ORIGINAL ARTICLE

Cytokine profile of patients with leptospirosis in Sabah, Malaysia

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ABSTRACT

Background: Leptospirosis is a zoonotic disease with symptoms ranging from a mild, febrile illness to a severe form with multiorgan failure. Severe leptospirosis may require medical interventions in the form of dialysis and/or mechanical ventilation and often leads to mortality. An exaggerated host immune response—in particular, a "cytokine storm"—that causes endothelial and organ damage is associated with the disease severity and mortality.

Methods: Microscopic agglutination test (MAT)-positive and MAT-negative human serum samples (n=30) from patients with leptospirosis were obtained from the Public Health Laboratory, Kota Kinabalu, Sabah, Malaysia and control serum samples (n=10) were obtained from healthy student volunteers. We estimated the levels of IL-1β, IL-6, IL-8, IL-10, and TNF-α in serum samples by a Luminex assay.

Results: The levels of IL-6, IL-8, and IL1-β were significantly higher in 13% of the patients with leptospirosis compared to the healthy controls, while the levels of IL-10 and TNF-α were not elevated in either group.

Conclusion: Our data suggest that elevated levels of IL-6, IL-8, and IL1-β may be associated with leptospirosis disease severity, which requires patient follow-up for confirmation.

Key WORdS:
Leptospirosis, disease severity, cytokine, Luminex assay

INTRODUCTION

Leptospirosis is a zoonotic disease caused by pathogenic species of *Leptospira interrogans*.[1] It occurs throughout the world, and outbreaks of leptospirosis have been reported after sporting events and floods.[2] The initial symptoms of leptospirosis include fever, myalgia, nausea, skin rash, chills, and headache, all of which resemble symptoms of other acute febrile illnesses, and these symptoms can resolve spontaneously.[3] However, about 5-15% of patients with a mild form of the disease may progress to a severe form known as Weil's syndrome, which includes multi-organ failure with haemorrhages, splenomegaly, tubulointerstitial nephritis, jaundice, pulmonary damage, and septic shock.[4,5]

The risk factors for severe leptospirosis include smoking, the serovar of *L. interrogans*, an infectious dose, differences in the host immune response, and a delay in antibiotic therapy.[3,6] The mortality rate for severe leptospirosis has been reported to be 5-10%, and it can rise to 50% in patients with a pulmonary haemorrhage.[7] Although the pathogenic mechanism of leptospirosis is not fully understood, the current evidence shows that a prolonged host immune response could play a role in the disease severity.[7,8] The inflammatory process resulting from the activation of the immune response that occurs during the clearance of *Leptospira* from the circulation is associated with organ damage, as observed in patients with severe clinical manifestations.[6,9,11] Several reports have demonstrated that severe cases of leptospirosis can be differentiated from mild cases by the presence of a "cytokine storm".[6,9,11] Patients with leptospirosis show elevated levels of TNF-α, IL-1β, IL-6, IL-8, and IL-10 compared to healthy individuals.[6,12] Higher levels of these cytokines are associated with disease pathogenesis such as lung injury in severe pulmonary haemorrhagic syndrome (SPHS), hepatitis, and others and are considered to be predictors of mortality.[6,8,11,13,14] Because the cytokine profile of leptospirosis patients in Borneo region is not known, we estimated the serum levels of five cytokines that were shown to be associated with the disease severity in leptospirosis patients and compared the levels to healthy controls.

MATERIALS AND METHODS

Clinical samples

The present study was part of an epidemiologic study to determine the etiological agents of nonmalarial acute febrile illnesses. The study was approved by the Medical Research and Ethical Committee, Ministry of Health, Malaysia, and the study number is NMRR-13-677-15713. Microscopic agglutination test (MAT)-positive and MAT-negative human serum samples were provided by the Public Health Laboratory, Kota Kinabalu, Sabah, Malaysia. Control serum samples were obtained from healthy student volunteers from the same area. All the serum samples were stored at -20°C until they were tested.

Determination of cytokines by Luminex assay

The serum samples were sent to Biomarketing Services Sdn. Bhd., Kuala Lumpur, Malaysia, for the estimation of cytokine levels. The levels of IL-1β, IL-6, IL-8, IL-10, and TNF-α in the

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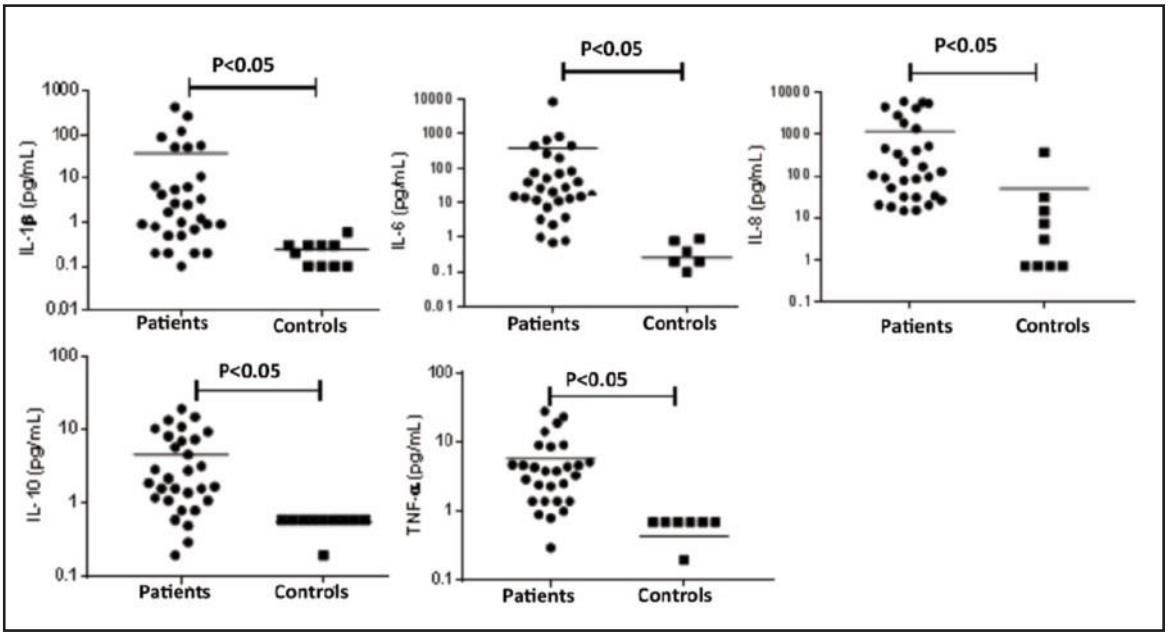

Fig. 1: Serum concentration of cytokines in leptospirosis patients and healthy controls. The P value shows the difference between the patients and controls, as calculated by the Mann-Whitney U test.

patient and control samples were measured by a Luminex assay (BD Biosciences, USA). The serum samples were diluted 1:2 in the kit diluent, and the assay was performed according to the manufacturer's instructions. The results were expressed in pg/mL.

Statistical analysis

A significant difference in the level of cytokines between the patient and control samples was determined using the Mann-Whitney *U test* in the SPSS program (2013).

RESULTS

Three MAT-negative and 27 MAT-positive samples from leptospirosis patients who were positive for both *Leptospira*-specific IgM and IgG by ELISA and 10 samples from healthy controls were tested for the presence of five cytokines. The control samples contained undetectable or very low levels of IL-1β, IL-6, IL-8, IL-10, and TNF-α, with the exception of one subject with 383 pg/mL of IL-8 (Figure 1). Although the levels of all these five cytokines were significantly higher in patients compared to the control group ($p<0.05$), the levels of IL-10 (0-19.2 pg/ml) and TNF-α (0-27.6 pg/ml) were low in all the patients. Of the 30 samples tested from patients with leptospirosis, only four (13%) showed elevated levels of IL-1β (92-446 pg/mL), IL-6 (270-8621 pg/mL), and IL-8 (348-6273 pg/mL). In addition, two patients showed higher levels of IL-6 (200 and 670 pg/mL) and IL-8 (1412 and 4381 pg/mL), one patient had a higher level of IL-6 (460 pg/mL), and eight other patients showed elevated levels of IL-8 (169-6004 pg/mL). The cytokine levels were not elevated in 15 patients (50%). Overall, 47%, 23%, and 13% of the patients showed elevated levels of IL-8, IL-6, and IL-1β, respectively.

DISCUSSION

Although inflammatory cytokines are necessary for bacterial eradication, the extensive release of pro-inflammatory cytokines causes pathological inflammatory disorders, tissue injury, and organ failure.[9,15] It has been reported that components of *Leptospira* including glycolipoprotein and lipopolysaccharide are released after bacterial lysis. They inhibit Na/K-ATPase, which may directly cause tissue injury or increased plasma levels of non-esterified fatty acids, which can stimulate the production of inflammatory mediators. This leads to the exacerbation of the immune response associated with the multi-organ dysfunction that is observed in the severe form of the disease.[16] Severe cases of leptospirosis can be differentiated from mild cases by a "cytokine storm" process and certain cytokines are associated with immunopathology and increased patient mortality.[6,11,17] Previously, ELISA kits were used to estimate the concentrations of cytokines in patients, but that assay requires a large volume of serum.[11,14] Recent studies have reported the use of a cytometric bead array (CBA) and a Bio-Plex Multiplex cytokine assay, both of which have a higher analytical capacity and require smaller sample volumes.[6,13,18] Other studies have reported the use of quantitative PCR and microarrays for analysing cytokine expression in humans and animals.[4,19-21] In this study, we used a Luminex assay that requires only a small volume of serum for the measurement of multiple cytokines.

It was reported earlier that higher levels of IL-10 (>140 pg/mL) and TNF-α are associated with leptospirosis fatalities and that a high IL-10:TNF-α ratio can serve as a predictor of the disease severity and mortality in leptospirosis.[4,6,11] Elevated levels of TNF-α were shown to be associated with kidney, liver, and lung involvement.[20,23] The levels of TNF-α and IL-10 were in the range of 0-28 pg/mL and 0-19 pg/mL,

respectively, in our study patients. This is similar to a few studies where no significant difference in the levels of TNF-α and IL-10 was observed between patients who died and those who survived, and the percentage of CD4+ T cells producing IL-10 did not differ between healthy controls and patients with severe or mild symptoms.[4,6,24]

On the other hand, 47%, 23%, and 13% of our study patients showed elevated levels of IL-8, IL-6, and IL-1β respectively. A Brazilian study using a CBA showed that the concentrations of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17A, and TNF-α were significantly higher in patients with severe disease compared to mild disease. Among the severe patients, the levels of IL-6 and IL-8 were higher in the nonsurvivors than in the survivors, while higher levels of IL-6 were associated with pulmonary haemorrhage.[6] A study in Thailand demonstrated that patients with organ involvement showed significantly higher levels of IL-6, IL-8, and IL-10 in acute serum samples and only IL-8 in convalescent serum samples, compared to patients without organ involvement.[12] Wang et al.[22] demonstrated that IL-1β, IL-6, and TNF-α were the main proinflammatory cytokines in the sera of patients with leptospirosis. Higher levels of IL-6 and IL-8 were shown to be associated with disease severity and mortality.[13,14,17] In contrast, other studies showed no significant difference in the IL-β and IL-6 levels between mild and severe cases of leptospirosis and the concentrations of IL-6 and IL-8 did not seem to affect the disease outcome.[4,11] Based on our assay results, only four out of 30 patients (13%) showed elevated levels of IL-6, IL-8, and IL-1β, suggesting that these patients may have the possibility of developing the severe disease, while the majority of our patient population had only a mild form of leptospirosis. This needs to be confirmed using clinical presentation, which requires patient follow-up. IL-8 is a proinflammatory mediator that induces chemotaxis in neutrophils and associated with leptospiral hepatitis, while IL-6 causes endothelial barrier dysfunction and associated with SPHS.[6,24] These two cytokines may have a role in the pathophysiology of Weil's syndrome.[6,11,12] Undetectable or very low levels of all the five cytokines were seen in control subjects and this could be due to prolonged storage of the serum samples.

It has been demonstrated that treatment with emodin, thymol, and astragalin inhibited the expression of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 by regulating NF-κB and mitogen-activated protein kinase signalling pathways in leptospira-infected uterine and endometrium epithelial cells of mice.[25] Immunomodulatory therapy with glucocorticosteroids is recommended for patients with severe symptoms and organ failure.[26,27]

CONCLUSION

This is a preliminary data suggesting elevated levels of IL-6, IL-8, and IL1-β may be associated with leptospirosis disease severity. Clinical history of our study patients could not be obtained for correlating with disease severity and this is the limitation of this study. A detailed study with larger sample size and patient follow-up is necessary to better understand to the role of inflammatory cytokines in disease severity of leptospirosis.

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