The aim of this study was to investigate the renal function of patients with visceral leishmaniasis (VL) and post-kala-azar dermal leishmaniasis (PKDL) by means of the specific marker cystatin C and related to circulating immune complexes and cytokine production. Forty patients with VL (23 with sub-acute disease and 17 with acute disease), 17 patients with PKDL, and 22 healthy controls were included. Cystatin C, but not creatinine, was significantly raised in VL ($P = 0.004$). The highest levels of cystatin C were found in those with acute disease ($P < 0.0001$). In VL, cystatin C levels were positively correlated to circulating immune complexes and production of granulocyte-macrophage colony stimulating factor (GM-CSF), but negatively correlated to aspartate aminotransferase and lactate dehydrogenase. We conclude that cystatin C is a superior marker of glomerular function in leishmaniasis and that immune complex deposition and GM-CSF are two functions that most likely are causally involved in the mechanisms leading to glomerular dysfunction in leishmaniasis.

INTRODUCTION

Visceral leishmaniasis (VL) is a disease of public health importance in Sudan and many other tropical countries and is considered to be one of the main health foci of these countries. VL is a disease caused by protozoa of the Leishmania donovani complex ($L.$ donovani, $L.$ archibaldi, and $L.$ infantum/chagasi). Leishmania, which are introduced into their human hosts by sand flies, rapidly invade macrophages, where they multiply inside phagolysosomes. Human infections may be asymptomatic (sub-clinical) or may cause a severe visceral disease that is called kala-azar (KA). The clinical manifestations include recurrent fever, hepatosplenomegaly, general lymphadenopathy, pancytopenia, and anemia. Death occurs in the absence of appropriate chemotherapy. Post-KA dermal leishmaniasis (PKDL) is a complication of VL and is characterized by a macular, maculopapular, and nodular rash in patients who have recovered from VL and who are otherwise well. The rash usually starts around the mouth, from where it spreads to other parts of the body depending on severity. It is mainly seen in Sudan and India, where it follows treated VL in 50% and 5–10% of cases, respectively. The interval at which PKDL follows VL is 0–6 months in Sudan and 2–3 years in India. During infection with leishmania, various host organs are affected, including the liver, spleen, and kidney. VL-related nephropathy is known both in humans and animals. However, in humans, most studies are based on very few cases, which showed different manifestations such as acute proliferative glomerulonephritis (GN), collapsing focal segmental glomerulosclerosis, acute interstitial nephritis, tubular cell necrosis, and tubulitis, as well as acute renal failure. In mice, renal involvement appeared as a diffuse inflammatory process composed of mononuclear cells in the kidney, and in canine VL, the kidneys showed diffuse membranoproliferative glomerulonephritis. In humans, renal involvement was identified by the presence of hematuria and proteinuria, but also in some cases, by rising serum creatinine or urea levels. The mechanisms underlying renal disease in VL may be many, but the involvement of circulating immune complexes and their deposition in the kidney has been shown to incriminate in the pathogenesis of VL.

Cystatin C is a cysteine proteinase inhibitor with a relative molecular weight of 13,250 da and is formed by all nucleated cells studied. Because cystatin C is formed at a constant rate and freely filtered by the healthy kidney, this protein is a good marker of renal function. Serum concentrations of cystatin C are almost totally dependent on the glomerular filtration rate (GFR). A reduction in the GFR causes a rise in cystatin C concentration. Cystatin C is not affected by factors such as muscle mass and nutrition, factors that have been shown to affect creatinine and urea values. Furthermore, the levels of cystatin C are independent of age and sex. In contrast to creatinine that requires a fall in GFR of $> 50\%$ before the levels start to increase, cystatin C seems to be particularly useful in cases with minor impairments of the glomerular filtration rate. The aim of this study was to investigate the renal function in subjects with VL and PKDL by means of cystatin C and relate the findings to circulating immune complexes. For comparison, we also studied liver function by various biochemical tests.

MATERIALS AND METHODS

Study area. The study was carried out at Tabarakalla rural hospital in Gedarif state. The reception area for the hospital is situated along the lower Atbara River in Gallabat Province, eastern Sudan. The area is located ~70 km southeast from Gadarif town. It is endemic for $L.$ donovani, and the main leishmania vector in the area is Phlebotomus orientalis. Patients enrolled in the study mainly came from the Tabarakalla and Barbar Elfogara villages.

Clinical history and examination. A detailed clinical history was obtained from each studied patient. Particular emphasis was made regarding previous or any form of leishmaniasis. Subjects were questioned about their ethnic and geographic origin and were examined for clinical manifestations of VL and PKDL. A general clinical examination was conducted with particular reference to hepatosplenomegaly, position and number of enlarged lymph nodes, and presence of scars of previous cutaneous leishmaniasis. Liver size was measured...
in the mid-clavicular line from the costal margin; the spleen size was assessed by measuring the distance between the costal margin in the anterior axillary line to the tip of the spleen. Lymphadenopathy was classified as localized if found only at one site and generalized if at two or more sites. The oral and nasal mucous membranes were examined for evidence of mucosal leishmaniasis. Both thick and thin blood films were examined for malaria parasites for all individuals with fever or splenomegaly.

**Diagnosis.** Inguinal lymph node aspiration was performed on those clinically suspected of having VL (i.e., all individuals with any of the following clinical findings: fever for > 2 months, left upper quadrant pain, lymphadenopathy, splenomegaly, or wasting). All individuals with clinically suspected VL but with negative results on inguinal lymph node aspiration were subjected to bone marrow aspiration from the superior posterior iliac crest. Bone marrow smears were fixed with methanol, stained with Giemsa, and examined under an oil-immersion lens for *L. donovani* bodies. Severely ill patients with VL admitted to the hospital because of need for further medical care were classified as acute VL. Patients not severely ill and treated as outpatients with daily injections of sodium stibogluconate were classified as sub-acute VL. PKDL was diagnosed on clinical grounds as the appearance of rash with typical distribution after treatment of VL with sodium stibogluconate. The interval between VL and the occurrence of PKDL and the duration of the rash was estimated from the patients’ oral statement.

**Patients and sample collection.** Venous blood drawn from leishmaniasis-infected patients and healthy controls was separated by centrifugation and frozen in liquid nitrogen within 2 hours of sampling. Serum and plasma samples were stored and transported frozen in liquid nitrogen until analyzed in Uppsala, Sweden. Samples were obtained from 42 patients with VL (32 men and 10 women; mean age, 21 years), 17 patients with PKDL (13 men and 4 women; mean age, 10 years), and 22 healthy controls (17 men and 5 women; mean age, 21 years). Ethical approval for this study was obtained from the ethical committee of the Faculty of Medicine, University of Khartoum, and from the Ministry of Health, Gedaref State, Sudan. Informed consent was obtained from all the adults who participated in the study. For younger children, consent was obtained from their parents.

**Laboratory assays.** Plasma levels (EDTA-plasma) of cystatin C, creatinine, uric acid, gamma-glutamyl transferase (γ-GT), albumin, lactate dehydrogenase: (LD), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (BIL-T), and conjugated bilirubin (Con-BIL) were all measured on the Architect instrument (Abbott Laboratories, Abbott Park, IL) at the routine department of Clinical Chemistry, University Hospital, Uppsala, Sweden, according to the instructions of the manufacturer. Levels of circulating immune complexes (CIC) in the serum samples were determined by a commercially available kit (Bindazyme C1q binding CIC EIA; Binding Site, Birmingham, UK).

In a parallel report we described studies on in vitro cytokine production induced by immune complexes (IC) obtained from serum of leishmaniasis-infected patients (A. Elshafie and others, unpublished data), in analogy with our earlier report on systemic lupus erythematosus patients.24 Briefly, serum was precipitated by 5% polyethylene glycol (PEG) 6000. The precipitate was incubated for 20 hours at 37°C with peripheral blood mononuclear cells (PBMCs) from healthy subjects in serum-free conditions, whereupon cytokines (granulocyte-macrophage colony stimulating factor [GM-CSF], interleukin 1 receptor antagonist [IL1ra], IL1β, IL6, IL10, tumor necrosis factor-α [TNF-α], and tumor necrosis factor receptor [TNFR] in the supernatants were measured by ELISA as earlier described.25

**Statistics.** For the comparison of more than two groups, we used analysis of variance (ANOVA), and for the comparison between two groups, we used the non-parametric Mann-Whitney test for unpaired samples. To estimate correlations between variables, we used the Spearman non-parametric test. All calculations were made by means of the software Statistica for Windows, v 7.0 (Tulsa, OK).

**RESULTS**

**Biochemical markers of kidney and liver functions.** In Table 1, the plasma levels of creatinine and some liver function tests are shown. The levels of creatinine were not elevated in subjects with VL compared with controls, whereas the levels in subjects with PKDL were significantly lower compared with controls (*P < 0.001*). The levels of the liver enzymes ALT, AST, and LD were elevated in VL compared with controls and compared with PKDL.

In subjects with acute VL, the creatinine levels were elevated compared with the cohort with sub-acute disease (*P = 0.03; Table 2). Also, the levels of γ-GT and bilirubin were elevated in the acute form of disease, but with no differences for the other liver enzymes.

**Cystatin C.** Cystatin C was measured in plasma in subjects with VL and PKDL and in control subjects. Overall, there was a statistically significant difference between the groups (*P = 0.0004, ANOVA; Figure 1). As shown in the figure, the levels in VL were significantly raised compared with subjects with PKDL (*P = 0.001*) and also compared with healthy controls.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 23)</th>
<th>VL (n = 40)</th>
<th>PKDL (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-GT (µkat/L)</td>
<td>0.30 ± 0.14</td>
<td>1.20 ± 2.40</td>
<td>0.27 ± 0.10</td>
</tr>
<tr>
<td>ALT (µkat/L)</td>
<td>0.15 ± 0.15</td>
<td>0.46 ± 0.65*</td>
<td>0.35 ± 0.23†</td>
</tr>
<tr>
<td>Bilirubin, total</td>
<td>5.7 ± 3.3</td>
<td>7.6 ± 10.6</td>
<td>3.7 ± 1.9*</td>
</tr>
<tr>
<td>AST (µkat/L)</td>
<td>0.44 ± 0.12</td>
<td>1.75 ± 2.12†</td>
<td>0.75 ± 0.29†</td>
</tr>
<tr>
<td>LD (µkat/L)</td>
<td>7.5 ± 4.5</td>
<td>13.1 ± 11.9*</td>
<td>5.1 ± 1.8</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>88 ± 90.3</td>
<td>97 ± 94*</td>
<td>62 ± 20†</td>
</tr>
</tbody>
</table>

Results are given as means ± SD. Statistical differences between controls and either of the two diseased groups are given by *P < 0.05, †P < 0.01 and **P < 0.001. Statistical differences between the two diseased groups are given by §P < 0.05, ¶P < 0.01 and ***P < 0.001. Mann-Whitney U test was used.

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Subacute VL (n = 23)</th>
<th>Acute VL (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-GT (µkat/L)</td>
<td>0.98 ± 0.87</td>
<td>1.51 ± 1.59</td>
</tr>
<tr>
<td>ALT (µkat/L)</td>
<td>0.47 ± 0.73</td>
<td>0.45 ± 0.56</td>
</tr>
<tr>
<td>Bilirubin, total (µmol/L)</td>
<td>4.7 ± 5.3</td>
<td>11.5 ± 14.5*</td>
</tr>
<tr>
<td>AST (µkat/L)</td>
<td>1.70 ± 2.33</td>
<td>1.84 ± 1.89</td>
</tr>
<tr>
<td>LD (µkat/L)</td>
<td>12.9 ± 12.3</td>
<td>13.4 ± 11.8</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>77 ± 17</td>
<td>123 ± 141*</td>
</tr>
</tbody>
</table>

Results are given as means ± SD. Statistical differences between the two groups are given by *P < 0.05 and †P < 0.01. Mann-Whitney U test was used.
However, the levels of cystatin C were similar among controls and subjects with PKDL. When the cohort of subjects with VL was divided in acute and sub-acute disease, the levels of cystatin C were significantly higher among those who had an acute disease ($P < 0.0001$; Figure 2).

**Relations between circulating immune complexes and renal or liver functions.** In a separate report, we showed that the circulating immune complexes are elevated in VL and PKDL and have the highest levels in the acute form of VL (A. Elshafie and others, unpublished data). To test the hypothesis that circulating immune complexes might be involved in the impairment of multiple organ functions, we correlated the CIC levels with the biochemical markers of kidney and liver functions. As shown in Figure 3, the levels of the circulating immune complexes were significantly correlated with the levels of cystatin C in subjects with VL ($r_s = 0.52$, $P < 0.001$). A significant correlation was also seen in the subgroup of subjects with acute VL ($r_s = 0.52$, $P < 0.05$), whereas no such relationships were found in the group of sub-acute VL or in the cohort with PKDL (data not shown).

For the whole group of subjects with VL or PKDL, we did not observe any correlations between the other biochemical markers of kidney and liver functions or circulating immune complexes. In the cohort of acute VL, however, we saw significant negative correlations to LD ($r_s = -0.52$, $P = 0.04$) and AST ($r_s = -0.56$, $P = 0.02$; data not shown).

**Relations between cytokine productions and renal or liver functions.** In a parallel report, we showed that IC-induced productions of a number of pro- and anti-inflammatory cytokines are increased in leishmania-infected patients compared with Sudanese controls. Among the studied cytokines, the production of GM-CSF was the only cytokine found to be raised in acute VL (A. Elshafie and others, unpublished data). A relationship between the markers of organ dysfunctions and the production of these cytokines was studied. Only the production of GM-CSF showed significant correlations with cystatin C in the whole group of leishmaniasis ($r_s = 0.30$, $P = 0.03$) and in the sub-group of patients with acute VL ($r_s = 0.54$, $P = 0.04$). In the subgroup with acute VL, a correlation was also seen to AST ($r_s = -0.57$, $P = 0.02$).

**DISCUSSION**

This study has shown that subjects with VL very often have impaired glomerular filtration and that this problem was almost invariably found in those with an acute disease. By measuring the new and more sensitive and specific marker of glomerular filtration rate, cystatin C, our results re-emphasize the importance of renal dysfunction in VL and confirm earlier case reports in this matter.8–11 Our study also suggests that the renal dysfunction may be directly related to the amount of circulating immune complexes and to the induction of GM-CSF production.

As noted in the introduction, VL is a major healthy problem in endemic areas of Sudan, with a high mortality rate if left untreated. In our study, the diagnosis of VL was based on the state-of-the-art principles, which include the demonstra-
tion of the actual parasite in aspirate materials and typical clinical findings. The differentiation in acute and sub-acute disease was somewhat arbitrarily based on the clinical condition of the patients and their need of hospital care. However, the very clear distinction between these two sub-groups in many of the measured variables supports the criteria for the allocation of the subjects into the two diagnostic sub-groups. One caveat in the interpretation of our data is the fact that all subjects with PKDL were children. However, as will be discussed below, this caveat is probably of minor importance in the interpretation of our cystatin C data, but should be borne in mind in the interpretation of many of the other biochemical variables.

In previous studies on leishmaniasis, glomerular function has been estimated by serum creatinine and urea levels. These reports have shown varying results and have mostly been based on case studies. Creatinine and urea levels are both confounded by several unrelated factors, which seriously may hamper the detection of any reduction in glomerular filtration. It is well documented that serum creatinine levels are dependent on muscle mass, and therefore, very different levels are found in men and women, but also in children. In addition, many of the subjects with VL were in poor condition, some with extensive muscle wasting, which would give rise to false-negative creatinine levels. The estimation of glomerular filtration by means of urea is even more complicated, because urea, among other things, is dependent on diet and diuresis. With the introduction of cystatin C, an accurate and sensitive means of the estimation of GFR has become available, which is unaffected by all the factors influencing creatinine and urea levels. Until now, only GFR has been shown to determine the circulating levels of cystatin C. Therefore, our findings of highly raised levels of cystatin C in the acute form of VL very clearly indicate that glomerular filtration is generally impaired in this condition as opposed to the patients with sub-acute VL and as opposed to those having PKDL.

In this study, we also measured several other biochemical markers to evaluate other organ involvements in leishmaniasis. ALT and γ-GT are both fairly specific markers of liver function, and the finding of raised levels of both these enzymes in VL indicates liver involvement, which was expected based on previous reports and based on the clinical findings with hepatosplenomegaly in many cases. However, the ALT levels were similar in acute and sub-acute disease in contrast to γ-GT and bilirubin, which indicate that liver disease is less of a destruction of the liver cells and more of a swelling and obstructive disease. The raised levels of AST in VL could have the same explanation, but could also be of muscle origin. Lactate dehydrogenase is found in the cytoplasm of all cells of the body, and the high levels in VL could have multiple origins, such as liver, muscle, and red blood cells. In children with PKDL, the only sign of liver involvement was slightly raised ALT and AST, whereas the other biochemical markers were unaltered or even lower than the controls. However, as mentioned above, we lack in this study an age-matched control group, which hampers the interpretation of this data.

One aim of this report was the study of the mechanisms of organ impairment in VL. It was therefore of considerable interest that we found very close correlations between the amount of circulating immune complexes and GFR as estimated by cystatin C but not as estimated by creatinine levels. Thus, immune complex deposition in the glomeruli seems to be a likely cause of renal dysfunction in acute VL. Indeed, immune complex deposition has been shown previously in some cases with VL and also in animals infected by leishmania. In a separate report, we showed raised production of GM-CSF and some other cytokines by PBMCs after incubation with serum precipitates obtained from the subjects with VL with acute disease. However, of the studied cytokines, only the production of GM-CSF was correlated to cystatin C. This was an interesting finding, because GM-CSF has been implicated recently in the pathogenesis of glomerulonephritis in animal studies. One putative chain of reactions is that circulating immune complexes stimulate the production of GM-CSF by monocytes/macrophages and that GM-CSF enhances the production and activation of neutrophil granulocytes, which in turn react to deposited immune complexes in the glomeruli with ensuing destruction of the tissue. Another possibility is that immune complex deposition in the glomeruli directly induces GM-CSF production by the mesangial cells in the glomeruli, with consequent activation of neutrophils. Seemingly odd findings were the negative correlations between LD and AST on one hand and the amount of circulating immune complexes and GM-CSF production on the other hand. Recent studies showed the detrimental effect of IgG production against the parasite antigen, which seemed to prevent the efficient eradication of the parasite. The mechanisms likely involved were the immune complex induced production of the anti-inflammatory cytokine IL-10. These findings seem to reject the prevalent theory, because the current dogma is that increments in specific IgG would enhance elimination and protect the subject from the adverse consequences of the infection. In our cases, we could argue that efficient immune complex formation is protective against some of the adverse effects of the parasite on organs such as the liver, whereas other organs, such as the kidney, suffer from immune complex formation because of the deposition at sites that are particularly vulnerable.

We conclude that cystatin C is a superior marker of glomerular function in subjects with leishmaniasis and that immune complex deposition and GM-CSF are two functions, which most likely are causally involved in the mechanisms leading to glomerular dysfunction in this disease. In leishmaniasis immune complex formation may be good to the host by the reduction of antigen deposition in certain organs, but also bad to the host by deposition in vulnerable structures such as the glomeruli of the kidney.

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