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Reduced High Density Lipoprotein Concentration Modulates Increased Interleukin-10 and Decreased Interferon-Gamma in Visceral Leishmaniasis Patients

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Abstract

Visceral leishmaniasis (commonly called kala-azar) is an immune-compromised parasitic protozoan disease. Hypocholesterolemia is one of the major observations in visceral leishmaniasis. The aim of this study was to evaluate and correlate total cholesterol and lipoproteins with gamma-interferon, a protective cytokine and interleukin-10, a disease progressive cytokine in visceral leishmaniasis patients. We conducted correlation studies among lipid profile, interleukin-10 and gamma-interferon on visceral leishmaniasis patients (n = 34). Lipid profile was analyzed on chemistry analyzer according to standard procedure. Gamma-interferon and interleukin-10 were analyzed quantitatively and qualitatively by enzyme linked immunosorbent assay and Fluorescence activated cell sorter respectively (n = 34). The releasing ability of interleukin-10 and gamma-interferon was assayed by fluorescence activated cell sorter (n = 10). We observed a positive correlation between total cholesterol and gamma-interferon (R = 0.364, p = 0.034) and between high density lipoprotein and gamma-interferon (R = 0.628, p = 0.0001). While negative correlation between total cholesterol and interleukin-10 (R = -0.399, p = 0.019) and between high density lipoprotein and interleukin-10 (R = - 0.526, p = 0.001) were observed. The releasing ability of interleukin-10 was increased and gamma-interferon was decreased at lower concentration of high density lipoprotein (p < 0.05). Thus, the present work suggests the correlation of total serum cholesterol and high density lipoprotein with gamma-interferon and interleukin-10 release in protection and disease progression respectively in visceral leishmaniasis.

Introduction

Visceral leishmaniasis (VL, commonly called kala-azar), occurs mostly in several Mediterranean countries and Indian sub-continent which includes India, Nepal and Bangladesh. *Leishmania donovani* (*Ld*), a parasitic protozoa, is responsible for the disease, which is transmitted to humans by the sand fly, *Phlebotomus argentipes*. India is among the highest for VL incidence in the world [1,2]. Bihar is one of the major states in India for VL. Presently, 28 out of 37 districts of Bihar are endemic and contribute about 90% of total Indian VL cases. Marked hepatosplenomegaly with moderate to severe anemia, pancytopenia are the common symptoms of VL [3].

Cholesterol has been earlier reported for its role in parasitic infections [4]. Several studies advocated about lipid disorders in children with active VL [5-9]. Decreased serum cholesterol and grossly reduced high density lipoprotein (HDL) have also been reported in pediatric VL cases [10]. VL is an immune-compromised disease but no observation is available about its relationship with lipid profile and the immune function in VL patients. However, there are reports about the immune function in VL which depicts that interleukin-10 (IL-10) is increased and interferon-gamma (IFN-γ) is reduced in serum of Indian VL patients [11]. It has also been reported that the induction of IFN-γ is critical for resistance and cure in all forms of leishmaniasis [12].

HDL is best recognized for its abilities to promote the efflux of cholesterol from cells, i.e. reverse cholesterol transport (RCT) which result in the net movement of cholesterol from peripheral tissues back to the liver via the plasma [13]. Besides the ability of HDL to modulate cholesterol bioavailability, it also affects the cells property involved in the innate and adaptive immune response and tunes inflammatory responses along with antigen presentation functions in macrophages and also activates T and B cells [14]. HDL which carries sphingosine-1 phosphate (S1P), a major active sphingolipid, modulates macrophage and lymphocyte functions in the pathogenesis of several immune-inflammatory disorders [15]. HDL composition varies to a large extent during inflammation and also functions as a reservoir for a number of biologically active substances which may impact the immune system [16]. In humans, HDL level and functions are altered in several immune-mediated disorders, such as Cohn's disease, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus and as well as during inflammatory responses [17-20]. Few studies even identified its major role in apoptosis, inflammation, infection, thrombosis and vasodilation [21-25]. The disease activity score and C-reactive protein (CRP) level are inversely associated with decreased number of small HDL particles [26]. During infection, both innate and adaptive immunities are involved in the inflammatory processes and as such HDL can be considered as a major player for immunity and toxin clearance. Since India is in a more pathogen and toxin rich environment, it is

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probably desirable to have more of HDL than our body's natural level. Hence, in the light of above assertions, the present study is aimed to see any association of total cholesterol and lipoproteins with protective immunity and disease progression in VL.

Materials and Methods

Patients and sample

The present study included 34 parasitological confirmed adult VL patients aged between 18 and 55 yrs who were admitted in the inward of Rajendra Memorial Research Institute of Medical Sciences (Indian Council of Medical Research), Patna, India, for antileishmanial treatment by Amphotericin B deoxycholate. Whole blood (5 ml) was collected by venipuncture in plain or heparin containing vaccutainer from all the study subjects. The serum was extracted from whole blood for lipid profile assay while whole blood was utilized for both quantitative (sandwich ELISA) and qualitative (Flow cytometry) assessments of IL-10 and interferon-gamma IFN- γ. The baseline characteristics of the study subjects (n = 34) are mentioned in Table 1.

Lipid profile analysis

Lipid profile was assayed on automated chemistry analyzer (Microlab-300™, The Netherlands) following the instructions of diagnostic kits (Merck-Labkit™, Spain). LDL-cholesterol was calculated by the Friedwald formula [27].

Quantitative cytokine analysis by sandwich ELISA

The IFN-γ and IL-10 levels were measured by ELISA as described elsewhere [28]. The ELISA kits used to measure the cytokine activities were obtained from BD Biosciences, USA. The results were expressed as picograms (pg) of cytokine/ml, based on the standard curves of the respective cytokine provided in the kit. The lower detection limits were 4.7 pg/ml for IFN-γ and 2 pg/ml for IL-10.

Lipoprotein separation

Lipoprotein fractions were obtained from a pool of 5 sera of healthy subjects with hypertriglyceridemia by preparative ultracentrifuge (Himac CP 100WX ™, Hitachi, Japan) on NaCl gradient [29]. The different concentrations of HDL were obtained and used to see the releasing ability of IL-10 and IFN- γ on fluorescence activated cell sorter.

Qualitative cytokine analysis by fluorescence activated cell sorter (FACS™)

Heparinized whole blood was stimulated for 6h at 37°C and 5% CO_2 with 50 ng/mL of soluble leishmania antigen (SLA) with 15 mg/dL, 20 mg/dL, 25 mg/dL, 30 mg/dL, 35 mg/dL of HDL- cholesterol separated by ultracentrifuge from hyperlipidemic healthy subjects and phorbol 12-myristate-13-actate (PMA), (Sigma, USA). During the last 4h, 10 μg/mL of Brefeldin A (BD, India) was added to arrest cytokine secretion. Erythrocytes were lysed with FACS lysing solution for 10 min (BD, India). Cell were washed twice with stain buffer (1% PBS, 0.5% filtered BSA and 0.1% NaN_3). Cells were washed and then incubated with monoclonal antibodies conjugated to FITC-CD4 for 30 minutes. The fixed cells permeabilized with 125 μL FACS buffer containing 0.1% saponin (BD, India) for 10 minutes at room temperature and then added the intracellular monoclonal antibodies conjugated to PE Interleukin-10 (IL-10), PE Interferon-γ (IFN-γ), Incubated for 30 minutes at 4°C in dark, Cells were washed once with 1 ml of stain buffer and analyzed by flow cytometer.

Characteristics	Value
Age (mean years)	31.5
Gender	
Male	16
Female	18
Clinical	
Fever (mean °C)	38.4
Spleen size below costal margin (mean cm)	6.5
Weight (mean kg)	35.5
Laboratory	
Hemoglobin (g/dl, mean)	8.0
Leucocytes (cells/nL, mean)	3.1
Thrombocytes (cells/nL, mean)	115
Serum creatinine (mg/dl, mean)	0.98
Serum albumin (g/dl, mean)	3.25
Serum alanine aminotransferase (U/L, mean)	29
Serum aspartate aminotransferase (U/L, mean)	34
Serum sodium (mmol/L, mean)	142.3
Serum potassium (mmol/L, mean)	3.8

Table 1: Baseline characteristics of 34 patients with visceral leishmaniasis.

Statistical analysis

The mean of data were compared using two tailed student t-test and Pearson's correlation coefficient with p-value was calculated. All data were expressed as Mean ± SEM and analyses were performed using Statistical Package for Social Sciences (SPSS) program version 15 (SPSS, Inc., North Carolina, USA). A p-value <0.05 was considered statistically significant.

Ethics approval was obtained from the ethics committee of the institute and patients have signed their informed consent for participation in this study.

Results

All of the studied VL patients (n = 34) revealed hypocholesterolemia and decreased high density lipoprotein (mean ± SD; 68.44 ± 4.09 mg/dl and 20.60 ± 0.94 mg/dl respectively) before the start of anti-leishmanial treatment as compared to normal reference range of lipid profile. The normal reference range of total cholesterol and HDL ranged between 130-160 mg/dl and 35-55 mg/dl respectively. We observed a positive significant correlation between total cholesterol and IFN-γ (R = 0.364, p = 0.034) (Figure 1A) and between HDL and IFN-γ (R = 0.628, p = 0.0001) (Figure 2A). The result revealed that IFN-γ concentration increases as the total cholesterol and HDL concentration increases. We also observed a significant negative correlation between total cholesterol and IL-10 (R = -0.399, p = 0.019) (Figure 1B) and significant negative correlation between HDL and IL-10 (R = -0.526, p = 0.001) (Figure 2B). The result suggested that IL-10 concentration decreases as the HDL concentration increases. We did not observed any
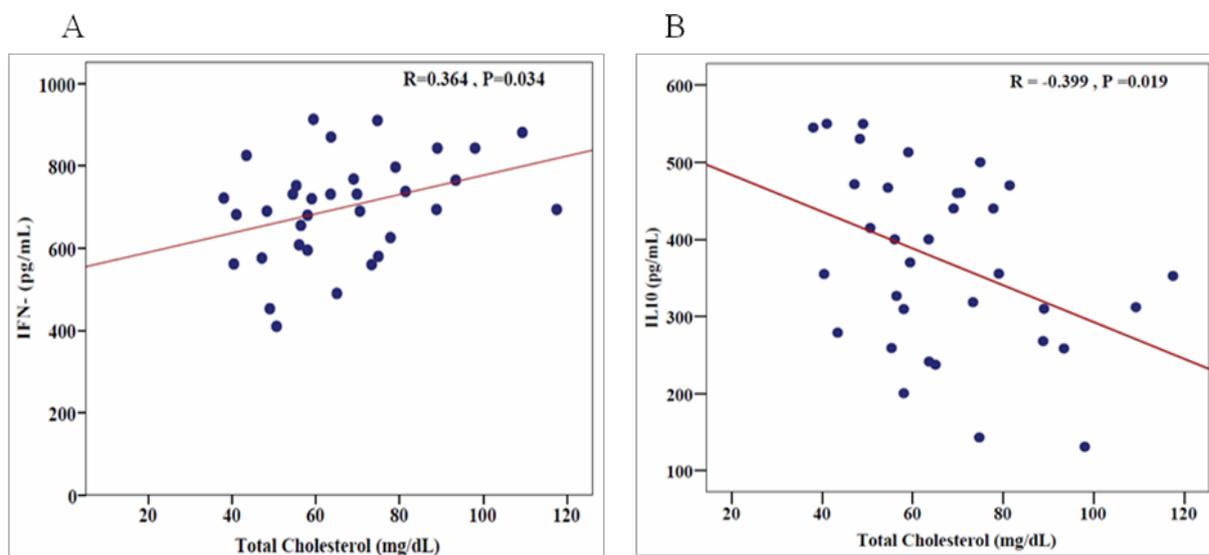
Figure 1: Scatter plot showing a reciprocal relationship between total cholesterol with IFN-γ and IL-10 in VL patients. Positive correlation between serum total cholesterol and IFN-γ (R = 0.364, p = 0.034); (Figure 1A) and negative correlation between total cholesterol and IL-10 (R = - 0.399, p = 0.001); (Figure 1B). P < 0.05 was considered statistically significant.
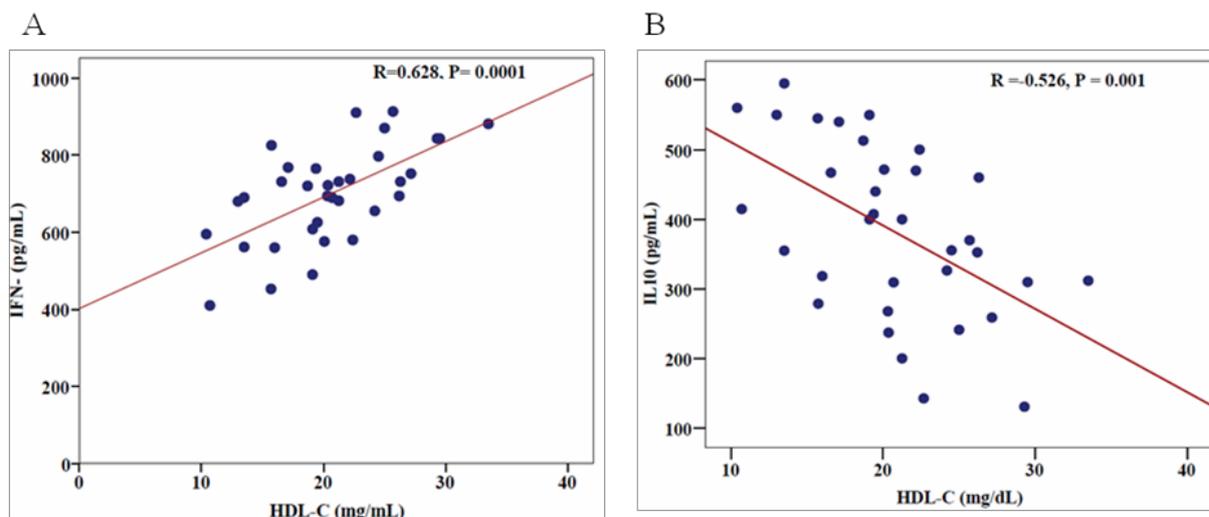

Figure 2: Scatter plot showing a reciprocal relationship between HDL with IFN-γ and IL-10 in VL patients. Positive correlation between serum HDL and IFN-γ (R = 0.628, p =0.0001) ; (Figure 2A) and negative correlation between HDL and IL-10 (R = -0.526, p = 0.001); (Figure 2B). p < 0.05 was considered statistically significant.

significant correlation between LDL and IFN-γ (R = 0.024, p = 0.892) and triglyceride and IFN-γ (R = -0.094, p = 0.597). The present results suggest the significant association of total cholesterol and HDL with disease protection as well as disease progression in VL.

The releasing ability of IL-10 and IFN-γ in the presence of HDL at different concentrations in VL patients revealed that at the lowest concentration of HDL (< 20 mg/dl), the percent releasing ability of IL-10 and IFN-γ were 13.85 ± 0.93 and 3.23 ± 0.33 respectively. Whereas, at higher concentration of HDL (>30 mg/dl), the % releasing ability of IL-10 and IFN- γ were 2.12 ± 0.34 and 14.38 ± 1.17 respectively (P < 0.05)

(Figure 3). This observation suggests that IFN-γ release increases as the concentration of HDL increases in the VL patients. Further, our present result also revealed that IL-10 release decreases as the concentration of HDL increases in the VL subjects. The representative illustration of flow diagram of cultured PBMNCs stimulated with *L. donovani* in presence of brefeldin-A and stained with anti-human CD4 FITC and anti human IFN-γ-PE and Anti human IL-10 PE of a VL patient showed an increase in frequency of IFN-γ producing CD4 cells and decrease in frequency of IL-10 (upper right of the quadrant) in an increasing concentration of HDL in VL patient (Figure 4). The above results clearly suggest that concentration of HDL in VL subjects modulates the releasing ability of
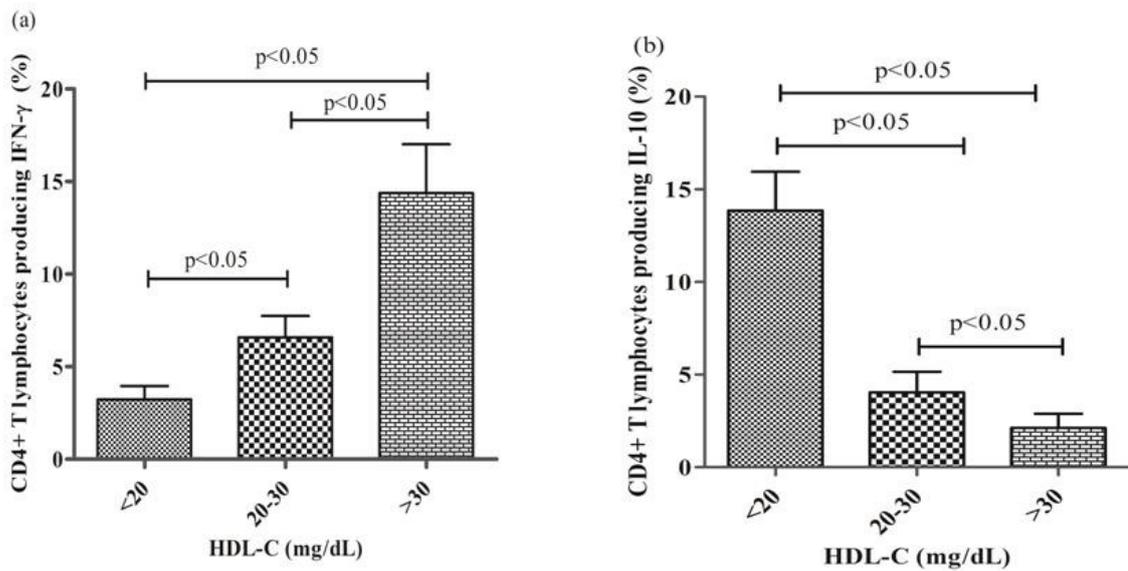
Figure 3: CD4+ T lymphocytes producing IL-10 & IFN- γ at different concentration of HDL-C in VL patients. Detection of IFN-γ and IL-10 in patients at different concentration of HDL was measured by Flow cytometry. (Figure 3a) showing comparison of CD4+ T lymphocytes producing IFN- γ at HDL <20 mg/dl (n=10), 20-30 mg/dl (n=10) and >30 mg/dl (n=10) in VL patients and (Figure 3b) showing comparison of CD4+ T lymphocytes producing IL-10 at HDL <20 mg/dl (n=10), 20-30 mg/dl (n=10) and >30 mg/dl (n=10) in VL patients. $p < 0.05$ was considered statistically significant.
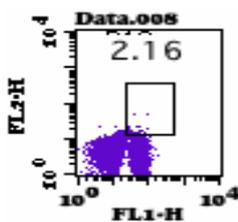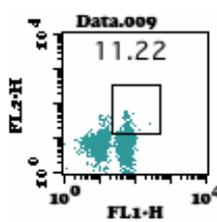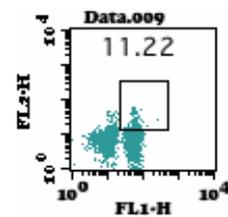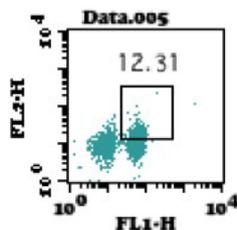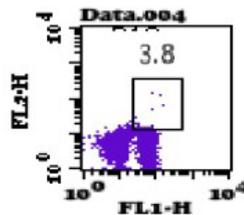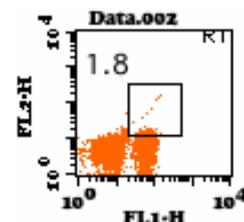

Figure 4: Detection of intracellular cytokines produced by lymphocytes of VL patients and contribution of CD4^{+} T cells. The representative illustration of flow diagram of cultured PBMNCs stimulated with *L. donovani* in presence of brefeldin-A and stained with anti- human CD4 FITC and anti human IFN-γ-PE and Anti human IL-10 PE of a VL patient showed an increase in frequency of IFN-γ producing CD4 cells and decrease in frequency of IL-10 (upper right of the quadrant) in an increasing concentration of HDL in a VL patient.

IFN-γ and IL-10.

Discussion

This is the first report indicating significant correlation of serum cholesterol and HDL with disease progression and protection in VL. The positive correlation of total cholesterol and HDL were observed with IFN-γ and IL-10 in VL patients.

Hypocholesterolemia and decreased HDL are documented in VL patients [10]. HDL is known to affect the cell property involved in the innate and adaptive immune response and tunes the inflammatory responses but none has ever studied the correlation between magnitude of cholesterol and HDL with IFN-γ and IL-10 in order to see its possible role in disease progression and protection in VL patients. One of the past studies reported about difference in immune system with less T-cell, TH1 and TH2 cells in hypocholesterolemic men than hypercholesterolemic men [30]. Earlier studies of our group reported that total serum cholesterol level in VL patients was inversely proportional to the *Leishmania* parasite burden [31]. This suggests the significance of total cholesterol concentration with disease severity in VL. The present study also revealed that at lower concentration of total cholesterol, the release of IFN-γ (a signature TH1 cytokine which is involved in protection) is less and vice-versa in VL. This suggests that higher total cholesterol concentration provides protection from VL. The clinical resolution of VL depends on a protective IFN-γ dominant TH1 from CD4$^+$ T-cells.

The TH1 immune response remains suppressed through decreasing production of IFN-γ and IL-12, which is critically regulated by IL-10, a pleiotropic cytokine secreted by many cell types including macrophage. The present present study revealed that at lower total cholesterol concentration, the IL-10 production is more, which suggest that it help to promote the disease progression. The study done on BALB/c mice revealed that IL-10 secretion by T cells influence immune activation early after infection and is sufficient for susceptible to an uncontrolled *Leishmania major* infection and also during the course of disease while IL-10-deficient BALB/C mice were able to control the infection [32].

The possible hypothesis about the role of HDL in immune modulation can be explained because B-cell receptors (BCR) and T-cell receptors (TCR) are located in lipid rafts. Removal of cholesterol from BCR lipid rafts by HDL, affects several modes of B-cell activation, including BCR-initiated signal transduction, endocytosis of BCR-antigen complexes, loading of antigenic peptides on to MHC-II, MHC-II-associated antigen presentation to T cells, and detection of helper signals via the CD40 receptor [33]. The HDL-induced cholesterol efflux from macrophages also affects antigen presentation to T cells as well as TCR signaling [34-36].

Our present work clearly suggests the possible role of HDL in disease protection as well as disease progression in VL. However, additional studies are required to answer several questions about HDL cholesterol and visceral leishmaniasis with regard to reduced plasma HDL-cholesterol levels as potential pathogenic cause of diseases. Further, HDL-Cholesterol consumption and its consequences versus benefits for protection against the disease and altered HDL function in the disease need to be undertaken.

In conclusion, it can be hypothesized that cholesterol has a significant role in the immune system and HDL plays the central role towards the protective immunity and disease progression in VL patients. Good cholesterol (HDL) diet can be considered as helpful in improving immunity and protection against VL infection and may possibly contribute a significant role in VL elimination programme of Govt. of India

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