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Circulating proinflammatory cytokines (IL-1β, TNF-α, and IL-6) and IL-1 receptor antagonist (IL-1Ra) in fulminant hepatic failure and acute hepatitis

K. D. SEKIYAMA*†‡, M. YOSHIBA‡ & A. W. THOMSON*†§ *Pittsburgh Transplantation Institute and Departments of †Surgery and §Molecular Genetics & Biochemistry, University of Pittsburgh Medical Center, Pittsburgh, USA and ‡Division of Gastroenterology, Department of Medicine, Showa University Fujigaoka Hospital, Yokohama City, Japan

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SUMMARY

Fulminant hepatic failure (FHF) is characterized by massive necroinflammation of the liver tissue and is associated with high mortality. Serum concentrations of IL-1β, tumour necrosis factor-α (TNF-α), IL-6 and IL-1 receptor antagonist (IL-1Ra) were measured in 30 patients with FHF and in 23 patients with acute hepatitis (AH) before start of treatment and in 23 healthy controls. Levels of all four molecules were increased significantly in FHF compared with AH, in which values were higher than in the healthy controls. High serum levels of IL-1β and a significantly reduced ratio of IL-1Ra to IL-1β (IL-1Ra/IL-1β) were observed in FHF patients who subsequently died compared with subjects who survived. TNF-α and IL-6 concentrations were correlated with levels of human hepatocyte growth factor (hHGF), an index of hepatocyte regeneration. Although serum cytokine levels varied considerably between patients within each group studied, it is suggested that the striking elevation in proinflammatory cytokine levels in FHF may reflect both the insufficiency of hepatitis virus elimination and a failure to control a vicious cytokine cascade leading to overwhelming hepatocyte destruction rather than regeneration. The high cytokine levels observed in these patients and the significantly elevated IL-1Ra/IL-1β ratio in FHF patients who survived compared with those who did not suggest the possible therapeutic use of cytokine antagonists for the control of this life-threatening disease.

Keywords cytokines IL-1 receptor antagonist fulminant hepatitic failure

INTRODUCTION

Activation of the host immune system in response to viral infection results in the production of many cytokines that act as mediators of disease activity. In particular, proinflammatory cytokines, such as IL-1, tumour necrosis factor-α (TNF-α), and IL-6 appear to play important roles in the pathophysiology of liver disease [1,2]. These cytokines have been studied extensively with respect to their immunobiological effects and their relationship to pathological processes in chronic viral hepatitis [3–6] but to a much lesser degree in acute viral hepatitis. In these conditions, disease activity is influenced by the competence of host T cell-mediated immunity, the extent and persistence of viral replication, and the severity of liver injury [7].

Fulminant hepatic failure (FHF) is a serious condition associated with massive necroinflammation of the liver and a very high mortality rate of 64–85% [8]. In this disease, increased monocyte production of TNF-α and IL-1 has been reported, with higher levels of both cytokines observed in subsequently deceased FHF patients compared with survivors [9]. The activity of these proinflammatory cytokines as mediators of necroinflammation within the liver may be closely related with viral replication, disease activity, and prognosis in virally induced FHF.

Cytokines such as IL-1 and TNF not only regulate hepatocyte destruction [1,2] but also elicit antiviral activity [10–12], stimulate synthesis of acute phase proteins in liver cells [13,14], and mediate hepatocyte regeneration [15–17]. Thus, stimulatory effects on hepatocyte growth have been described for TNF-α and IL-6 [18,19], whereas IL-1β has been shown to be a potent growth inhibitor of rat hepatocytes [15]. Interleukin-1 receptor antagonist (IL-1Ra)—a 22 kD protein—is a specific inhibitor of IL-1 produced mainly by human monocytes, that blocks the triggering of the IL-1 receptors without agonist effects [20–22]. These proinflammatory cytokines and IL-1Ra may play important roles in the regulation of hepatocyte regeneration in FHF.

In this study, we have examined the relationship between serum levels of IL-1β, TNF-α, IL-6, and IL-1Ra and clinical

Correspondence: Dr A. W. Thomson, Pittsburgh Transplantation Institute, W1544 Biomedical Science Tower, Department of Surgery, University of Pittsburgh Medical Center, 200 Lothrop Street, Pittsburgh, PA 15213–2582, USA.

and biochemical parameters in patients with FHF and acute hepatitis (AH). Significant increases in each of these cytokines in FHF compared with AH and healthy controls were observed. Furthermore, the ratio of IL-1Ra and IL-1β in patients with FHF who subsequently died was significantly decreased compared with that in subjects who survived. In addition, serum levels of TNF-α and IL-6 were closely correlated with the serum concentration of hepatocyte growth factor (HGF)—an index of hepatocyte regeneration. These findings provide insight into regulatory mechanisms in the immunopathology of FHF and may prove helpful both in disease monitoring and in the development of cytokine-directed therapeutic strategies.

MATERIALS AND METHODS

Patients

The subjects were 53 adult patients with acute liver disease studied on admission to Showa University Fujigaoka Hospital, Yokohama City, during the last 5 years. Thirty patients were diagnosed with FHF according to the criteria by Trey & Davidson [23]. Twenty-four of them were in grade I or II, four were in grade III and two in grade IV hepatic encephalopathy on admission to Fujigaoka Hospital. After development of hepatic encephalopathy, these FHF patients received intensive liver support treatment [24] consisting of plasma exchange in combination with haemodiafiltration using high-performance membranes. Fifteen patients survived and 15 died. Major clinical characteristics and laboratory values of these patients are described in Table 1. In 22 of the cases, the cause of FHF was laboratory proven hepatitis virus infection. The causal virus was type B in seven patients, acute exacerbation of HB carrier in two, type C in six, coinfection with type A and type B in one, type C and type A or type B in six. Eight patients were classified as non-A, non-B, non-C hepatitis.

Twenty-three patients with AH were also studied. The causal virus was type A in 12 patients, type B in two, type C in three, and coinfection with type A and type B in two. Four patients were classified as non-A, non-B, non-C hepatitis. Serum levels of cytokines were also measured in 23 healthy, age- and sex-matched adult controls.

Diagnostic studies

Virological markers and laboratory parameters were analysed in the clinical laboratory of Showa University Fujigaoka Hospital.Type A hepatitis was diagnosed by positive IgM antibody against hepatitis A antigen (anti-HA). Type B hepatitis was diagnosed by positive hepatitis B surface antigen (HBsAg), IgM antibody to hepatitis B core antigen (anti-HBc) or HB virus DNA by polymerase chain reaction (PCR). Diagnosis of type C hepatitis was based on a positive result for either antibody to hepatitis C virus (HCV), determined by an enzyme-linked immunosorbent assay (Ortho-HCV, Ortho Diagnostic Systems, Raritan, NJ), or detection of hepatitis C virus RNA by PCR [25]. Type non-A, non-B, non-C hepatitis was diagnosed by exclusion of type A, B, and C hepatitis, and negative results for anti-hepatitis D antigen, herpes virus, cytomegalovirus, Epstein–Barr virus, and autoantibodies (both anti-mitochondrial antibody and anti-nuclear factors). Drug-induced and alcoholic aetiologies were also excluded. Histological diagnosis of hepatitis was confirmed by liver biopsy or at autopsy.

Blood sampling

Serum samples were obtained from FHF patients on the day of onset of encephalopathy (day 0), either on admission to Showa

Table 1. Clinical characteristics and laboratory values of patients with fulminant hepatic failure (FHF) and acute hepatitis (AH): medians (range)

Parameter	FHF		AH ($n = 23$)
	Deceased ($n = 15$)	Surviving ($n = 15$)	
Age (years)	56 (30–78)	51 (22–70)	34 (17–64)
Sex (M/F)	8/7	7/8	11/12
Causal virus¶ (A,B/C,NANB)	3:12†	6:9	16:7
AST (IU/*l*)	372 (95–7432)	493 (75–6820)	190 (26–3149)
ALT (IU/*l*)	436 (31–4285)‖	727 (69–4481)	367 (24–2700)
Total bilirubin (mg/dl)	26·9 (12·6–44·2)*§	10·5 (3·2–31·7)†	5·4 (1·0–36·5)
Prothrombin time (%)	24·4 (9·2–85)*	35·2 (6·4–100)*	89·1 (33·3–144)
Albumin (g/dl)	3·2 (2·6–3·6)‖	3·4 (2·5–4·1)	3·5 (2·2–4·6)
WBC ($\times 10^9/l$)	9·6 (3·7–23·0)	9·7 (1·5–15·5)†	6·1 (2·9–10·0)
Platelet count ($\times 10^9/l$)	13·7 (3·7–31·5)†	12·8 (1·9–25·4)*	21·8 (8·9–41·7)
CRP (mg/dl)	2·3 (0·2–8·8)†	1·7 (0·2–5·8)‡	0·4 (0·2–6·5)
Endotoxaemia	3/15	1/15	0/23
hHGF (ng/ml)	4·78 (1·4–69·8)	5·74 (0·51–39·3)	ND
AFP (ng/ml)	4·2 (0·1–52·0)‖	11·3 (3·4–656)	ND

ND: not done.

* $P < 0{\cdot}001$; † $P < 0{\cdot}01$; ‡ $P < 0{\cdot}05$ compared with AH; § $P < 0{\cdot}01$, ‖ $P < 0{\cdot}05$ compared with surviving FHF patients.

¶ Patients were positive for hepatitis A or B or hepatitis C or non-A, non-B (NANB) hepatitis.

Normal ranges for AST and ALT were 9–32 and 5–35 IU/*l*, respectively; total bilirubin 0·2–1·0 mg/dl, prothrombin time 70–140% and albumin 3·6–4·8 g/dl.

Table 2. Serum levels of cytokines in patients with fulminant hepatic failure (FHF), acute hepatitis (AH) and healthy controls: median (range)

Subjects	Cytokine concentration (pg/ml)			
	IL-1β	TNF-α	IL-6	IL-1Ra
FHF ($n = 30$)	301 (10–1041)*¶	1914 (71–6447)*§	585 (35–4728)*‖	846 (112–8349)*¶
deceased ($n = 15$)	337 (123–569)*§**	1829 (71–6447)*§	632 (169–4728)*§	757 (304–8108)*¶
surviving ($n = 15$)	131 (19–1041)‡	1998 (272–5115)*§	358 (35–2712)*	1286 (112–8349)*¶
AH ($n = 23$)	150 (28–943)†	82 (14–686)	290 (54–1080)*	494 (173–1750)*
Healthy controls ($n = 23$)	79 (13–227)	80 (42–203)	72 (13–149)	203 (112–294)

* $P < 0.001$; † $P < 0.01$; ‡ $P < 0.05$ compared with healthy controls; § $P < 0.001$; ‖ $P < 0.01$; ¶ $P < 0.05$ compared with AH; ** $P < 0.01$ compared with surviving FHF patients.

University Fujigaoka Hospital or from the referring hospital, before the start of liver support treatment. In addition, serum samples were obtained from six patients 7 days before the day of onset of the hepatic encephalopathy (day −7). For AH patients, all serum samples were also obtained on the day of admission to hospital, before conventional treatment commenced. Serum was isolated as soon as possible from clotted blood and stored at −70°C until assayed. Processing of the blood was identical for all samples from each of the patients. Septicaemia was considered present when two positive blood cultures for the same microorganism were reported. Endotoxin was measured in heparinized plasma samples using limulus amoebocyte lysate assay kits (Seikagaku Kogyo, Endotoxin test D: Tokyo, Japan).

Hepatocyte growth factor (HGF) assay

Serum human hepatocyte growth factor (hHGF) level was determined by ELISA (Otsuka Assay Laboratories, Tokushima, Japan) using a monoclonal anti-hHGF antibody as the solid phase and polyclonal rabbit antibody to hHGF as the liquid phase [26].

Cytokine assays

Cytokine levels in serum samples were assayed in duplicate using ELISA kits with MoAbs specific for human IL-1β (T Cell Diagnostics, Inc., Cambridge, MA), TNF-α, IL-6, interferon-gamma (IFN-γ) (Genzyme, Cambridge, MA), and IL-1Ra (R&D Systems, Minneapolis, MN). The sensitivity limits of the assays (determined by the kit manufacturers) were as follows: IL-1β, 4·3 pg/ml; TNF-α, 10 pg/ml; IL-6, 18 pg/ml; IFN-γ, 100 pg/ml, and IL-1Ra, 22 pg/ml.

Statistical analysis

All results are expressed as median (range). Differences between groups were analysed using the Mann–Whitney U-test, χ^2 test or Fisher's exact test. The correlation between different parameters was determined by linear-regression analysis. A P value of less than 0·05 was considered statistically significant.

RESULTS

Patient groups

The major clinical characteristics and laboratory values of the FHF and AH patients are summarized in Table 1. Differences in causal virus were significant between subsequently deceased FHF patients (low incidence of hepatitis A or B) and AH patients, but not between deceased and surviving FHF patients. Patients with FHF had significantly increased total bilirubin and C-reactive protein (CRP), and decreased prothrombin time (PT) and platelet count compared with AH patients. Age, sex, and the liver enzymes AST and ALT did not differ significantly between FHF and AH patients. Two AH patients with either AST or ALT within the normal range each showed elevations in total bilirubin and albumin and reduced PT. Positive results of endotoxin assay or blood culture were found in four cases of FHF (three of whom subsequently died), but in no patients with AH. Subsequently deceased FHF patients had significantly lower ALT and α-fetoprotein (AFP) levels, but significantly higher total bilirubin levels than survivors. Serum levels of hHGF were similar in the two FHF patient groups.

Serum cytokine levels

Highly significant increases in serum levels of IL-1β, TNF-α, IL-6, and IL-1Ra were found in FHF compared with both AH and healthy controls (Table 2). In the patients with AH, there were significant increases in IL-1β, IL-6, and IL-1Ra levels compared with healthy controls. IL-1β levels in subsequently deceased FHF patients were significantly higher than in survivors ($P < 0.01$). Moreover, in subsequently deceased FHF patients, the median ratio of IL-1Ra to IL-1β (3·1 (0·5–24·1)) was threefold lower than that (10·3 (0·2–43·3)) in survivors ($P < 0.05$, Fig. 1). TNF-α and IL-6 levels were not significantly different between subsequently deceased and surviving FHF patients. We also measured serum levels of IFN-γ in patients with FHF and found that these values did not differ significantly between non-survivors and survivors (331 (294–360) and 339 (302–457) pg/ml, respectively).

Serum samples obtained from six FHF patients before the onset of encephalopathy were also available for study. Between day 7 and the day of onset of encephalopathy (day 0), levels of IL-1β decreased in two FHF patients who survived but increased slightly in four patients who later died (Table 3). The reductions in IL-1β levels were accompanied by marked decreases in IL-1Ra in survivors, whereas there was no consistent pattern in those who died. Serum levels of TNF-α and IL-6, however, showed rather similar overall patterns both in the surviving and non-surviving patients.

Correlations between different cytokines

Table 4 shows the correlation coefficients (r) and significance

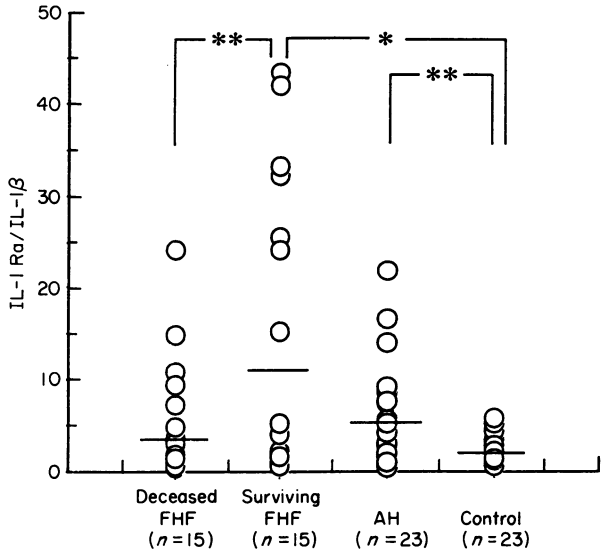

Fig. 1. The ratio of IL-1Ra to IL-1β (IL-1Ra/IL-1β) in patients with fulminant hepatic failure (FHF), acute hepatitis (AH), and healthy controls (control). Median ratios are indicated by horizontal bars. The median ratio in subsequently deceased and surviving patients with FHF is 3·1 and 10·3, respectively, in AH 5·2, and in healthy controls 2·0. n = number of subjects. * $P < 0.01$; ** $P < 0.05$.

values (P) between the four cytokines studied in all 53 patients (30 FHF and 23 AH) and 23 controls. Serum levels of TNF-α and IL-6 were the most closely correlated (Fig. 2). Both TNF-α and IL-6 were correlated with IL-1Ra and with IL-1β.

Correlations between different cytokines and liver function tests

Table 5 shows the correlation coefficients (r) and significance values (P) between the four cytokines and various indices of liver dysfunction and regeneration in all 53 patients studied. TNF-α and IL-1Ra but neither IL-1β nor IL-6 correlated with AST and ALT. Contrastingly, TNF-α, IL-6 and IL-1Ra were inversely correlated with PT. TNF-α, IL-6, and IL-1Ra were each correlated with CRP concentration.

Table 3. Changes in cytokine levels in six FHF patients during the pre-encephalopathy period (day 7) and at the onset of hepatic encephalopathy (day 0)

	Cytokine concentration (pg/ml)							
	IL-1β		IL-1Ra		TNF-α		IL-6	
Patient	d−7	d0	d−7	d0	d−7	d0	d−7	d0
Surviving								
1	158	26	7208	397	264	1423	298	566
2	722	389	6930	1552	230	698	162	262
Deceased								
3	170	327	1045	606	182	260	420	495
4	263	355	2441	3360	446	2617	350	224
5	145	407	4410	717	351	71	206	1134
6	173	312	1360	437	399	2049	212	1983

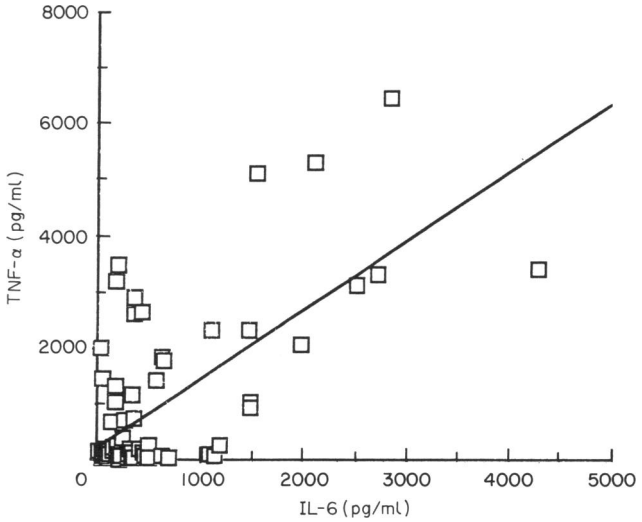

Fig. 2. Correlation between serum levels of TNF-α and IL-6 in 53 patients (30 FHF + 23 AH) and 23 healthy controls.

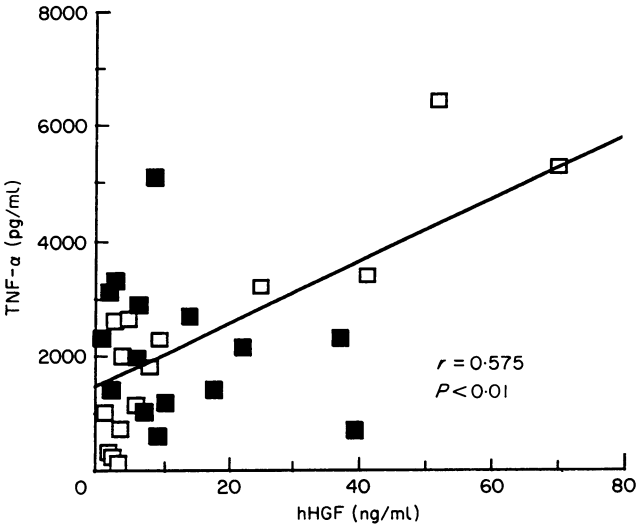

Fig. 3. Correlation between serum levels of TNF-α and hHGF in the 30 patients with FHF included in this study. □, Surviving; ■, subsequently deceased patients.

Table 4. Correlation coefficients and significance values between different cytokines in all 53 liver disease patients and 23 healthy controls

Parameter A	Parameter B	r	P
IL-1β	TNF-α	0·257	<0·05
IL-1β	IL-6	0·266	<0·05
IL-1β	IL-1Ra	0·034	NS
TNF-α	IL-6	0·668	<0·001
TNF-α	IL-1Ra	0·463	<0·01
IL-6	IL-1Ra	0·471	<0·01

r, Correlation coefficient; P, significance value.

Table 5. Correlation coefficients and significance values between cytokine levels and indices of liver dysfunction and regeneration (based on the 53 liver disease patients, except for HGF and AFP which were quantified only in FHF patients)

Parameter	IL-1β	TNF-α	IL-6	IL-1Ra
AST	NS	0·353*	NS	0·515*
ALT	NS	0·287†	NS	0·313†
Total bilirubin	NS	NS	NS	NS
Prothrombin time	NS	−0·587*	−0·300†	−0·268†
Albumin	NS	NS	NS	NS
WBC	NS	0·399*	NS	NS
Platelet count	NS	NS	NS	NS
CRP	NS	0·391*	0·348*	0·528*
hHGF	NS	0·575*	0·457*	NS
AFP	NS	NS	NS	NS

* $P < 0·01$; † $P < 0·05$.

Correlation between TNF-α with hHGF levels in FHF

Analysis of the relationship between serum levels of various cytokines and indices of liver regeneration (HGF and AFP) for the 30 patients with FHF, revealed that TNF-α and hHGF were closely correlated (Fig. 3). However, there was no significant correlation between IL-1β or IL-6 and HGF or between any of the cytokines studied and AFP.

DISCUSSION

In this study, we found that serum cytokine levels varied very considerably between patients within each group studied. Nevertheless, significant increases in serum IL-1β, TNF-α, IL-6, and IL-1Ra were found in patients with FHF compared with AH and healthy controls. Serum levels of TNF-α, IL-6, and IL-1Ra correlated significantly with parameters of hepatocyte injury and acute hepatic protein synthesis in the patients with FHF and AH. TNF-α and IL-6 levels were also related to serum level of HGF—an indicator of hepatocyte regeneration. That circulating cytokine levels correlate with amounts of these molecules in the liver and reflect cytokine-mediated tissue damage, however, is difficult to establish given the variations in cytokine concentrations observed within patient groups. Moreover, cytokine determinations by ELISA often do not reflect levels of biologically active cytokines. Caution must also therefore be exercised in interpreting, for example, the apparent relationship established at a single time point between the prognosis of FHF and both the serum level of IL-1β and the ratio of IL-1Ra to IL-1β. We did not find any relationship between peripheral endotoxaemia and serum levels of any of the cytokines measured.

Poor prognosis in FHF patients has been attributed to a lack of liver regeneration in non-A, non-B hepatitis [27]. Notably however, a stimulatory effect of sera from patients with FHF has been reported on rat hepatocyte DNA synthesis in primary culture [28]. Furthermore, no significant difference has been shown between the expression of proliferating cell nuclear antigen in FHF and non-fulminant acute hepatitis [29]. These findings suggest that the initial mechanisms of hepatocyte regeneration are activated in FHF. Indeed, HGF purified from the plasma of FHF patients [30], or recombinant hHGF has been shown to be a potent stimulator of hepatocyte DNA synthesis [31]. Recombinant hHGF also stimulates hepatocyte growth in mouse liver and has an antihepatitis effect [32]. Stimulatory effects on hepatocyte growth have also been described for TNF-α and IL-6 [18,19,33]. A dual effect of IL-1β, i.e. a stimulatory effect at low and an inhibitory effect at higher concentrations on normal cells has been observed [15]. In the present study, serum levels of TNF-α and IL-6 were correlated significantly with the serum level of HGF, suggesting their possible importance in hepatocyte regeneration in FHF. Activity of TNF-α and IL-6 may trigger hepatocyte regeneration at the same time as destruction of infected hepatocytes. In the case of widespread and persistent viral replication, an over-responding host immune reaction may lead to overwhelming hepatocyte destruction, rather than to stimulation of hepatocyte regeneration. The mechanisms of toxicity of A, B and C virus may however not be identical. Further studies are needed to elucidate the relationship between proinflammatory cytokines, the production of HGF and hepatocyte regeneration.

In a large number of patients with chronic liver diseases, increased circulating levels of IL-1Ra have been reported and shown to be related to disease activity [34]. High IL-1Ra levels have also been reported in patients with experimental endotoxaemia [35] and high-dose exogenous IL-1Ra has been highly effective in reducing the lethality of endotoxin-induced shock in animals [36,37]. This supports the contention that endogenous IL-1Ra may not be adequate to significantly neutralize the effects of endogenous IL-1 in individuals that succumb to septic shock [38]. Under *in vitro* conditions, 90% inhibition of the response of T cells to IL-1 requires 100- to 1000-fold greater molar concentrations of IL-1Ra [39,40]. We found a significantly higher ratio of IL-1Ra to IL-1β in surviving FHF patients than in patients who died—the median ratio in surviving patients was about 10 : 1. At best, this might be expected to neutralize 10% of active IL-1. Thus, while IL-1Ra seems to be an index of disease activity, the overall IL-1Ra/IL-1β balance alone would appear inadequate to influence the clinical outcome in FHF.

We also studied six FHF patients in the pre-encephalopathy period and found a reduction in IL-1β levels coupled with much higher initial IL-1Ra levels in two surviving patients compared with subsequently deceased individuals whose IL-1β levels uniformly increased. Serum levels of IL-1Ra as high as those found in Patient 1 (Table 3), where the IL-1Ra/IL-1β ratio was $> 40 : 1$, would be expected to inhibit the action of circulating IL-1β or its production. While the two surviving FHF patients were diagnosed as type non-A, non-B, non-C hepatitis with no HCV-RNA detectable by PCR, three of the four deceased patients were diagnosed as type C hepatitis detected by the presence of HCV-RNA. Therefore there may be a close relationship between persistent viral replication, elevation of cytokine levels, and prolongation of liver injury. It would clearly be of interest to study serially the levels of these factors in a larger number of patients.

In patients with FHF, insufficient viral elimination and inadequate control of a vicious cytokine cascade lead to progressive hepatocyte destruction. The high cytokine levels observed in patients in this study suggest evaluation of the possible benefit of cytokine antagonists at an early clinical stage to control this serious, life-threatening disease.

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