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# Serum stimulatory activity and polymorphonuclear leucocyte movement in patients with fulminant hepatic failure

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## SUMMARY

Serum from 27 patients with fulminant hepatic failure and grade IV encephalopathy had reduced ability to stimulate the movement *in vitro* of normal polymorphonuclear leucocytes. All patients had a deficiency of serum complement factors C3 and C5 and there was a significant positive correlation between C5 and serum stimulatory activity. However, in addition to this complement defect, serum from 22% of patients contained an antagonist to normal serum stimulatory factors. This antagonism was attributed to at least two different substances in the serum on the basis of differences in heat lability, dialysability and action on complement factor C5a. Polymorphonuclear leucocytes from eight of 13 patients had reduced movement toward serum, but serum from only one patient contained an antagonist acting on the cells; this was probably related to an underlying carcinoma of the breast. During the early stages of clinical recovery, serum stimulatory and complement activity returned to normal. These serum and cellular defects have not been reported previously in patients with fulminant hepatic failure and represent major defects in the body's defences against bacterial infection.

## INTRODUCTION

Bacterial infection is a common complication of patients with fulminant hepatic failure, with bacteraemia occurring in up to 36% (Mummery, Bradley & Jeffries, 1971; Nusinovici *et al.*, 1977; Wyke *et al.*, 1982). In one series of patients from this unit bacterial infection was considered a major factor contributing to death in 9·1% of patients, some of whom developed major sepsis including abscess formation (Gazzard *et al.*, 1975). An essential part in the body's defence against infection is the clearance of bacteria from the portal and systemic circulation by the reticuloendothelial cells, in particular the Kupffer cells, but in patients with fulminant hepatic failure the phagocytic function of these cells is severely impaired (Canalese *et al.*, 1981). With this failure in phagocytic function, infection may spread to other organs and into the tissues. The clearance of bacteria from these sites depends on the stimulated directional movement of polymorphonuclear leucocytes (chemotaxis) in response to serum stimulatory factors (chemoattractants), in particular complement factor C5a. Defects of either the cellular or serum functions are associated with an increased susceptibility to

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infection (Ballow *et al.*, 1975) and have been reported in patients with alcoholic liver disease (De Meo & Andersen, 1972; Van Epps, Strickland & Williams, 1975) but not in patients with fulminant hepatic failure.

We have investigated the ability of serum from patients with fulminant hepatic failure to affect the movement *in vitro* of normal polymorphonuclear leucocytes.

## PATIENTS AND METHODS

Twenty-six patients (15 women, 11 men) with fulminant hepatic failure and one with subacute hepatic necrosis were studied. Their ages ranged from 15 to 60 years with a mean of 34·6 years. Hepatic necrosis was attributed to paracetamol self-poisoning in 12, halothane associated hepatitis in five, type A viral hepatitis in four (IgM anti-HAV$^+$), non-A, non-B hepatitis in four (by absence of HBsAg, HBsAb and IgM anti-HAV) and isoniazid-induced hepatitis in one. All patients received full supportive therapy including intravenous dextrose, cimetidine, dexamethasone (for cerebral oedema), vitamin supplements, lactulose orally and magnesium sulphate enemas. All patients received artificial liver support by daily 4–6 hr periods of haemodialysis with polyacrylonitrile membrane, and eight of these patients were also treated by charcoal haemoperfusion. Nine patients had renal failure (creatinine $>200$ $\mu$mol/l) and seven had culture proven bacterial infection which contributed to death in the three patients who died. Venous blood samples were obtained daily before periods of haemodialysis or charcoal haemoperfusion, from the time of the first signs of grade IV encephalopathy until death or recovery. Blood was allowed to clot for 2 hr at room temperature before separation of serum, which was either used immediately or stored in aliquots at $-70$°C until the time of assay. Fifteen healthy medical and laboratory staff aged between 18 and 40 years without evidence of liver disease or illness known to impair polymorphonuclear leucocyte function acted as controls. A control serum pool, derived from the sera of six controls, mixed in equal proportions, was stored in aliquots at $-70$°C. All serum samples were thawed once only.

*Preparation of polymorphonuclear leucocytes.* Polymorphonuclear leucocytes were isolated from heparinized venous blood by dextran (average molecular weight 150,000) sedimentation of erythrocytes and Ficoll-Triosil gradient centrifugation), as previously described (Wyke *et al.*, 1980). After 'shock lysis' of residual erythrocytes the pellet contained 98% polymorphonuclear leucocytes, of which at least 90% were viable as judged by exclusion of trypan blue. A working suspension of $2 \times 10^6$ polymorphonuclear leucocytes/ml was made with Hank's balanced salt solution (HBSS).

*Measurement of polymorphonuclear leucocyte movement.* Polymorphonuclear leucocyte movement was measured by a minor modification of a method described by Agett *et al.* (1979). Briefly, the lower compartment contained either 5% test serum or HBSS and was separated from the upper compartment, which contained the polymorphonuclear leucocytes, by a membrane filter of 3 $\mu$m pore size. Duplicate chambers were used for each test and the distance travelled by the polymorphonuclear leucocytes was measured by the leading front method. In each experiment, the distance moved by the cells towards HBSS was subtracted from that moved towards the test serum, to derive a measure of stimulated movement. The movement of patient's polymorphonuclear leucocytes was expressed as the distance travelled in $\mu$m, whereas results for serum stimulatory activity were expressed as a percentage of the activity of the control serum pool.

*Serum complement studies and preparation of C5a.* Serum concentrations of immunoreactive C3 were determined by radial immunodiffusion against rabbit antiserum, and concentrations of C5 by rocket immunoelectrophoresis against specific rabbit antiserum (Behring Diagnostics, Hoechst UK Ltd, Hounslow, UK) in 16 patients. Results were expressed as the percentage activity of the control serum pool included as a standard in each plate.

A preparation of C5a was made by the method of Beebe, Ward & Spitznagel (1980): fresh normal serum pool was treated with zymosan and $\varepsilon$-amino caproic acid to activate complement maximally, separated by centrifugation and filtration, heated at 56°C for 30 min and stored in aliquots at $-70$°C. The stimulatory activity of the preparation was blocked by anti-C5 but not anti-C3, whereas serum pool heated at 56°C for 30 min had no stimulatory activity. This preparation was used in a final concentration of 5% in HBSS.

## RESULTS

Movement of normal polymorphonuclear leucocytes towards sera from all patients was considerably reduced, although values ranged from 0 to 83%. The mean value (26·9 ± 23·6, s.d.) was significantly different from the mean of 98·1 ± 6·3 for sera from 15 controls ($P < 0{\cdot}01$, Wilcoxon's Rank Sum Test) (Fig. 1). The severity of the serum defect was not related to the aetiology of the hepatic necrosis; prolongation of prothrombin time or abnormalities of liver function tests including levels of serum albumin which were normal in all but two patients; or presence of renal failure, culture proven bacterial infection, or final outcome.

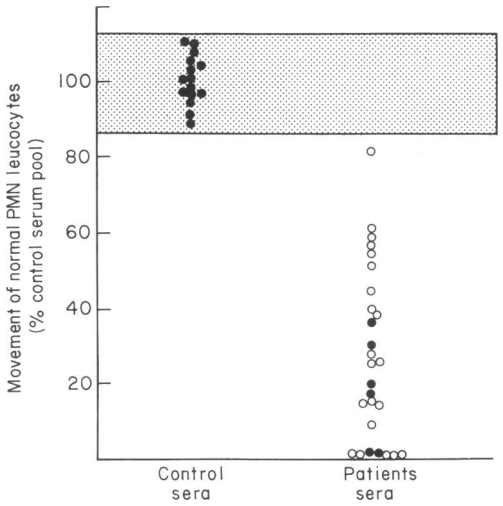


**Fig. 1.** Movement of normal polymorphonuclear (PMN) leucocytes towards sera from controls and patients with fulminant hepatic failure (● = survivors; ○ = non-survivors; ⊠ = normal range. mean ± 2 s.d.).

*Relation between serum complement and defect of serum stimulatory activity*

Concentrations of serum complement factor C3 and C5 measured at the same time as serum stimulatory activity, were reduced in all patients, with a mean of 17·2 ± 6·6, s.d. and 28 ± 19·9% of the activity of the control serum pool respectively. There was a significant positive correlation ($r = 0{\cdot}62$, $P < 0{\cdot}05$) between the serum stimulatory activity and levels of C5; levels of C3 and serum stimulatory activity were not significantly correlated ($r = 0{\cdot}51$, $P > 0{\cdot}05$).

Serial measurements during the 7 days after admission in encephalopathy in the four patients who recovered (one hepatitis A, three paracetamol overdose) showed an improvement in serum stimulatory activity, and in two patients complement functions had returned to normal although serum stimulatory activity was still slightly impaired. Despite recovery of consciousness both of these patients were very ill: the one with hepatitis A still had very abnormal liver function tests and the other had developed a septicaemia from which a group B haemolytic streptococcus was isolated (Fig. 2).

*Studies of polymorphonuclear leucocytes from patients*

In eight of 13 patients, movement of polymorphonuclear leucocytes towards control serum pool was reduced compared with that for cells from 15 controls (Fig. 3). This defect of patients' polymorphonuclear leucocytes was not related to the aetiology of the hepatic necrosis or the presence of culture proven bacterial infection or renal failure. The distance moved by the polymorphonuclear leucocytes was not related to prolongation of the prothrombin time or levels of serum albumin or bilirubin.

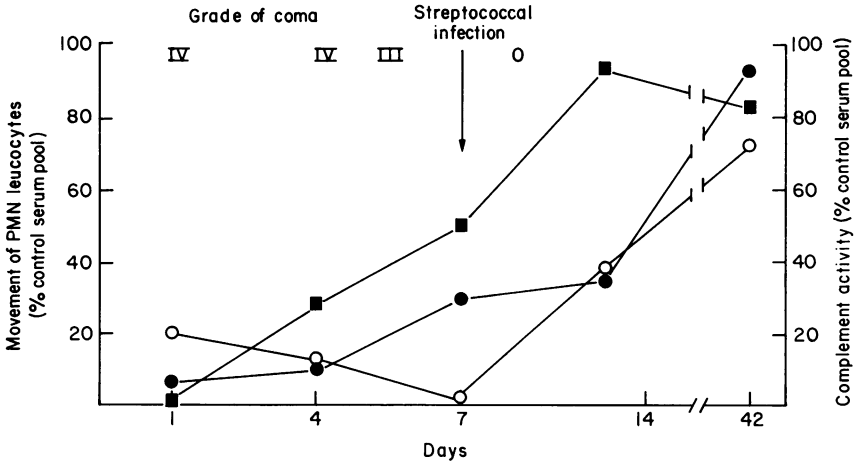


**Fig. 2.** Serial values of serum stimulatory activity and complement levels in a patient with paracetamol overdose while in grade IV encephalopathy and during the acute stages of recovery. (○ = serum stimulatory activity; ● = complement factor C3; ■ = complement factor C5).

*Characteristics of the serum abnormality*

To test for the presence in the patients' serum of a factor(s) acting directly on the cells (cell directed antagonist), normal polymorphonuclear leucocytes were pre-incubated for 90 min at 37°C in serum from 12 patients. The movement of normal polymorphonuclear leucocytes towards control serum pool was reduced after pre-incubation in serum from one of the 13 patients whereas pre-incubation in serum from 18 normal subjects had no effect. The pre-incubation studies were repeated after the serum with antagonistic activity had been heated at 56°C for 30 min; normal polymorphonuclear leucocytes moved 52% of the distance of control cells compared with 32% before heat treatment. These pre-incubations were also performed after the serum had been dialysed *in vitro* for 12 hr against HBSS at 4°C. The dialysed serum had a decreased antagonistic effect: after dialysis the pre-incubated cells moved 67% of the distance of control cells, compared with 32% before dialysis.

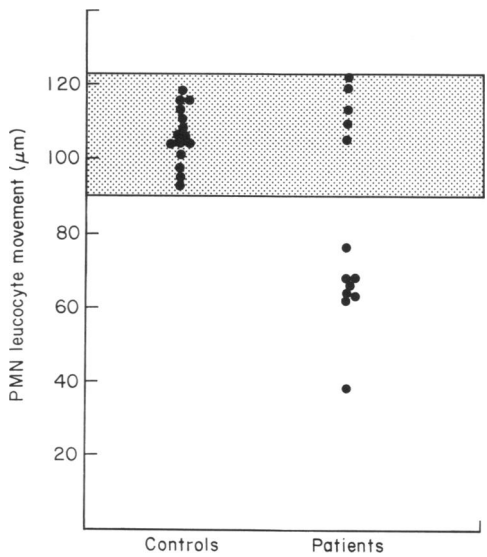


**Fig. 3.** Movement towards serum pool of polymorphonuclear (PMN) leucocytes from patients and controls. (▨ = normal range for control polymorphonuclear leucocytes, mean ±2 s.d.).

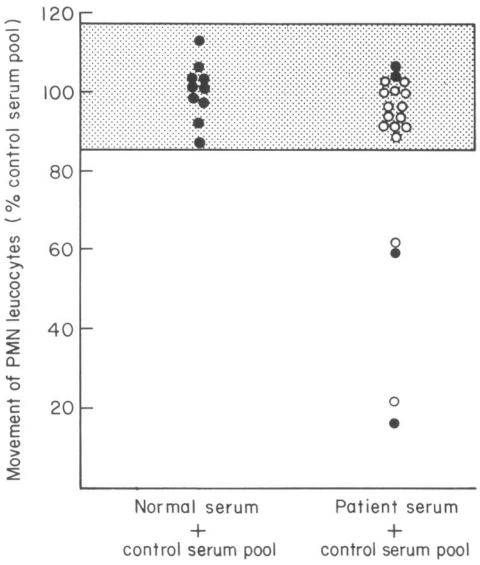


**Fig. 4.** Effect of patients' serum on the serum stimulatory activity of the control serum pool. (● = survivors; ○ = non-survivors; ⊠ = normal range, mean ±2 s.d., for 5% control serum pool + 5% control serum).

Since ascorbic acid can potentiate neutrophil function (Anderson & Theron, 1979) together with a serum factor the effect on this cell directed antagonist was examined. Normal polymorphonuclear leucocytes were pre-incubated at 37°C for 30 min in test serum with or without ascorbic acid in a final concentration of 250 μM. The cells were washed, resuspended in HBSS, and their movement towards control serum pool and HBSS determined.

The antagonistic effect of patients' serum on the movement of normal polymorphonuclear leucocytes towards control serum pool was completely abolished when ascorbic acid was added to the pre-incubation mixture, whereas polymorphonuclear leucocytes pre-incubated in control serum pool were unaffected by the addition of ascorbic acid. The serum with antagonistic activity was from a 56 year old woman who had had a mastectomy for carcinoma of the breast 5 weeks before the onset of fulminant hepatic failure due to type A viral hepatitis. There was no evidence of bacterial infection, renal failure, or abnormalities of serum immunoglobulins. Polymorphonuclear leucocytes, isolated from this patient at the same time as the serum, had reduced movement towards control serum pool compared with controls.

To determine whether patients' serum was either deficient in the normal stimulatory factors or contained a substance(s) which antagonized or inactivated these factors, sera from 19 patients and 10 controls were each mixed with control serum pool in a ratio 1:1, and a 10% concentration of these mixtures used as a stimulant for normal polymorphonuclear leucocytes. In 15 of 19 patients (78%) the defect of serum stimulatory activity was entirely corrected by adding normal serum, which suggests deficiency of stimulatory factors in the patients' serum. However, in the other four (22%) the stimulatory activity of normal serum was significantly reduced (Fig. 4) which suggests the presence, in these four sera, of an inhibitor of the stimulatory factor of normal serum (humoral directed antagonist). There was no significant difference in the distance moved by normal polymorphonuclear leucocytes towards patients' sera, in the absence of normal sera, between the 15 sera with apparent deficiency (28·7 ± 21·9 s.d.) and the four sera with demonstrable antagonistic activity (19·8 ± 28·6) ($P > 0{\cdot}05$, Wilcoxon's rank sum test). Also, the presence of either deficiency or humoral directed antagonism was not related to the aetiology of hepatic necrosis or severity of abnormalities of liver function, including levels of serum albumin and prolongation of prothrombin time, serum complement levels, final clinical outcome or presence of either bacterial infection or renal failure.

When sera from the four patients with humoral directed antagonism were heated at 56°C for 30

**Table 1.** Characteristics of humoral directed antagonist in serum from four patients

| Patient No. | Aetiology | Stimulatory activity (as % control) | | | | |
|---|---|---|---|---|---|---|
| | | Patient's serum alone | Serum pool + Patient's serum | Serum pool + Patient's serum (heated at 56°C for 30 min) | Serum pool + Patient's serum (dialysed *in vitro* for 12 hr) | Patient's serum + C5a |
| 2 | Paracetamol | 1 | 17 | 98 | 97 | 105 |
| 3 | Paracetamol | 17 | 59 | 96 | 91 | 103 |
| 4 | Halothane | 61 | 22 | 54 | 70 | 62 |
| 5 | Halothane | 0 | 62 | 24 | 71 | 75 |
| Control range (mean ± 2 s.d.) | | 87–112 | 86–118* | 86–118 | 86–118 | 87·9–112† |

* Normal range for 5% serum pool and 5% control sera.
† Normal range for 5% C5a and 5% control sera.

min and then mixed with the control serum pool the effect was abolished in two cases of paracetamol-induced hepatic necrosis, slightly reduced in one of the two other cases (halothane associated hepatitis), and increased in the other (Table 1). When these four sera were dialysed *in vitro* against HBSS at 4°C for 12 hr and then mixed in the control serum pool, the antagonistic effect was abolished in the two patients with paracetamol-induced hepatic necrosis, considerably reduced in one of the two cases with halothane associated hepatitis, and in the other case there was no change (Table 1).

Since C5a is considered the most active and stable complement stimulatory factor (Beebe, Ward & Spitznagel, 1980) the effect of mixing sera from the four patients with humoral directed antagonistic activity with a preparation of C5a in the ratio of I:I was examined. Sera from the two patients with halothane associated hepatitis impaired the stimulatory activity of C5a, while sera from the two patients with paracetamol self-poisoning had no effect (Table 1).

## DISCUSSION

The commonest cause of the defect in serum stimulatory activity for polymorphonuclear leucocytes, found in all the patients in this series, was a deficiency of serum stimulatory factors. The most important serum stimulatory factor is complement, especially C5a, and the severe reduction in complement components C3 and C5, with significant positive correlation between C5 and the serum stimulatory activity seen in these patients, suggests that the complement deficiency was responsible for the serum defect. Very low concentrations of serum proteins can affect polymorphonuclear leucocyte movement, but the defect of serum stimulatory activity and C5 were not related to serum levels of total protein or albumin which were normal in all but two patients. Both congenital and acquired deficiencies of C3 and C5 have been associated with impaired serum stimulatory activity for polymorphonuclear leucocyte movement and increased susceptibility to infection (Ballow *et al.*, 1975). The return to normal of serum complement factors and stimulatory activity in patients who survived indicates that the defects are acquired rather than congenital. Similar findings of severe deficiencies of complement factors of the classical and alternative pathway in association with impaired opsonization of bacteria and yeasts have been reported in patients with fulminant hepatic failure while in coma (Wyke *et al.*, 1980).

Although a deficiency of serum stimulatory factors was the explanation for the serum defect in most patients, in 25% of patients, mixing normal serum with patients' serum resulted in antagonism of normal serum function. This effect could not be explained by differences in the severity of the defect of serum stimulatory activity or levels of complement factors in the patients' serum and

suggests that a substance(s) which antagonises or inactivates the normal stimulatory factors may be present in the serum. This activity is known as humoral directed antagonism and has been reported in patients with cirrhosis, primary hepatocellular carcinoma, lepromatous leprosy and Hodgkin's disease (Van Epps *et al.*, 1975; Yousif-Kadaru *et al.*, 1982; Ward, Govalnick & Bullock, 1976; Ward & Berenberg, 1974).

In patients with alcoholic liver disease, humoral directed antagonistic activity has been associated with raised levels of serum IgA and IgG. However, in all but one of our patients the levels of immunoglobulins were within the normal range and there was no significant difference in immunoglobulin levels between those with and those without antagonistic activity.

Physicochemical characterization of the humoral directed antagonists showed two distinct types. In the two patients with paracetamol-induced hepatic necrosis the antagonist was heat labile, dialysable and had no effect on the stimulatory activity of C5a. Conversely, serum from the two patients with halothane associated hepatitis contained a heat stable non-dialysable antagonist which had an adverse effect on the C5a preparation. In the former group the mechanism of action of the antagonist is difficult to explain because of the adverse effect on normal serum was not shown for C5a. It is possible that serum from these patients has anti-complementary activity, known to occur in patients with liver disease (Rizzetto *et al.*, 1976), which may interfere with the production of C5a by antagonism of earlier factors in the complement cascade. The failure of dialysis to remove the humoral directed antagonistic activity could be because the substance is of high molecular weight, or low molecular weight but protein bound or present in high concentrations, requiring repeated periods of dialysis for complete removal. The patients' serum may contain more than one humoral antagonist, as has been reported in patients with cirrhosis (Van Epps *et al.*, 1975).

The defect in serum stimulatory activity could, however, be due to a cell directed antagonist in the patients' serum, although this is unlikely because, with one exception, control polymorphonuclear leucocytes functioned normally when pre-incubated in patients' serum. The heat stable cell directed antagonist found in the serum of one patient was probably related to a pre-existing carcinoma of the breast; cell directed antagonism has been reported in sera from patients with carcinoma of the breast and bronchus (Maderazo, Anton & Ward, 1978). The mechanism for this antagonism is not known, but the ability of ascorbic acid to abolish this effect *in vitro* in our case suggests oxygen-mediated inhibition of the polymorphonuclear leucocytes (Anderson & Jones, 1982).

Although polymorphonuclear leucocytes from eight (61%) of the patients had impaired stimulated movement, serum from only one of these eight exhibited cell directed antagonism which may have contributed to the defect. This impairment of polymorphonuclear leucocyte function in the other patients was probably secondary to the hepatic necrosis because none had a previous history of susceptibility to infection which would suggest a congenital defect. Circulating immune complexes impair the movement of polymorphonuclear leucocytes but they are unlikely to be responsible since patients with paracetamol overdose, whose levels of complexes are not raised, had a similar defect of cell movement (Canalese *et al.*, 1981).

Patients with fulminant hepatic failure have defects of other host defence mechanisms against bacterial infection, with prolonged clearance of $^{125}$I-heat-aggregated albumin by the reticuloendothelial system (Canalese *et al.*, 1981) and impaired serum opsonization of bacteria and yeast owing to deficiency of complement (Wyke *et al.*, 1980). The defects of polymorphonuclear leucocyte movement and serum stimulatory activity are further abnormalities of host defences and may add to the increased susceptibility to infection in these patients.

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