr>
<tr>
<td></td>
<td>&gt;18</td>
<td>14</td>
<td>80 ± 19</td>
</tr>
</tbody>
</table>

LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein.

$^a$Statistical significance of differences between VL patients and age-matched healthy individuals, as determined by the paired Student’s t-test.

$^b$Mean concentration in mg/dL ± standard deviation of the mean.

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The lipoproteins did not significantly affect the production of IL-12 (Figure 1j, k, l), with the exception of a reduction when the cells were incubated with 35 mg/dL of HDL (Figure 1j).

DISCUSSION

As shown herein, VL patients have high circulating levels of triglycerides and VLDL, and markedly reduced levels of total cholesterol, LDL, HDL and apolipoprotein A (which is mainly involved in the composition of HDL), and a modest decrease in apolipoprotein B (which participates in the composition of both LDL and VLDL). These results are in agreement with those published by Bertoli et al., who reported hypertriglyceridaemia in 28 patients with VL (29), by Bekaert et al., who reported decreased plasma levels of HDL, LDL, apolipoproteins A-I and A-II and increased triglycerides levels in 17 Tunisian patients with VL (28), and by Liberopoulos et al., who reported hypocholesterolaemia in a patient with VL (30). VLDL plays a role in the transfer of triglyceride and cholesterol from liver to muscular and adipose tissue. The mechanisms controlling its production are poorly understood and its acquirement by tissues is dependent upon the activity of enzymes such as endothelial lipoprotein lipase, which may be impaired in patients with VL (41). A decrease in VLDL metabolism to intermediate density lipoprotein (IDL) may contribute to the decreased LDL formation in VL patients. The low rate of hydrolysis of VLDL triglyceride by lipoprotein lipase would also decrease the availability of unesterified cholesterol, phospholipids and various lipoproteins that enter the constitution of HDL. Additionally, a reduced synthesis of apolipoprotein A in liver and intestine, and an enhanced activity of the LDL receptor, may be triggered by pro-inflammatory cytokines, such as IL-6 (28,30,42).

In this study, both infected and uninfected macrophages were shown to produce IL-6 and IL-10 when incubated with physiological concentrations of HDL and LDL in vitro. The production of IL-6 and IL-10; however, was not affected by infection of the macrophages by Leishmania. The spontaneous production of IL-6 and IL-10 by macrophages, in the presence of physiological concentrations of plasma lipoproteins, may reflect a real phenomenon, i.e. it would also occur in macrophages that are not stimulated in vivo, or may result from cell activation because of in vitro conditions. Increased concentrations of both IL-10 and IL-6 have been found in the plasma of VL patients (15,16,19). This work provides evidence that the simple infection of human monocyte-derived macrophages by Leishmania does not lead to the production of these cytokines. In the case of IL-10, this has been confirmed by studies that show that the production requires the presence of anti-Leishmania antibodies (43) or bacterial lipopolysaccharide (44), although one study reported its production in the absence of additional stimulus (45). An absence of effect of Leishmania infection on IL-10 production by macrophages is consistent with the fact that T cells have been found to be a source of that cytokine in VL patients (46). On the other hand, in some reports, the in vitro infection of macrophages by Leishmania was shown to promote the synthesis of IL-10, IL-6, TNF-α and/or their correspondent mRNA, even in the presence of sub-physiological concentrations of plasma lipoproteins, i.e. the concentrations present in medium containing 10% normal serum (45,47–50). This is in disagreement with the data presented herein, in which infection of human macrophages by Leishmania stationary phase promastigotes, in the absence of physiological concentrations of lipoproteins, did not induce the production of these cytokines. This discrepancy may be explained by differences in the Leishmania and macrophage-donor species that were used in different studies, because, with the exception of one work showing increased synthesis of IL-10, all the reports referred above describe results obtained using murine macrophages, and none of them used L. infantum. In fact, the expression of IL-1 by infected macrophages varies in accordance with the Leishmania species and the mouse strain used (51).

Contrasting with the findings described above for IL-6 and IL-10, physiological amounts of LDL, or even the reduced amounts found in some patients with VL, synergize with L. infantum infection to induce TNF-α production by macrophages. The synergism between infection and LDL was obligatory, neither of them alone led to the production of high concentrations of the cytokine. The results described above, therefore, may support the hypothesis that LDL is required for the production of high levels of TNF-α in VL. In fact, the possibility that infected macrophages, in the presence of LDL, are a major source of TNF-α, could explain the apparently paradoxical findings of high plasma levels of TNF-α and low numbers of circulating TNF-containing monocytes in VL patients (19): infected splenic macrophages, and not uninfected circulating monocytes, would be the main source of TNF-α. TNF-α levels may be further augmented in VL patients by the presence of increased numbers of macrophages in inflammatory infiltrates. For instance, the spleen, which is usually markedly enlarged in those patients, is populated by large numbers of infected and uninfected macrophages (52,53), and is very likely the major infected organ in VL. Splenic cells are all in ample contact with plasma lipoproteins, favoured by characteristics of the splenic blood circulation. An increase in TNF-α
production occurs in the course of severe forms of VL, and has been associated with disease progression (13) and lymphoid tissue disorganization (14,54) indicating that, when TNF-α is present in high levels in VL patients, its pathogenic effect may prevail upon the benefits that would arise from its controlling intracellular parasite growth (54). On the other hand, it is shown in this work that lipid concentrations, in the range of those found in the plasma of many VL patients, and therefore in their spleens, affect the production of cytokines by macrophages in vitro. An impairment of the local immune response in the spleen could well make the difference in parasite proliferation or control. The importance of this fact in the course of the disease deserves further investigation.

It is interesting to note that none of the lipoproteins affected the production of IL-12 by infected or uninfected macrophages, indicating that lipoprotein levels do not directly influence the IL-12-dependent production of IFN-γ (55,56) in VL patients. These findings also indicate that the lipoprotein preparations were not contaminated by bacterial lipopolysaccharide (LPS), as LPS induces IL-12 production by human macrophages (57).

As shown herein, the pattern of lipoprotein concentrations that is found in VL patients (low total cholesterol, low HDL, low LDL and high VLDL levels) leads to decreased productions of TNF-α and IL-6 and normal production of IL-10 by macrophages in vitro, a situation that would tend to reduce inflammation. Would this constitute a negative feedback mechanism, in which increased levels of IL-6 and/or TNF-α would lead to increased inflammation and decreased lipoprotein levels, which, in their turn, would increase the threshold for production of IL-6 and TNF-α by macrophages, leading to a reduction in their levels and in the consequent inflammation? Consistent with the view that low cholesterol levels reduce inflammation, the results of this study may have implications for the efficacy of a suggested treatment of VL. It has been suggested that it should be of clinical advantage to reduce plasma cholesterol levels in VL patients by means of specific medications (37). The data presented herein pose some doubts on the desirability of that procedure; as cholesterol levels are already much lower in VL patients than in healthy, age-matched individuals. It would nevertheless be worthwhile to carry out prospective studies on VL patients to see if there is any correlation between plasma lipid levels and disease outcome.

In this work, the effect of lipoproteins on macrophages that had been previously infected by Leishmania promastigotes was shown. Further work would be required to find out if infection by amastigotes would produce the same results. It would also be worthwhile to investigate whether the effect of LDL on Leishmania-infected macrophages has any degree of specificity for the parasite, or if it would also occur in the case of infection by other intracellular pathogens, or even in the case of phagocytosis of inert particles. Could LDL enhance the defense against intracellular pathogens?

It is provided herein evidence that plasma lipids could play a role in the development or maintenance of the immune response found in active VL, in so far as the presence of LDL would be required for the production of TNF-α by L. infantum-infected macrophages, and that the levels of lipoproteins found in some VL patients would be below the threshold required for the maximal production of TNF-α and of IL-6. Plasma lipid alterations in VL could therefore act as one additional factor in a very likely complex interplay of parasite-, environmental- and host-dependent factors (58–60), the net result of which is the usually irreversible progression to lethality of untreated VL patients.

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