Research Article

Sera from Visceral Leishmaniasis Patients Display Oxidative Activity and Affect the TNF-α Production by Macrophages In Vitro

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1. Introduction

Zoonotic visceral leishmaniasis (ZVL), caused by the intracellular protozoan parasite Leishmania infantum, is a tropical disease that is often fatal when untreated [1]. ZVL patients are usually unable to mount an effective immune response against the parasite and indeed appear to be immunosuppressed. This suppression has strong nonspecific and specific components mediated by serum factors and leishmanicidal activity of infected macrophages, respectively. The lipid profile has been shown to be altered in ZVL patients’ sera. This work aimed at (i) determining the HDL, Apo A1, LDL, and VLDL concentrations in ZVL patients’ sera; (ii) investigating the oxidative effect of ZVL patients’ sera on the β-carotene matrix; (iii) measuring IL-10, IL-6, IL-12p40, and tumour necrosis factor-α (TNF-α) concentrations in the macrophage cultures, to which 10% of ZVL patients’ serum had been added. Levels of HDL, LDL fraction, and apolipoprotein A1 in ZVL patients’ sera were lower than those of healthy individuals’ sera, except for the mean level of VLDL. The matrix of β-carotene and linoleic acid system was oxidized in the presence of ZVL patients’ sera. The presence of ZVL patients’ sera did not modify the cytokine production of IL-6, IL-12p40, and IL-10 by human macrophages in vitro but TNF-α production was altered, probably due to lack of macrophage stimulation by lipoprotein.
2. Methods

2.1. Sera Samples. A total of 15 blood samples, for serum preparation, were collected before chemotherapy, from 15 parasitologically diagnosed patients with ZVL, aged over 18 and seen at the Endemic Diseases Unit, Pirajá da Silva in Jequié (PIEJ), Bahia, Brazil. Control sera were collected from 15 age- and sex-matched (seemingly healthy) of same endemic region individuals, who did not have detectable anti-Leishmania antibody by ELISA [18]. All patients and volunteers were previously informed of the nature of the research and agreed to participate in the study. The project was approved by the Committee of Ethics in Research of the Gonçalo Moniz Research, Oswaldo Cruz Foundation.

2.2. Evaluation of Oxidant Activity of the ZVL Patients’ Sera Using the β-Carotene and Linoleic Acid System. Oxidant activity was assessed according to the methodology described by Marco (1968) [19] and modified by Fernández-Miranda et al. (1998) [20]. Fifteen ZVL patients’ and 15 healthy individuals’ sera were tested. The β-carotene solution was prepared by dissolving 1 mg of β-carotene in chloroform in a round-bottom flask containing 20 mg of linoleic acid and 200 mg of the emulsifier Tween 20 (β-carotene system). After removal of the chloroform by rotary evaporator at 50°C, 50 mL of distilled water was added under vigorous stirring. Aliquots (5 ml) of this emulsion were transferred to a series of test tubes containing in duplicate 0.2 mL of sera and initial absorbance (Abs) was read at 470 nm (time zero) in a spectrophotometer (BIOSPECTRO SP-220). The samples were then incubated in a water bath at 50°C for 120 min and the absorbances were read again at 470 nm. The oxidant activity was calculated considering 100% of absorbance in β-carotene system without serum (control) using the following formula:

\[
\text{Abs initial of sample} - \text{Abs final of sample} \times 100.
\]

2.3. Quantification of Sera Lipids. The serum levels of triglycerides, cholesterol, and fraction, and apolipoprotein A1 were determined with the use of commercially available kits (Labtest Diagnostica S.A., Belo Horizonte, Brazil), using enzymatic methodologies or Trinder’s final reaction (quinone imine formation).

2.4. Cell Culture. Healthy donors’ PBMC were separated using Ficoll-Paque (Sigma Chemical Co., St. Louis, 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% CO₂. After 48 hours of culture, the wells were washed with RPMI at 37°C for removal of nonadherent cells and incubated in complete RPMI medium. On the seventh day 100 μl (10% v/v) of ZVL patients’ or healthy individuals’ sera was added to wells and the cultures were incubated for 24 hours at 37°C and 5% CO₂. The culture supernatants were then collected and stored at −20°C.

2.5. 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.), and IL-10 (Human ELISA Set kit, BD Biosciences), following the manufacturers’ instructions.

2.6. Statistical Analyses. Analyses of the statistical significance of differences in lipid fractions and cytokines production found in ZVL patients and in normal individuals’ sera were carried out using the unpaired Student’s t-test. The correlation between oxidant activity of the ZVL patients’ sera and concentrations of HDL fraction was determined by Spearman test. Differences were considered as statistically significant when \( p < 0.05 \).

3. Results

Low levels of HDL, LDL fraction, and apolipoprotein A1 were found in ZVL patients’ sera. The mean level of HDL in those sera was 4.4 times lower than that of healthy individual sera. Conversely, the mean level of VLDL was higher in ZVL patients’ sera than that found in healthy individuals’ sera (\( p < 0.05 \), Table 1).

The matrix of β-carotene and linoleic acid system was oxidized to a higher degree in the presence of ZVL patients’ sera than in the presence of healthy individuals’ sera (\( p <
The percentage of oxidation had a negative correlation with the serum HDL-cholesterol concentration ($r = -0.86$, $p < 0.05$). The system in vitro used here did not show differences in the IL-6, IL-12p40, and IL-10 production by macrophages of healthy individuals in presence of ZVL patients’ sera or normal human sera. However, TNF-α production by macrophage in the presence of ZVL patients’ sera was inhibited, perhaps by the low concentration of LDL, two times lower than those found in normal individuals’ sera. A minimal concentration of LDL could be necessary to stimulate the TNF-α production by the macrophage infected by *Leishmania* in vitro. We have previously shown that LDL stimulates the production of TNF-α by infected PBMC-derived macrophage, and not by uninfected macrophage [4]. The synergy between infection and LDL was obligatory, and neither of them alone led to the production of high individuals’ sera, as well as low values of Apo A1 and LDL.

## Table 2: Oxidant activity of 15 zoonotic visceral leishmaniasis (ZVL) sera and 15 healthy individuals’ sera on the β-carotene and linoleic acid and linoleic acid system.

| Number of individuals | Oxidant activity (%) | Concentrations of HDL fraction (mg/dL)\(^a\) |
|-----------------------|----------------------|--------------------------------------------|
|                       | ZVL patients         | Healthy individuals                        | LV patients | Healthy individuals |
| 1                     | 93                   | 32                                         | 7           | 37                    |
| 2                     | 52                   | 33                                         | 5           | 36                    |
| 3                     | 86                   | 24                                         | 7           | 39                    |
| 4                     | 85                   | 23                                         | 6           | 41                    |
| 5                     | 26                   | 36                                         | 18          | 32                    |
| 6                     | 52                   | 30                                         | 8           | 34                    |
| 7                     | 11                   | 51                                         | 16          | 48                    |
| 8                     | 41                   | 32                                         | 9           | 59                    |
| 9                     | 25                   | 36                                         | 15          | 32                    |
| 10                    | 27                   | 32                                         | 10          | 37                    |
| 11                    | 51                   | 21                                         | 7           | 53                    |
| 12                    | 94                   | 29                                         | 6           | 42                    |
| 13                    | 30                   | 48                                         | 9           | 58                    |
| 14                    | 92                   | 34                                         | 5           | 35                    |
| 15                    | 25                   | 42                                         | 11          | 31                    |

Mean\(^b\) $52.7 ± 29.7$ \(33.5 ± 8.4\) \(9.3 ± 4.1\) \(40.9 ± 9.3\)

Correlation between oxidant activity of the ZVL patients’ sera and \(r = -0.86\), $p < 0.05$. \(^b\) Mean concentration in mg/dL$^{-1}$ ± standard deviation of the mean.

## Discussion

Lipid disorders have been described in patients with active ZVL [4]. The serum lipid profile of these patients is characterized by hypertriglyceridemia with reduced levels of HDL and apolipoprotein A1. In the present work, it is shown that concentrations of HDL and its major apolipoprotein, Apo A1, and LDL were markedly reduced in ZVL sera when compared with those concentrations in the sera of normal individuals living in the same area. These results are in agreement with Bekker and collaborators (1989) [21], who reported decreased sera levels of HDL and Apo A1 in 17 Tunisian patients with ZVL, and with Liberopoulos and collaborators (2002) [22], who reported a case of a man with ZVL and severely decreased HDL-cholesterol serum levels. Soares and collaborators (2010) [4] described values of HDL in ZVL patients’ sera approximately six times lower than in normal individuals’ sera, as well as low values of Apo A1 and LDL.
concentration of the cytokines [4]. 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 subphysiological concentrations of sera lipoproteins, that is, the concentrations present in a medium containing 10% normal serum [33–37].

Evidence has provided herein that ZVL patients’ sera oxidize the matrix of β-carotene and linoleic acid system but do not modify the production of IL-6, IL-12p40, and IL-10 by human macrophages in vitro. The TNF-α production in the presence of ZVL patients’ sera was inhibited, probably due to the low concentration of LDL in ZVL patients’ sera.

**Conflicts of Interest**

The authors declare that there are no financial or commercial conflicts of interest.

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