Original Article

Role of IL-8, IL-10 and TNF-α level in pathogenesis of leptospiral acute hepatitis syndrome

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Abstract

Background: Leptospirosis is the most widespread zoonosis occurring worldwide. Severity of disease may range from inapparent infection to fulminant, fatal disease. Besides, direct injury by microbial factors, cytokines produced in response to infection have been proposed to be involved in the pathogenesis of leptospirosis.

Objective: This study was done to assess the impact of cytokines IL-8, IL-10 and TNF-α in pathogenesis of leptospirosis and to establish their role as diagnostic markers in disease severity.

Methods: Forty six patients with signs and symptoms of acute hepatitis who were positive for leptospira by IgM ELISA along with 30 control individuals were included in the study. Concentrations of circulating IL-8, IL-10 & TNF-α were measured in pg/ml by ELISA.

Results: There were two to threefold rise in liver enzymes in patients with leptospirosis. Concentrations of IL-8, IL-10 were found to be elevated in 41 (89.1%), 26 (56.52%) patients while that of TNF-α were largely suppressed in patients with leptospirosis. Mean levels of IL-8, IL-10 & TNF-α was 175.96 pg/ml, 32.9 pg/ml and 46.49 pg/ml respectively. Higher levels of IL-8, IL-10 and lower levels of TNF-α were found to be associated with better prognosis. IL-10/TNF-α ratio was significantly high in our study.

Conclusion: Ratios of IL-10/TNF-α may play a role in predicting disease prognosis as high IL-10/TNF-α ratios are associated with good prognosis.

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Introduction

Leptospirosis is a well-known, worldwide zoonotic disease with a much greater incidence in tropical countries.[1] The incidence rates of leptospirosis are usually underestimated partly due to the lack of awareness of the disease and due to the paucity of easy, rapid and accurate tests to permit early diagnosis of the disease.[2] Protean and non-specific presentations of leptospirosis have often led to misdiagnosis, especially in malaria, dengue and viral hepatitis endemic regions of South Asia, South America and the Caribbean.[3,4,5] Although there have been some reports of leptospirosis from Chandigarh, Delhi and Mumbai,[6,7,8] leptospirosis continues to remain largely undetected in most parts of India. Common symptoms of leptospirosis in humans are sudden onset of fever, headache, chills, severe myalgia, and conjunctival suffusion.[9]

Although several components of this organism have been identified, the molecular mechanisms underlying pathogenesis of this infectious disease are still poorly understood.[10] Besides, direct injury by microbial factors, cytokines produced in response to infection have been proposed to be involved in pathogenesis of leptospirosis. Tumour Necrosis Factor alpha (TNF-α) is a cytokine involved in early systemic inflammation that stimulates the acute phase reaction.[11] Elevated plasma concentrations of TNF- α have been associated with poor prognosis in patients with leptospirosis.[12] Anti-inflammatory effectors play an important role to counter regulate the effects of pro-inflammatory cytokines. Interleukin-10 (IL-10) is classically described as an anti-inflammatory cytokine with pleiotropic effects in immunoregulation and inflammation by down-regulating the expression of Th1 cytokines.[13] An early imbalance of IL-10 in sepsis was shown to be associated with death despite TNF alpha production.[14] IL-8 is produced by several host cells including monocytes, macrophages, kupffer cells and hepatocytes. IL-8 is a potent chemo-attractant for neutrophils and is known to contribute to acute liver inflammation. While several studies have demonstrated elevation of serum IL-8 levels in conditions like alcoholic hepatitis[15] and non-alcoholic steatohepatitis[16] few reports have emerged regarding its role in leptospirosis. This study focussed on the potential role of IL-8, IL-10 and TNF alpha in pathogenesis of leptospiral acute hepatitis syndrome and their individual impact on prognosis of disease.

Materials and Methods

Study design

Patients with symptoms of acute hepatitis attending the Medicine Outpatient Department or admitted in the Medicine Wards of J.N. Medical College over a 10-month period from June 2009 to March 2010 were enrolled in the study. A written informed consent was obtained from each patient. Detailed clinical history was elicited from them and details of their liver function tests were recorded. Patients with autoimmune hepatitis, alcoholic hepatitis and drug-induced hepatitis, renal, pulmonary disorders, other acute or chronic inflammatory diseases or patients who had history of surgery, trauma within the preceding 2 months were excluded from the study. ELISA for HAV, HBV, HCV and HEV were performed to rule out common viral etiology of hepatitis. IgM antibodies to leptospira were detected by ELISA (DRG, USA).

Thirty healthy blood donors who had no history of fever in the last 6 months [22 male (73.34%) and 8 female (26.66%); mean age: 37 years] were selected from the blood bank and included in the study. They had no serological markers of HAV, HEV, CMV, EBV infection and liver functions were normal.

Collection and Transport of Sample

Blood was collected aseptically from all patients with acute liver disease within 1 week ± 3 days of onset of symptoms. Serum was separated by centrifugation, aliquoted and stored at -20°C till further tests were performed.

Serological Test for Leptospira

Patients negative for all the hepatitis viruses were tested for evidence of recent leptospiral infections by specific leptospira IgM antibody using the commercially available ELISA kit (DRG International, Inc.). The test procedure was performed according to the protocol provided along with the kit and absorbance was read at 450 nm. The results for leptospira IgM ELISA were interpreted according to the manufacturer's instructions, i.e. values, 0.0– 0.3 optical density (OD) units (DRG ELISA) were considered negative, 0.5-1.0 OD units were equivocal and >1.0 OD units were positive. For samples showing equivocal results, another blood sample was drawn after a period of 10 or more days, and the test was repeated. Negative and positive control sera were provided by the manufacturer and their absorbance were used for

the calculation of the cut-off and for determining the validity of the test.

Detection of IL-10, IL-8, TNF-α

Levels of IL-10, IL-8, TNF-α were estimated in the serum of all leptospira positive cases and healthy blood donors by ELISA (Orgenium, Finland). Absorbance was read at 450 nm in an automated ELISA plate reader. A standard curve of optical densities versus concentrations of IL-8, IL-10 and TNF-alpha was generated to determine their concentrations in serum samples. Sequential estimation of interleukins was not performed.

Statistical analysis

Receiver operating characteristics (ROC) curve was used to analyse the cytokines using Med Calc programme. The level of significance in all cases was set at a two tailed $p < 0.05$.

Result

Cytokine profile of 46 cases positive for leptospira by IgM ELISA was assessed. Among these 25 were males and 21 were females. The mean age of patients was 31.99±0.28 years.

Clinical features

All patients had fever. Jaundice and myalgia were present in 76% and 34.7% patients, respectively. Calf muscle pain was present in 37 (80%). Of 46 patients, 26 patients (57.2%) were oliguric. Subconjuctival suffusion was present in 13% of patients. Other clinical features, which were more commonly seen, were tachycardia (64.2%), diarrhoea (8.6%), renal tenderness (14.8%), tachypnoea (55.4%). Hepatomegaly (36.9%) and splenomegaly (10.86%) were less common findings in leptospira patients while these findings were common in viral hepatitis. The most important clinical presentations are shown in Table 1. The average stay of patients in hospital was 15 days.

The biochemical profile of patients with leptospirosis is shown in Table 2. All the parameters were mildly raised in leptospira patients in comparison to viral hepatitis. One mortality was observed due to leptospira.

Evaluation of IL-10, IL-8 and TNF- α

We compared the levels of IL-10, IL-8 and TNF-α in leptospira patients with that of healthy control group. Cytokine analysis was done in 30 healthy controls.

Table 1: Comparision of clinical features of leptospirosis and acute viral hepatitis (AVH).

Clinical features	Leptospirosis (%) (n=46)	AVH (A-E) (%) (n=142)	p value
Jaundice	35(76)	134 (94.36)	
Fever	45(97.8)	88(61.97)	
Headache	21(45.6)	22(15.49)	
Anorexia	27(58.6)	115(80.98)	
Nausea/vomiting	17(36.9)	120(84.5)	P<0.05
Myalgia	16(34.7)	39(27.46)	
Hepatomegaly	17(36.9)	142(100)	P<0.001
Splenomegaly	5(10.86)	82(57.7)	P<0.01
Conjunctival suffusion	6(13)	_	P<0.001
Diarrhoea	4(8.6)	_	
Abdominal pain	31(67.3)	52(36.6)	
Respiratory symptoms	9(19.5)	_	
Oliguria	26(57.2)	_	P<0.01
Chills	34(74.3)	26(19.7)	P<0.001
Calf muscle pain	37(80.5)	_	P<0.001
Arthralgia	3(7.2)	15(10.56)	
Tachycardia	30(64.2)	_	P<0.001
Tachypnea	26(55.4)	_	P<0.001
Renal tenderness	7(14.8)	_	
Acute onset	43(94.2)	112(78.87)	

Table 2: Biochemical profile of leptospirosis and acute viral hepatitis.

Parameters	Leptospirosis (mean value)	Acute viral hepatitis (mean values)	Reference range
AST	31.3	160.33	2-20 IU/L
ALT	32.61	127.16	2-15 IU/L
ALP	16.46	19.91	3-13 KAU/100 ml
Serum Bilirubin	2.84	13.66	0.2-1.0 mg/100ml
Albumin	2.75	3.06	3.4-5.4 g/dl
INR	1.64	2.48	0.8-1.2
ESR	44	18	M 12-19 F 18-21

(a) Healthy controls: The median values of IL-10, IL-8 and TNF-alpha in healthy controls were 7.80pg/ml, 31.71pg/ml, 158.53 pg/ml, respectively .The mean value of IL-10 in healthy controls was 12.79 pg/ml ranging between 0.2890

pg/ml and 52.978 pg/ml. In majority of the individuals IL-10 levels were clustered around 15pg/ml with few cases demonstrating higher levels around 50 pg/ml. The mean level of IL-8 in the control group was 53.75 pg/ml ranging from 11.78 to 164.99 pg/ml, with the highest level being observed in two individuals. There was no clustering of IL-8 levels around specific values as seen in IL-10. All values were randomly scattered between high and low levels. The mean serum TNF-alpha level in healthy controls was much higher at 167.02 pg/ml. TNF-alpha levels were usually higher as compared to other cytokines levels. The lowest value was 125.73 pg/ml while highest level was 256.06 pg/ml. Most of the TNF-alpha levels lay between 50-100 pg/ml.

(b) Study group: IL-10 and IL-8 levels were significantly elevated in comparison to the healthy controls as against TNF alpha which was largely suppressed. The median values of IL-10, IL-8 and TNF-alpha in patients group were 18.59pg/ml, 171.28pg/ml and 46.65pg/ml respectively. IL-10 was found to be elevated in 26 (56.52%; p <0.001) patients. The mean value of IL-10 in patients was 32.94 pg/ml which was significantly higher than healthy controls (p<0.01) (Figure 1). 14 patients (30%) showed values of IL-10 clustering between 20–50pg/ml with two patients demonstrating levels as high as 220.44 pg/ml and 232.44 pg/ml. The lowest level was 0.19 pg/ml with 232.44 pg/ml being the highest value in our study group. On examining ROC curve (Fig. 4) of IL-10 in relation to leptospiral hepatitis, the significant cut off was 19.71 pg/ml. The sensitivity was 79.4%, specificity was 82.3%, positive predictive value was 94.5%, negative predictive value was 59%, area under curve was 0.687 with 95% CI of 0.541 to 0.811. Higher levels of IL-10 corresponded with two to threefold rise in liver enzymes.

IL-8 levels were elevated in a larger majority, 41 (89.1%) patients with leptospirosis. Although there was a wide gap between lowest (0.20 pg/ml) and highest (288.19 pg/ml) concentration, the majority of the patients had significantly elevated IL-8 levels. The mean level of IL-8 in leptospira positive patients was 175.96 pg/ml (Figure 2).

There was no specific clustering of IL-8 levels but most of the IL-8 levels were above 95 pg/ml. Higher levels of IL-8 corresponded with three- to fourfold rise in levels of liver enzymes. Patients with elevated IL-8 levels also presented with a more severe spectrum of clinical signs and symptoms.

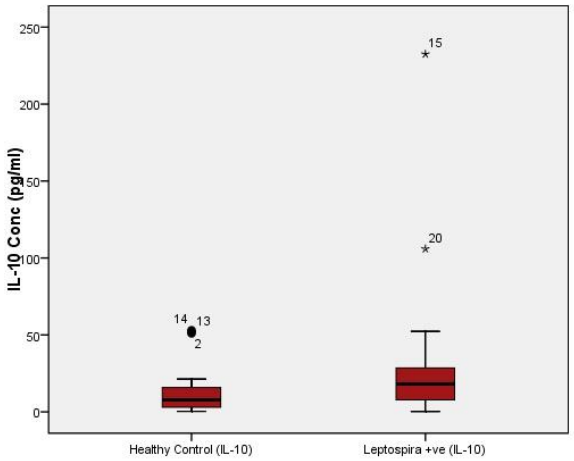

Fig 1: Box plot of IL-10 in healthy controls and patient group

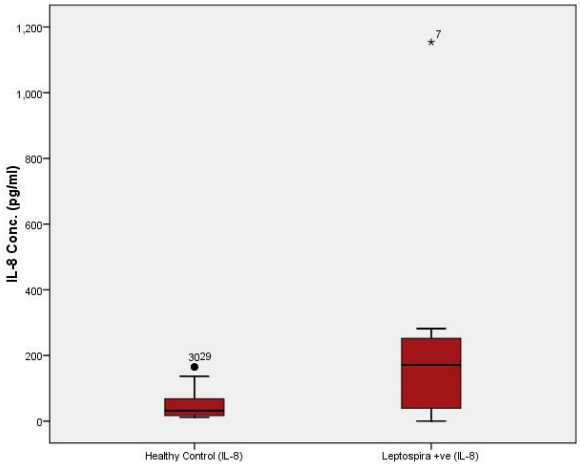

Fig 2: Box plot of IL-8 in healthy controls and patient group

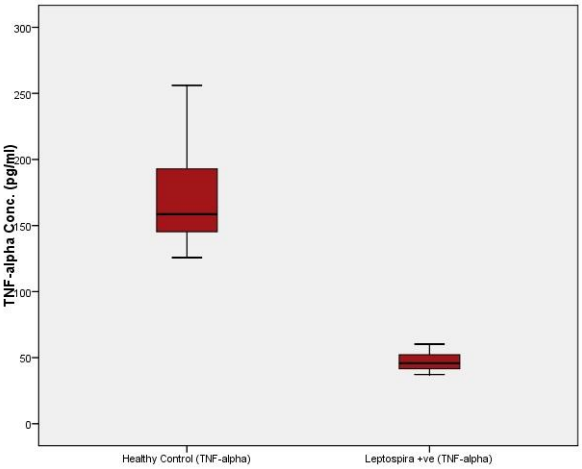

Fig 3: Box plot of TNF-α in healthy controls and patient group

Seventy-eight percent had hepatomegaly, 84% had jaundice, 90% had nausea and vomiting and 84%

complained of abdominal pain. On examining the ROC curve of IL-8 in relation to leptospiral hepatitis, the significant cut-off level of IL-8 was 31.73 pg/ml. The sensitivity was 77.8%; specificity was 85.7%; positive predictive value was 93.3%; negative predictive value was 60%; area under curve was 0.833 with 95%CI of 0.631–0.951 (P<0.001).

Surprisingly, TNF-alpha levels were largely suppressed in all the patients infected with leptospira-induced hepatitis. Elevated TNF-alpha level was seen only in the patient who expired. The mean level of TNF-α in leptospira positive individuals were 46.49 pg/ml (Figure 3). There was not much difference between lowest (37.21 pg/ml) and highest (60.25 pg/ml) value. Most of the values lay predominantly between 40-50 pg/ml. Patients with such relatively low values of TNF-alpha also demonstrated three to fourfold rise in liver enzymes. The significant cut-off for TNF-alpha was 53.16 pg/ml by ROC analysis. Both sensitivity and specificity were 100%; area under curve was 1 with 95% CI of 0.86–1 (P<0.001).

The ratio of mean values of IL-10 to TNF-α in the study was 0.74, which was significant (p<0.001). Difference between areas under curve of IL-10 and IL-8 was 0.250, 95% CI of 0.0753 to 0.425. This finding was significant (z-statistic 2.805, p<0.005).

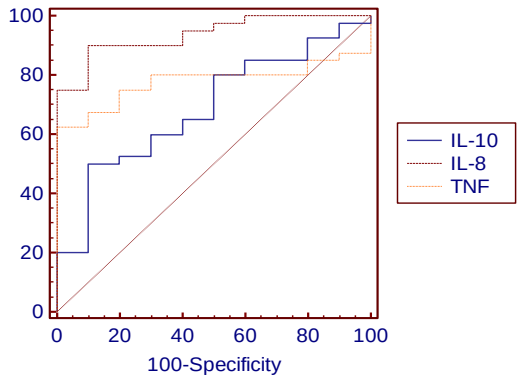

Fig 4: ROC curve showing levels of IL-8, IL-10 and TNF-α in healthy controls and leptospira positive patients.

Difference between areas of IL-10 & TNF-α was not significant. The difference between areas under curve of IL-8 and TNF-α was significant at p<0.03, area under curve being 0.160. Figure 4 shows ROC curve of IL-8,IL-10 and TNF-α in leptospira positive patients and healthy controls.

Discussion

Leptospirosis significantly contributes to acute disease in Southeast Asia and can mimic hepatitis, dengue and even febrile illnesses. Co-infection of viral hepatitis with leptospira may also occur.[17] For prompt and effective management it is therefore important to differentiate leptospirosis from other causes of acute febrile illnesses.

Fever (97.6%) was the commonest presentation in our study, followed by jaundice in 76.4% of patients. This is in concordance with a study conducted in Maharashtra [6] and Chandigarh [1] where fever and jaundice were the most common presentation, in contrast to the Barbados hospital admission, where 97% of patients had jaundice.[18] A clinic-epidemiological study [19] carried out in the North Eastern states of India reported headache as predominant symptoms (84.21%) followed by fever (73%).

Symptoms and signs like extreme muscle tenderness and suffusion of conjunctiva, which are considered as cardinal signs of leptospirosis, occurred in a proportion of patients and were helpful in making a provisional clinical diagnosis. Conjunctival suffusion was found to be low (13%) in our study as compared to Bharadwaj *et al.*[6] and Sethi *et al* [1] but higher than that reported by Manocha *et al.*[20] Frequency of oliguria, muscle pain, tachypnoea, tachycardia was also high in our study compared to previously published studies.[1] Only one mortality was observed which was very low as compared to another study.[6]

Among the biochemical profile of patients with leptospirosis all the parameters were mildly deranged as compared to patients with viral hepatitis. Leptospirosis should be considered in patients having clinical features similar to viral hepatitis but with mildly deranged liver function tests. Detection of IgM antibodies to leptospira by ELISA is now widely used in diagnosis of leptospirosis.

In this study, we assessed the role of IL-8, TNF-alpha and IL-10 as diagnostic markers of disease severity. Their role in immuno-pathogenesis was also analysed. IL-8 was observed to be uniformly elevated in all the patients infected with leptospirosis. We compared IL-8 levels between healthy controls and leptospira-infected patients. Significantly lower IL-8 levels were observed in healthy controls (P<0.01). Few studies have reported the role of IL-8 in the

pathogenesis of leptospirosis.[15] IL-8 appears to play a dominant role in hepatitis as it was observed to be elevated in 89% patients. Patients with elevated IL-8 levels also showed a more severe spectrum of clinical signs and symptoms. One patient died after developing severe hepatitis and acute renal disease. IL-8 levels were extremely raised in this patient. These findings suggest that high IL-8 levels are associated with severe disease. Similar observations were made by Wagenaar et al. [16] where IL-8 was considered a significant predictor of mortality. However, Kyriakidis et al. [15] compared IL-8 levels in survivors and non-survivor of Weil's disease and reported no significant difference between the two groups. Elevated levels of IL-8 with viral and non-viral hepatitis suggest that this chemokine may play a role in both viral and non-viral pathogenesis of liver disease.

The TNF-alpha level, on the other hand, was low in majority of cases except in the one case who expired in whom it was considerably raised. Low levels of TNF-alpha, a pro-inflammatory cytokine could be the cause of non-progression of disease to Weil's disease. TNF-α production is one of the earliest events in many types of liver injuries, and it triggers the production of other cytokines that together recruit inflammatory cells, kill hepatocytes and initiate a hepatic healing response that includes fibrogenesis. High TNF-alpha levels has been associated with severity of disease and increased mortality among patients with leptospirosis.[15,21] Thus, TNF alpha and IL-8 levels could serve as a valuable prognostic indicator in leptospirosis for monitoring progression of disease. However, mortality was observed in only one case with elevated IL-8 and TNF alpha levels which is a limitation of this study. More studies should be conducted to analyze this correlation further.

The concentration of IL-10 in pg/ml in patients was higher than in healthy controls. IL-10 was found to be elevated in significant majority 56.52% (26/46) patients as compared to 2% healthy controls. Not much work has been done on IL-10 association with leptospira prognosis. Bozkaya et al. [22] showed insignificant rise of IL-10 in HBV and HCV patients. High levels of IL-10 are usually seen in non viral hepatitis which may be the reason for the decreased elevations of ALT and AST and lower levels of necrotizing inflammation in our study group. IL-10 is essential in the control of sepsis-like infection and regression of tissue lesion.[23] High levels of IL-10 may be related to correspondingly low level of TNF-α as it is known that IL-10 is capable of inhibiting synthesis of pro-inflammatory cytokines. The average ratio of IL-10/TNF-α was significantly higher in our study group. This corresponds with the findings of Tajiki et al. [12] who reported that high IL-10/TNF-α ratio are associated with good prognosis. However, another study [14] reported this high ratio in non survivors than survivors in febrile illness. These conflicting results may indicate that when massive amounts of both types of mediators are released in persons with multiple organ dysfunction syndromes, the balance between these forces cannot be restored. In our study, one patient died after developing severe hepatitis and acute renal disease. Both IL-10 and TNF-α level were raised in this patient. We propose that severe disease manifestations with fatal outcome are accompanied with elevated levels of both IL-8, IL-10 & TNF-alpha. There is no published data till date relating the levels of IL-8, IL-10 & TNF-α to progression of leptospirosis in human subjects. However, studies on hamsters models [24] and human cell lines [25] have shown the correlation of cytokine gene expression as prognostic markers in leptospirosis, thus predicting the progression of disease towards multiple organ failure. A single point evaluation of IL-10, IL-8 and TNF-α was carried out in our study which may represent a limitation.

Conclusion

Interleukins appear to play an important role in pathogenesis of leptospirosis and more studies should be done to establish their impact on disease severity and prognosis. This was a preliminary study to establish the role of cytokines in leptospirosis. However, attributing precise role to any given cytokine in the context of leptospira, pathogenesis or clinical progression is complicated. Further studies should be conducted to assess the correlation between IL-8, IL-10 and TNF-alpha with disease progression, as high IL-10/TNF-α ratio seems to be associated with good prognosis in human leptospirosis. Intervention leading to elevated IL-10 levels may be beneficial in suppressing the pro-inflammatory cytokines and reducing liver damage.

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Competing Interests

None declared

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