SHORT COMMUNICATION

Human visceral leishmaniasis: decrease in serum cholesterol as a function of splenic parasite load

Kala azar or human visceral leishmaniasis (VL) is a debilitating disease associated with hepato-splenomegaly, anaemia, thrombocytopaenia and immunosuppression (Pearson *et al.*, 1983). The predominant causative agent, *Leishmania donovani*, replicates within the reticulo-endothelial system of the liver, and the liver parenchyma, although initially unaffected, is slowly damaged as the disease progresses (Alsaffar and Al Mudhaffar, 1979). Consequently, hepatic dysfunction — showing as coagulation defects and changes in the serum concentrations of several liver-specific enzymes — is typical of VL (Chakroborty *et al.*, 1949).

As the liver is the main source of cholesterol biosynthesis in mammals (Tennent *et al.*, 1957), hepatic dysfunction may lead to low serum concentrations of cholesterol and this may lead to further morbidity. Hypocholesterolaemic men tend to have significantly fewer circulating lymphocytes, total T cells, helper T-cells and CD8+ cells than hypercholesterolaemic men (Muldoon *et al.*, 1997). In their meta-analysis of 19 cohort studies covering 68,406 deaths, Jacobs *et al.* (1992) found an inverse correlation between blood concentrations of cholesterol and mortality from respiratory and gastro-intestinal diseases (most of which are of infectious origin). Subsequently, in a 15-year follow-up study of >120,000 individuals, Iribarren *et al.* (1998) found a strong inverse association between blood concentrations of cholesterol and the risk of being admitted to hospital because of an infectious disease. It appears that hypercholesterolaemia may confer a survival advantage in many, if not all, infectious diseases. In an experimental study, Netea *et al.* (1996) showed that

mice deficient in receptors for low-density lipoprotein (LDL) and with endogenous hypercholesterolaemia were protected against infection with Gram-negative micro-organisms, the lower cholesterol levels observed being associated with increased mortality. In tuberculosis, serum concentrations of cholesterol, high-density lipoprotein and LDL can be used as indirect markers of disease severity, with relatively low levels indicative of advanced disease (Rao, 2009). Åkerlund *et al.* (1986) described how, in patients with severe bacterial infections, total serum cholesterol concentrations were lowered during the acute stage of their disease.

Decreased serum cholesterol has already been reported in patients with VL (Lal *et al.*, 2007). In experimentally infected hamsters, Banerjee *et al.* (2009) not only demonstrated a significant decrease in membrane cholesterol during the active stage of *L. donovani* infection but also found that the liposomal delivery of cholesterol offered significant protection. These observations led to the present study, which was focused on cholesterol and not other lipids. In this study, since cellular cholesterol and serum cholesterol are in dynamic equilibrium (Chobanian *et al.*, 1962), serum concentrations of cholesterol in Indian patients with VL were determined as surrogate markers of cellular cholesterol. Subsequently, the relationship between the observed serum concentrations of cholesterol and the parasite burdens in the patients' spleens was explored.

PATIENTS AND METHODS

As part of the routine procedure for VL diagnosis, splenic aspirates and blood samples

were collected from suspected cases of VL who presented at the Rajendra Memorial Research Institute of Medical Sciences, Patna, India, and gave their informed consent. Amastigote burdens in the splenic aspirates were graded on a logarithmic scale, from 0 (no amastigotes in 1000 microscopic fields) to 6 (>100 amastigotes/microscopic field) (Chulay and Bryceson, 1983). Serum concentrations of total cholesterol were determined (again, as part of the routine investigation), in a Microlab-200 chemistry analyser (Merck, Darmstadt, Germany), using a commercial enzymatic colorimetric assay (Merck Diagnostic, Mumbai, India) based on the 'CHOD-PAP' method (Deeg and Ziegenhorn, 1983).

To be enrolled in the study as a VL case, a patient had to be found seropositive for leishmanial infection (i.e. for antibodies that reacted with the rK39 antigen) but seronegative for HIV-1 and HIV-2 in both a rapid test and ELISA. The control subjects who were similarly investigated, after giving their informed consent, appeared healthy, had none of the signs and symptoms of VL, had no detectable antibodies that reacted with rK39 and lived in areas of India where VL is endemic (the 'endemic controls') or where VL is not endemic (the 'non-endemic controls').

The effect of the grade given for the splenic burden of amastigotes (G) on the serum concentration of cholesterol (S ; mg/ml) in a patient aged Y years was examined using linear models of the form:

$$S = a + bG + cY$$

TABLE. Parameter estimates for the mathematical model (see main text), based on least-squares regressions and the data collected from female and male cases of visceral leishmaniasis

	Females		Males	
	Value and (95% confidence interval)	P	Value and (95% confidence interval)	P
Intercept (a)	86.18 (60.89–112.03)	<0.001	126.18 (102.81–149.54)	<0.001
Multiplier for grade of splenic burden (b)	–8.01 (–0.99–15.03)	0.03	–17.93 (–25.69–10.17)	<0.001
Multiplier for age in years (c)	0.03 (–0.56–0.62)	0.92	0.28 (–0.31–0.87)	0.36

Although not of primary interest, models were separately fitted for each gender and adjusted for age, because both gender and age are potentially important factors that modulate the level of LDL cholesterol in adults (Ulmer *et al.*, 2004; Lewington *et al.*, 2007). Interactions between gender, age and grade for the splenic parasite burden were also considered by including separate linear terms for males. Each model was fitted using standard least-squares regression (Rao, 1972) and also, to consider sensitivity to outliers, using quantile (median-based) regression (Koenker, 2005). The R software package (www.cran.r-project.org) was used for all of the data analyses.

RESULTS

The 100 subjects included in the study comprised 66 VL cases (35 males and 31 females; aged 1.5–55 years), 14 endemic controls (10 males and four females; aged 14–45 years) and 20 non-endemic controls (11 males and nine females; aged 10–58 years). The fasting blood sugar concentrations of the VL cases varied from 83–102 mg/dl.

A least-squares fit of the mathematic model to data from the VL cases (see Table) indicated that, in both the female VL cases ($P < 0.001$) and the male cases ($P = 0.03$), the serum concentrations of cholesterol decreased significantly with increasing grade for the splenic parasite burden. The mean rate of decrease was higher for the male cases (17.93 mg/dl for

FIG. A graph showing the serum concentration of cholesterol detected in each female (○) or male (Δ) subject plotted against the grade of splenic parasite load in that subject. The colour of each data-point indicates whether the subject was a known case of visceral leishmaniasis (black), a non-endemic control (red) or an endemic control (green). The two regression lines shown are fitted to the data for the female (dashed line) or male cases (solid line) of visceral leishmaniasis; the coefficients defining each of these lines are given in the Table.

each 10-fold increase in amastigote numbers) than for the females (8.01 mg/dl for each 10-fold increase in amastigote numbers). The age of the VL case appeared to have no significant effect on the serum concentration of cholesterol, whether the case was female ($P=0.36$) or male ($P=0.95$). As similar results were obtained when the model was fitted using median regression (data not shown), the results of the least-squares fit appeared insensitive to outliers. In addition, the results of the analysis of residuals did not indicate the presence of a significant non-linear trend.

The mean serum concentrations of cholesterol (and 95% confidence intervals) recorded for the female and male non-endemic controls were 145.21 (125.84–164.58) and 184.62 (163.53–205.71) mg/dl, respectively — significantly higher than the corresponding values recorded for the

female [109.45 (91.63–127.27) mg/dl; $P=0.004$] and male [115.16 (106.28–124.04) mg/dl; $P<0.001$] endemic controls.

Using the relevant fitted model for the VL cases (Table), the predicted serum concentration of cholesterol for a woman aged 22 years with no detectable amastigotes in her spleen ($G=0$) was 86.82 mg/dl. The corresponding 95% confidence interval (62.80–110.84 mg/dl) contains the mean cholesterol concentration recorded among the female endemic controls but not that recorded among the female non-endemic controls. Similarly, according to the model described for male VL cases in the Table, the predicted cholesterol concentration for a man aged 22 years with no detectable amastigotes in his spleen ($G=0$) was 132.27 mg/dl. The corresponding 95% confidence interval (106.30–158.24 mg/dl) contains the mean cholesterol concentration recorded among the male endemic

controls but not that recorded among the male non-endemic controls. These comparisons are made visually apparent in the Figure.

DISCUSSION

The present results indicate that in VL cases (at least, in VL cases from eastern India), serum concentrations of cholesterol are inversely correlated with the density of amastigotes in the spleen. As serum cholesterol and membrane cholesterol are in dynamic equilibrium (Chobanian *et al.*, 1962), cellular concentrations of cholesterol levels are presumably relatively low in patients with high amastigote burdens in their spleens. Cellular cholesterol is required for the assembly of membrane lipid rafts, and *L. donovani* is already known to affect the antigen presentation of macrophages by disrupting such rafts (Chakroborty *et al.*, 2005), probably by decreasing membrane cholesterol. This may explain why, in hamsters at least, the liposomal delivery of cholesterol can lead to the clearance of *L. donovani* infections (Banerjee *et al.*, 2009).

The role of membrane cholesterol in the interaction between T-cells and antigen-presenting cells (APC) has been well studied, in various systems. Cholesterol treatment enhances the antigen-presenting function of monocytes, for example, by up-regulating the expression of molecules of major histocompatibility complex (MHC) class II (Hughes *et al.*, 1992). In infections with *Helicobacter pylori*, it also promotes the phagocytosis of the bacteria by APC and enhances an antigen-specific T-cell response during bacterial challenge, leading to a T-cell-dependent reduction in the number of bacteria in the stomach (Wunder *et al.*, 2006). Treatment of APC with nystatin or methyl cyclodextrin, which are known quenchers of cholesterol from the membrane, reduces the cells' antigen-presenting ability without affecting their surface expression of class-II molecules (Anderson *et al.*, 2000). Thus, cholesterol may play a decisive role in the cell-mediated immune response of the

host. Defective cellular immunity is one of the main characteristics of VL (Pearson *et al.*, 1983). In the present study, all of the VL cases investigated had fasting blood sugar levels that were in the 'normal' range, indicating that *L. donovani* affects the cholesterol metabolism but not the general carbohydrate metabolism of its human hosts.

It is unclear why the mean serum concentration of cholesterol in the endemic controls was significantly lower than that in the non-endemic controls, although the possibility that some of the endemic controls were infected with *L. donovani* cannot be excluded. Although all of the endemic controls had been found negative for antibodies to the rK39 antigen, patients in the early stages of *L. donovani* infection may appear seronegative (Evans *et al.*, 1992).

The major question posed by the present results is 'how do intracellular parasites affect the serum-cholesterol profile?', especially since *L. donovani* in the liver of its human host replicates within Kupffer cells and not in the hepatic parenchyma. It is possible that parasite-derived factors carried in the host's circulation affect the metabolic activity of uninfected cells, perhaps enhancing cholesterol catabolism and/or decreasing cholesterol biosynthesis. An alternate possibility is that the parasites directly exploit cholesterol from their host, for their own growth. The mechanism by which *L. donovani* induces the dysregulation of its host's cholesterol metabolism, if any, is currently under investigation.

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