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# Increased tumour necrosis factor α production by neutrophils in patients with hepatitis B

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## Abstract

***Aims*—To investigate the role of serum and neutrophil tumour necrosis factor α (TNFα) in patients with viral hepatitis**

***Methods*—The activities of serum and neutrophil TNFα were measured using a bioassay of in vitro cytotoxicity against L929 cells in 57 patients with viral hepatitis and 20 healthy blood donors.**

***Results*—Both serum and neutrophil TNFα in patients with chronic active hepatitis (CAH) and subacute fulminant hepatitis (SAFH) increased compared with those in normal controls ($p < 0.01$). No such differences were seen in patients with acute hepatitis. Serum and neutrophil TNFα were obviously reduced in patients with CAH and SAFH during convalescence compared with the active period ($p < 0.05$; $p < 0.01$). Furthermore, serum TNFα was significantly increased in patients with SAFH and complications compared with those without ($p < 0.01$), and in patients with SAFH who died compared with those who survived ($p < 0.01$). Neutrophil TNFα was significantly higher in patients with SAFH and secondary bacterial infections ($p < 0.05$).**

***Conclusions*—Production of serum and neutrophil TNFα is increased in patients with CAH and SAFH, suggesting that neutrophil TNFα causes liver injury in these patients.**


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Tumour necrosis factor α (TNFα) is a cytokine which mediates many important biological actions.[1 2] Many experimental and clinical studies have shown that TNFα has a pivotal role in hepatocyte necrosis, including alcoholic hepatitis, viral hepatitis, and galactosamine liver damage.[3–5] Dubravec *et al* recently found that peripheral blood neutrophils can also synthesise and secrete TNFα.[6]

Although there have been some reports on the importance of monocyte or macrophage derived TNFα in liver cell necrosis,[3 7] the activity of neutrophil TNFα in patients with hepatitis and its role in the pathogenesis of liver injury in viral hepatitis are unknown. To evaluate the role of neutrophil TNFα in patients with viral hepatitis, we measured basal and lipopolysaccharide (LPS)-stimulated TNFα concentrations in peripheral blood neutrophils, a new source of TNFα production, using a bioassay.

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## Methods

Fifty seven inpatients (13 women, 44 men, 20–54 years old) with various clinical types of viral hepatitis were studied. All had hepatitis B (HBV) except nine subjects diagnosed with hepatitis A, all of whom had acute hepatitis. The serological profiles of patients with hepatitis B were HBsAg and HBeAg positive, or HBsAg and anti-HBc positive, or HBsAg, HBeAg, and anti-HBc positive. Serum HBV DNA was positive in 42 of 48 patients with HBV infection. The clinical types of patients were defined according to clinical signs and the results of liver function tests and liver histology.

Twenty healthy blood donors (11 women, nine men, 22–41 years old) with negative serum markers for hepatitis A, B, and C virus, and normal liver function tests were used as normal controls.

Peripheral blood neutrophils were isolated from anticoagulated venous blood by dextran sedimentation and Ficoll-Hypaque gradient and the residual erythrocytes were removed using hypotonic water lysis.[6] Neutrophils ($1 \times 10^6$ cells/ml) were suspended in RPMI-1640 medium containing 10% complement inactivated fetal calf serum, 20 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Neutrophil purity was 97% by Giemsa stain and the viability was 99% by tryptan blue exclusion.

Neutrophils ($1 \times 10^6$/Well) were seeded into 24-well cell culture plates. Duplicate cultures were incubated either in the above medium or medium plus lipopolysaccharide (Sigma, final concentration 5 μg/ml) at 37°C and a 5% $CO_2$ environment. After 24 hours culture supernatant fluids were collected and stored at −70°C for TNFα activity assay. Serum samples from patients and controls were obtained simultaneously and stored at −70°C.

Monocytes and macrophages were also isolated from normal controls by adherence of Ficoll-Hypaque-separated mononuclear cells to plastic Petri dishes for two hours at 37°C and stimulated in the same way except that they were cultured at a final concentration of

*Table 1 Mean (SD) TNFα activities in normal controls (U/ml)*

| *Age (years)* | *No of cases* | *Serum TNFα* | *Neutrophil TNFα* |
|---|---|---|---|
| 20 | 7 | 8·86 (3·98) | 6·0 (3·06) |
| 30 | 10 | 9·11 (3·02) | 6·44 (2·19) |
| 40 | 3 | 6·0 (2·0) | 4·67 (1·15) |
| Total | 20 | 8·50 (3·24) | 5·90 (2·38) |

*Table 2 Mean (SD) TNFa activities in patients*

| | No of cases | Mean age (years) | TNFa (U/ml) Serum | TNFa (U/ml) Neutrophil |
|---|---|---|---|---|
| Acute hepatitis | 15 | 33 | 8·27 (3·99) | 5·60 (2·29) |
| CAH | 20 | 38 | 17·20 (7·47)** | 12·40 (6·82)** |
| SAFH | 22 | 39 | 28·0 (18·02)** | 17·36 (9·94)** |

**p < 0·01 compared with controls.

*Table 3 Correlation between serum and neutrophil TNFa*

| | No of cases | r = | p Valve |
|---|---|---|---|
| Controls | 20 | 0·5803 | < 0·01 |
| Acute hepatitis | 15 | 0·7307 | < 0·01 |
| CAH | 20 | 0·8826 | < 0·01 |
| SAFH | 22 | 0·4468 | < 0·05 |

$2 \times 10^5$ cells per well. This population represents one fifth of the total number of neutrophils that were similarly stimulated.

The activities of serum and neutrophil TNF*a* were measured by bioassay of in vitro cytotoxicity against L929 cells.[6 8] Briefly, L929 cells ($5 \times 10^5$/ml) were seeded into 55-well cell culture plates in 0·1 ml per well and incubated at 5% $CO_2$, at 37°C for 24 hours. Spent medium was removed and L929 cells were washed. Sera or supernatant fluids of serial two-fold dilutions were added to each well in 0·1 ml, and actinomycin D (final concentration 1 $\mu$g/ml) was added to all wells at the same time. Control wells contained lipopolysaccharide and actinomycin D only. After 24 hours of incubation at 37°C in a 5% $CO_2$ atmosphere medium was removed and plates were stained with 0·2% crystal violet in 0·1 ml per well for 10 minutes. Excess stain was removed by washing in tap water. After drying, 1% sodium dodecyl sulphate was added to all wells, and absorbance determined at 570 nm using an enzyme linked immunosorbent assay (ELISA) reader (ME 891, China). Sample TNF*a* activities were calculated as the reciprocal of the dilution resulting in 50% cytotoxicity.

Data were expressed as mean (standard deviation) and analysed using Student's *t* test, the Mann-Whitney U test, and linear regression analysis.

*Table 4 Serial observations of mean (SD) TNFa in patients*

| | TNFa (U/ml) Serum | | TNFa (U/ml) Neutrophil | |
|---|---|---|---|---|
| CAH n = 6 | | | | |
| At admission | 20·0 (9·47) | p < 0·01 | 16·67(9·61) | p < 0·05 |
| Convalescence | 9·0 (4·69) | | 6·33 (3·32) | |
| SAFH n = 8 | | | | |
| At admission | 23·0 (11·66) | p < 0·05 | 17·50 (13·34) | p < 0·05 |
| Convalescence | 13·50 (8·26)* | | 6·75 (3·37) | |

* p < 0·05 compared with controls.

*Table 5 Association between TNFa and clinical course in patients with SAFH*

| | TNFa (U/ml) Serum | | TNFa (U/ml) Neutrophil | |
|---|---|---|---|---|
| Secondary infections: | | | | |
| Yes 11 | 39·27(19·08) | p < 0·01 | 22·36(12·03) | p < 0·05 |
| No 11 | 16·73(6·15) | | 12·36(2·80) | |
| Complications*: | | | | |
| Yes 8 | 43·0(19·57) | p < 0·01 | 20·75(8·94) | p > 0·05 |
| No 14 | 19·43(10·03) | | 15·43(10·27) | |
| Prognosis: | | | | |
| Died 8 | 43·0(19·57) | p < 0·01 | 20·75(8·94) | p > 0·05 |
| Survived 14 | 19·43(10·03) | | 15·43(10·27) | |

* Includes upper gastrointestinal haemorrhage, hepatorenal syndrome, and hepatic coma.

## Results

TNF*a* ACTIVITY OF NORMAL CONTROLS

The mean (SD) serum TNF*a* activities of normal men and women were 9·11 (4·14) U/ml and 8·0 (2·37) U/ml, respectively; the neutrophil TNF*a* was 6·67 (2·64) U/ml and 5·27 (2·05) U/ml, respectively. No significant differences were found between the two groups in serum or neutrophil TNF*a* (p > 0·05). There were also no differences between the different age groups for serum as well as neutrophil TNF*a* activities (table 1). Neutrophil TNF*a* concentrations were undetectable in culture supernatant fluids without lipopolysaccharide stimulation in 20 normal controls.

Secretion of TNF*a* by monocytes or macrophages was also detected. The results showed that a quantity of monocytes equal to a fifth of the neutrophils produced significantly less TNF*a*. It was confirmed that the TNF*a* determined in this study was produced by neutrophils.

PATIENTS' TNF*a* ACTIVITY

The activities of serum TNF*a* in patients with acute hepatitis A (n = 9) and with hepatitis B (n = 6) were 7·86 (3·62) U/ml and 8·63 (4·16) U/ml, respectively; neutrophil TNF*a* was 5·71 (2·78) U/ml and 5·59 (2·20) U/ml, respectively. There were no significant differences (p > 0·05) between the two groups of acute patients for serum and neutrophil TNF*a* activities. Both serum and neutrophil TNF*a* activities in patients with chronic active hepatitis and subacute fulminant hepatitis were increased compared with those found in normal controls. No such differences were found in patients with acute hepatitis (table 2). TNF*a* production of neutrophils without lipopolysaccharide stimulation was detectable in only three of 20 cases with CAH (4, 8, and 4 U/ml, respectively) and five of 22 cases with SAFH (6, 8, 8, 8, and 4 U/ml, respectively).

There was a significant correlation between serum and neutrophil TNF*a* activities (table 3).

Serum and neutrophil TNF*a* were obviously reduced in patients with CAH and SAFH during convalescence compared with the active period. However, serum TNF*a* in convalescing patients with SAFH was still much higher than that of normal controls (table 4).

Serum TNF*a* was significantly increased in patients with SAFH and complications, including secondary bacterial infections, upper gastrointestinal haemorrhage, hepatorenal syndrome and hepatic coma, compared with those free of these. This was also seen in patients with SAFH who died com-

pared with those who survived. However, neutrophil TNFα was significantly higher in patients with SAFH and secondary bacterial infections as the only complication (table 5).

No correlations were found between the concentrations of serum or neutrophil TNFα and patients' weight, serum albumin, or numbers of peripheral blood neutrophils.

## Discussion

The factors which cause liver cell necrosis in patients with hepatitis B are not fully understood, but clearly both the cellular and humoral immune responses of the hosts are important determinants.[9 10] Some studies have shown that TNFα, as a factor mediating liver injury, has a pivotal role in the pathogenesis of hepatic necrosis in patients with hepatitis B.[4 5 11] However, the importance of TNFα produced by neutrophils, and not monocytes or macrophages, has not been documented in such patients. We have shown that high activities of neutrophil TNFα as well as serum TNFα were present in patients with CAH or SAFH, and were closely associated with the severity of the patient's condition. Our results lend further support to the important role TNFα has in hepatocyte necrosis of hepatitis B. They also show that neutrophil TNFα is associated with liver injury. The concentrations of both serum and neutrophil TNFα were not increased in patients with acute hepatitis, including hepatitis A and B. This may be one of the explanations why severe hepatocyte necrosis occurs only in patients with CAH and SAFH and not in patients with acute hepatitis.

One of the most potent stimulators of TNFα release is endotoxin. Endotoxaemia occurs in patients with hepatitis, especially CAH and SAFH.[12] Furthermore, recent studies have shown that most of the effects of endotoxin are mediated through cytokines, such as interleukin 1 (IL-1) and TNFα.[5 13] We propose, therefore, that endotoxaemia or secondary bacterial infections may be one of the causes for increased activity of neutrophil TNFα in patients with CAH and SAFH. Our results showed that both serum and neutrophil TNFα activities were much higher in patients with secondary bacterial infections than those without, further supporting the notion that increased TNFα activity was at least partly induced by endotoxin stimulation.

Neutrophils from three patients with CAH and five with SAFH produced some TNFα without lipopolysaccharide stimulation. No evidence of bacterial infections was apparent in three patients with CAH, but there was spontaneous bacterial peritonitis in five with SAFH. This might be explained by the possibility that the neutrophils were pre-activated in vivo, as a result of endotoxaemia. The bacterial contamination of samples should not be a factor because basal neutrophil TNFα was not found in normal controls or patients with concomitant acute hepatitis. The increase in serum TNFα activity differed from the level of neutrophil TNFα activity in patients with complications and in those who subsequently died. One possible explanation is that serum TNFα activity was influenced by TNFα released from neutrophils in addition to that produced by monocytes or macrophages.

Despite numerous studies, the precise mechanisms for liver cell necrosis induced by TNFα remain obscure.[14] Several potential factors could be implicated. (i) TNFα, as an antiviral agent, can quickly lyse and cause necrosis of hepatocytes infected with HBV. When HBV is eradicated, massive liver cell necrosis occurs.[14] (ii) TNFα may promote the production of some oxygen free radicals,[15 16] and it has been confirmed that oxygen free radicals can cause liver injury and necrosis.[17] (iii) TNFα may stimulate release of other inflammatory cytokines, such as IL-1, IL-6, and IL-8, which can cause or aggravate liver damage.[18 19]


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