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Defective opsonisation and complement deficiency in serum from patients with fulminant hepatic failure

R J WYKE, I A RAJKOVIC, A L W F EDDLESTON, AND ROGER WILLIAMS

From the Liver Unit, King's College Hospital and Medical School, London

SUMMARY Serum from 23 of 26 patients with fulminant hepatic failure and grade IV encephalopathy had defective opsonisation of *E. coli* and yeast (*S. cerevisiae*). No toxic serum factors acting on the polymorphonuclear leucocytes or inactivators of the normal serum opsonisation factors were found. Complement deficiency was shown to be the most likely cause of the defect in opsonisation. The addition of a heat-labile fraction of normal serum at low concentration corrected the defect and factors of both the classical and the alternative pathways of complement were reduced to below 40% of the activity of the control serum. During the early stages of clinical recovery serum opsonisation and complement activity returned to normal with statistically significant correlations between tests of opsonisation and total haemolytic complement CH_{50}, C3 and total alternative pathway activity. Defective serum opsonisation and complement deficiency represent major defects in the body's defences against infection.

Bacterial infection is a common complication of fulminant hepatic failure[1] and has been implicated as the main cause of death in 11% of fatal cases.[2] Normally the removal of bacteria from the blood requires serum factors, immunoglobulin, and complement, which coat (opsonise) the bacteria before they are ingested and killed by the polymorphonuclear leucocytes. Congenital deficiency of individual complement factors or immunoglobulin markedly increases susceptibility to infection.[3–5] In this paper we describe investigations into opsonisation by serum from patients with fulminant hepatic failure. Two organisms, *Escherichia coli* and yeast (*Saccharomyces cerevisiae*), which have different opsonisation requirements were used. We also investigated the relationship between the opsonisation defect measured *in vitro* and the deficiency in complement factors in the same patients.

Methods

PATIENTS

Serum samples were obtained serially from 26 patients with fulminant hepatic failure after they had developed grade IV encephalopathy. The cause of their liver damage was paracetamol self-poisoning in 14, viral hepatitis in nine (two HBsAg-positive), and multiple exposure to halothane in three. Seven patients (two hepatitis, five paracetamol self-poisoning) recovered completely. Full supportive therapy was given including intravenous dextrose, cimetidine, dexamethasone (for cerebral oedema), vitamin supplements, lactulose orally, and magnesium sulphate enemas. Liver support by means of daily periods of four to six hours' haemodialysis with a polyacrylonitrile membrane was needed in all patients, expecially in 11 who developed renal failure (creatinine >200 μmol/l). Three patients developed bacterial septicaemia after admission and a fourth had *E. coli* septicaemia on admission. Three of the four patients with infection survived and in the other the immediate cause of death was cerebral oedema rather than the infection. Antibiotics were given to three patients with proven infection and to nine with suspected bacterial infection.

Venous blood samples were taken daily before haemodialysis, from the time of the first signs of grade IV encephalopathy until death or recovery. Blood was allowed to clot for two hours at room temperature before separation of serum, which was either used immediately or stored in aliquots at −196°C until the time of assay. Blood from 41 healthy medical and laboratory personnel aged between 18 and 40 years without evidence of liver disease or illness known to impair polymorphonuclear leucocyte function acted as controls. A

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control serum pool was derived from the sera of six controls, mixed in equal proportions, and stored in aliquots at −196°C.

PREPARATION OF POLYMORPHONUCLEAR LEUCOCYTES FOR *E. coli* AND YEAST OPSONISATION ASSAYS

After sedimentation of erythrocytes with dextran (average molecular weight 26,400), the leucocyte-rich plasma fraction was layered onto a Ficoll-Triosil gradients and centrifuged at 400–450 *g* for 20 minutes at 10–15°C. The pellet containing polymorphonuclear leucocytes and erythrocytes was washed twice with a 1:2 mixture of ordinary and modified Hank's balanced salt solution, separated by centrifugation and the residual erythrocytes 'shock-lysed' with distilled water for 10 seconds then made isotonic with equal volumes of 1·8% saline and ordinary Hank's balanced salt solution. The polymorphonuclear leucocytes were separated by centrifugation and resuspended in modified Hank's balanced salt solution. At least 90% of the cells isolated in this way were viable as judged by exclusion of trypan blue. The number of polymorphononuclear leucocytes was determined with a Neubauer chamber and a working suspension made with modified Hank's balanced salt solution.

Escherichia coli OPSONISATION ASSAY

Serum opsonisation of *E. coli* was measured by the method of Quie *et al.*[6] *E. coli* 089 in the late log-phase of growth, obtained by overnight culture at 30°C in trypticase soy broth, were washed twice with 0·9% saline at 4°C and the number of organisms estimated turbidometrically. The reaction mixture containing $2{\cdot}5\times10^6$ polymorphonuclear leucocytes, 1×10^6 *E. coli,* and 40 µl of test serum in a final volume of 1 ml of Hank's balanced salt solution was incubated in air in polystyrene tubes on a Matburn rotary mixer for one hour at 37°C. A 50 µl aliquot of reaction mixture was removed at zero and 60 minutes and the polymorphonuclear leucocytes lysed with 5 ml of cold distilled water. The number of viable *E. coli* cells was determined by plating out in duplicate with the Miles-Misra technique onto MacConkey's agar and the number of colony-forming units (CFU) counted after overnight incubation at 37°C.

Results were expressed as the opsonisation index:

$$\left[1-\frac{\text{Mean number of CFU [in duplicates] at 60 min}}{\text{Mean number of CFU [in duplicates] at zero min}}\right]\times100$$

Control tubes included in each experiment contained: (a) *E. coli*+buffer; (b) *E. coli*+test serum; (c) *E. coli*+polymorphonuclear leucocytes+normal serum; (d) *E. coli*+polymorphonuclear leucocytes. The numbers of *E. coli* in control tubes (a), (b) and (d) never varied by more than 2% during the hour of incubation. Serum bactericidal and bacteriostatic activity was excluded by controls (b). Control (c) was used both as a standard and to establish the normal range. Serum from patients who received antibiotics was dialysed against Hank's balanced salt solution for 12 hours at 4°C and was shown to be without bactericidal or bacteriostatic effects by incubation with *E. coli.*

In the presence of polymorphonuclear leucocytes and serum from one control, opsonisation studied on four separate occasions gave a mean opsonisation index of 98·72±SD 1·19. The variance for counting duplicate colony units was 3·16%.

YEAST OPSONISATION ASSAY

Yeast opsonisation was measured by the method of Levinsky *et al.*[7] with a suspension of heat-inactivated bakers' yeast (*Saccharomyces cerevisiae*) in Hank's balanced salt solution mixed with normal polymorphonuclear leucocytes (in a ratio of yeast: polymorphonuclear leucocytes 10:1) and 5% test serum in a final volume of 1 ml. After incubation at 37°C for 30 minutes in polystyrene tubes on a Matburn rotary mixer, the number of residual yeasts was counted with an electronic Coulter counter. Controls included in each experiment were (a) yeast+buffer; (b) yeast+polymorphonuclear leucocytes+buffer; (c) yeast+polymorphonuclear leucocytes+buffer+control serum pool. Control (a) provided a measure of the number of yeasts present in the reaction mixture after 30 minutes' incubation. Control (b) was used both to confirm the inability of polymorphonuclear leucocytes to ingest umoposonised yeasts and as a measure of the cellular content of the reaction mixture.

The opsonisation index=

$$\left[1-\frac{\text{Number of residual yeast cells after 30 min}}{\text{Number of residual yeast cells in control (b) after 30 min}}\right]\times100$$

Results for test serum were expressed as a percentage of the activity of the control serum pool-control tube (c).

In the presence of polymorphonuclear leucocytes from one control, the control serum pool (studied on eight separate occasions) gave a mean opsonisation index of 77·75±SD 6·25 SEM 1·11. With polymorphonuclear leucocytes from 13 controls the control serum pool opsonised a mean of 78·2±SD 4·03 SEM 1·11 of the yeasts.

SERUM COMPLEMENT AND IMMUNOGLOBULIN ASSAYS

Functional activity of C4, C5, total haemolytic complement (CH50), total alternative pathway, and factors B and D were determined by haemolytic diffusion in agarose.[8 9] Immunoreactive C3 concentrations were determined by radial immunodiffusion against rabbit antiserum (kindly provided by Professor D K Peters). Results were expressed as the percentage activity of the control serum pool included as a standard in each plate. For some experiments, aliquots of serum pool were depleted of active C3 (R3), factor B (RB) and C4 (R4) by the method described by Lachman *et al.*[8] Levels of serum immunoglobulin IgG, and IgM were measured in the serum of 19 of the patients with specific antisera (Behring Diagnostics, Hoechst UK Ltd, Hounslow, UK) and a laser nephelometer.

Results

Serum opsonisation indices for both *E. coli* and yeasts were reduced in all but three patients. *E. coli* opsonised by serum from 12 controls were almost all killed by normal polymorphonuclear leucocytes, mean opsonisation index being 98·5±SD 1·2. Opsonisation by serum from 22 patients was significantly reduced ($P<0.01$, Wilcoxon's rank sum test), although the results were spread over a wide range, mean 40·7±38·7. The mean yeast opsonisation index was also significantly reduced ($P<0.01$, Wilcoxon's rank sum test), although, in common with *E. coli* opsonisation, results for patients' serum were spread over a wide range, with a mean of 37±28·9 compared with 98·7±7·1 for the control sera (Table 1). No correlation was found between this defect in serum opsonisation index for either organism and the aetiology or clinical outcome of the hepatic necrosis.

The results of the serial measurements in 10 of the fatal cases showed a progressive reduction in opsonisation indices in eight while, in seven of the patients who recovered, serial studies were available during the period of improvement in five. In all cases serum opsonisation indices for both *E. coli* and yeasts returned to normal, in three patients between one and nine days after all signs of encephalopathy had disappeared (between two and 10 days after admission). There was, however, no correlation with the level of consciousness, some minor signs of encephalopathy still being present in two other patients when opsonisation indices had returned to normal.

Experiments with polymorphonuclear leucocytes from four patients, in the presence of autologous serum, showed the *E. coli* opsonisation index to be reduced, with a mean value of 31·5. In the presence of control serum pool the cells functioned normally, with a mean opsonisation index of 98·75.

CHARACTERISATION OF SERUM ABNORMALITY

Dialysis *in vitro* against Hank's balanced salt solution for 12 hours at 4°C had no effect; mean yeast opsonisation index in the five patients studied was 60·5 before and 53 after dialysis ($P>0.5$, paired *t*-test). Similarly, serum obtained immediately before and after treatment by polyacrylonitrile membrane dialysis in five patients gave mean opsonisation values for yeast of 56·3 and 39 respectively and for *E. coli* 88·3 and 76·3 respectively (Table 2). These findings indicate that the serum defect was not caused by a toxic substance of low molecular weight.

To examine for the action of a non-dialysable toxic serum factor, normal polymorphonuclear leucocytes were incubated for one hour at 37°C in a 10% dilution of serum from nine patients known to have defective opsonisation. When normal polymorphonuclear leucocytes treated in this way were incubated with *E. coli* or yeast cells already opsonised

Table 1 *Initial measurements of serum opsonisation (mean±SD) at time of grade IV encephalopathy*

	Opsonisation	
	Yeast %	*E. coli* %
Total series	37·1±28·9 (24)	40·7±38·7 (22)
Survivors	31·8±27 (7)	23·2±33·6 (6)
Fatalities	38·8±30·14 (18)	47·3±39·3 (16)
Paracetamol self-poisoning	38·6±26·5 (15)	31±30·3 (10)
Viral hepatitis	29·4±25·7 (8)	55±42·6 (9)
Controls	98·7±7·1 (38)	98·5±1·2 (12)

Figures in parentheses show number of patients studied.

Table 2 *Measurements of serum opsonisation before and after four hours of polyacrylonitrile haemodialysis in five patients*

Patient	*Aetiology*	*Opsonisation index*			
		Yeast		*E. coli*	
		Before	*After*	*Before*	*After*
1	Hepatitis	105	54	92	71
2	Halothane	—	—	91	67
3	Hepatitis	72	70	82	91
4	Hepatitis	0	0	—	—
5	Paracetamol	48	32	—	—
Mean		56·3	39	88·3	76·3

Table 3 *Effects of dilutions of control serum on yeast opsonisation by serum from three patients*

	Final concentration (%) serum in reaction mixture			
Control serum	0	0·5	1	2
Patients' serum	5	4·5	4	3
Serum of patient 1	56		100	99
Serum of patient 2	75	99	98	101
Serum of patient 3	75		104	105
Control serum diluted with buffer	0	14·3	53	100

Numbers do not denote the same patients as in Table 2

by preincubation in control serum pool, a mean of 99·3% *E. coli* and 100% yeast cells were phagocytosed, indicating no impairment of leucocyte function.

To determine whether the defect was due to a deficiency of normal opsonisation factors, serum from patients and from control serum pool were mixed together in different porportions. Severe defects of *E. coli* opsonisation by serum from three cases were totally corrected by the addition of control serum pool at concentrations of 0·25, 0·33, and 2%. As control serum pool in a concentration of 1% in the absence of patients' serum was only able to opsonise 35% of *E. coli*, the contribution by patients' serum in these experiments is greater than immediately apparent. Similar studies of yeast opsonisation by serum from three other patients showed that the defect could be totally corrected by addition of 0·5 and 1% control serum pool (Table 3), while control serum pool at a concentration of 0·5 and 1% in the absence of patients' serum opsonised only 14 and 53% of yeasts respectively (Table 4). Heating of control serum to 56°C for 30 minutes abolished the beneficial effect even at a concentration as high as 5%.

RELATIONSHIP BETWEEN SERUM COMPLEMENT AND DEFECTS OF OPSONISATION

When factors of the classical and alternative pathways of complement were measured at the same time as opsonisation indices, reduction in concentration were found in 24 of 25 cases with mean values of less than 40% of the activity of the control serum pool (Table 4). Levels of CH50, C3, C4, and C5 were higher in cases of viral hepatitis than in those with paracetamol self-poisoning, although only C4 reached statistical significance. The only serum with normal complement activity had normal opsonisation of *E. coli*, but impaired yeast opsonisation. Defective opsonisation was found in 22 of the 24 cases with complement deficiency. Serial studies in 11 of the fatal cases showed a further reduction in complement levels and opsonisation indices in 10. In the remaining case there was a 26% mean increase in factor B, CH50, C3, C4, and C5 after transfusion of 11 units of blood and 2 units of fresh frozen plasma over four days and in this case *E. coli* opsonisation also increased (from 0 to 99). However, five of the cases with progressive defects had also received transfusions but of smaller amounts (2 units of fresh frozen plasma or blood). The relationship between the changes in complement factors and opsonisation defect was examined by the ability of normal serum depleted of C3 (R3) or factor B (RB) or C4 (R4) to correct opsonisation *in vitro*. In three cases *E. coli* opsonisation was still defective after the addition of R3 or R4 but was fully corrected by the addition of RB.

In six of the seven patients (two hepatitis, five paracetamol self-poisoning) who regained consciousness, complement factor activity returned to normal in four cases between three and nine days and in two cases between time of discharge (two and nine days respectively after all signs of encephalopathy had disappeared) and follow-up at one and

Table 4 *Initial measurements of serum complement expressed as percentage activity of serum pool (mean±SD)*

Clinical groups	*Factor B*	*Factor D*	*Alternative pathway*	*Total haemolytic complement (CH50)*	*C3*	*C4*	*C5*
Total series	26·4±25·9 (25)	29·1±28·8 (16)	22·6±17·2 (25)	12·3±15·1 (23)	23·8±11·6 (25)	15·9±13·1 (24)	15·4±17·1 (15)
Fatalies	25·2±28·8 (18)	23±30·9 (10)	21·6±17·5 (18)	13·5±15·9 (17)	24·1±13·1 (18)	15·9±12·9 (18)	18·3±19·5 (10)
Survivors	29·7±17·6 (7)	39·2±23·8 (6)	25·6±17·5 (7)	9·3±13·7 (7)	23±7·4 (7)	16·2±14·8 (6)	9·6±10 (5)
Paracetamol self-poisoning	26·7±24·5 (14)	31·1±24·2 (9)	18·5±17·1 (15)	6·4±8·4 (14)	20·4±8·6 (14)	11·4±11·8* (14)	8·8±12·1 (9)
Viral hepatitis	32·1±31·7 (8)	37±37·7 (4)	26·8±20·1 (8)	26·4±18·6 (7)	32·3±14 (8)	23·9±12·1* (7)	33·8±18·9 (4)

Figures in parentheses represent number of patients studied.
*0·05>P>0·02 (Wilcoxon's rank sum test).

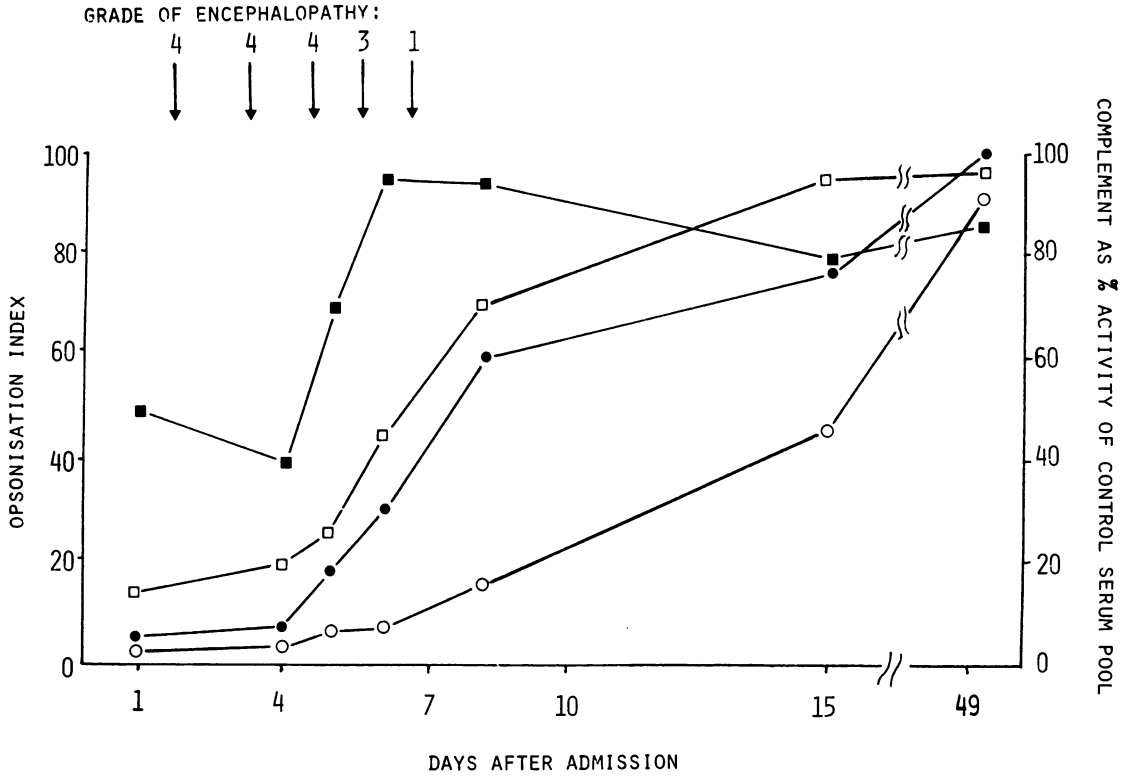

Figure *Serial values of serum opsonisation and complement levels in a case of paracetamol self-poisoning while in grade IV encephalopathy and during the acute stages of recovery.* □——□ E. coli *opsonisation.* ■——■ *Yeast opsonisation.* ●——● *Alternative pathway activity.* ○——○ *Total haemolytic complement (CH50).*

six months respectively. The activity of the alternative pathway tended to recover earlier and more rapidly than that of the classical pathway and was paralleled by a return to normal of opsonisation (Figure). The increase in *E. coli* opsonisation during recovery correlated with the activity of the total alternative pathway ($r=0{\cdot}81$, $P<0{\cdot}001$) and C3 ($r=0{\cdot}65$, $P<0{\cdot}001$), while recovery of yeast opsonisation correlated only with levels of the total haemolytic complement CH50 ($r=0{\cdot}63$, $P<0{\cdot}01$).

Discussion

In considering the possible causes for the defective *in-vitro* tests of opsonisation found in the present patients, a primary defect in the polymorphonuclear leucocytes can be excluded as the cells functioned normally when control serum was used in the assay. This contrasts with reported findings in other studies of leucocyte function in patients with fulminant hepatic failure[10,11] and in children who are carriers of hepatitis B surface antigen.[12] Saunders *et al.*[13] attributed impaired killing of *E. coli* by normal polymorphonuclear leucocytes in serum from patients with acute viral hepatitis to the action of a virus-related serum antagonist on the leucocyte. In our patients, however, normal polymorphonuclear leucocytes functioned normally after preincubation in patients' serum and the failure of both haemodialysis and *in-vitro* dialysis to improve opsonisation excludes the action of a toxic serum factor of low molecular weight. Furthermore, similar defects of opsonisation were found in sera from patients with fulminant hepatic failure caused by paracetamol self-poisoning and halothane-associated necrosis as in fulminant viral hepatitis. The low concentration of normal serum needed to correct the opsonisation defect indicates a deficiency in opsonisation factors rather than the action of an inhibitor or inactivator.

E. coli opsonisation requires the action of serum immunoglobulins IgM and IgG and complement C3, produced mainly by activation of the classical pathway,[14] whereas yeast opsonisation is dependent solely on complement, activated *via* the alternative pathway.[15] The heat lability and low concentration

of normal serum needed to correct the opsonisation defect is consistent with complement deficiency rather than immunoglobulin as the cause of the defective opsonisation. Additional evidence is the reduction of complement factors in the sera of patients at a time when opsonisation defects were most markedly affected and the good correlations between improvement in serum opsonisation and complement activity during the acute stages of recovery. The correlation between the recovery of the alternative pathway activity and *E. coli* opsonisation should be interpreted with caution, in view of the correction achieved by the addition of normal serum depleted of factor B (RB), which suggests a defect in the classical pathway.

The liver is the major site of synthesis of complement factors and the fact that serum complement and opsonisation returned to normal during recovery is evidence that these defects are not congenital but secondary to the hepatic necrosis. Markedly lowered complement levels have been reported in patients with acute hepatic necrosis[16] and less severe defects in viral hepatitis due either to reduced synthesis or increased catabolism.[17–19] In our patients consumption of complement may have been increased especially in cases with acute viral hepatitis in whom circulating immune complexes are raised. Similar defects in complement and opsonisation, however, were present in hepatic necrosis caused by paracetamol self-poisoning (D Vergani, personal communication) in which immune complexes are almost always absent. The complement deficiences in our patients are severe and affect several factors to a degree associated with impaired host defence against infection in other congenital and acquired complement deficiency states.[3–5 20]

The importance of these findings is shown by the occurrence of bacterial infection in four (15%) of the cases at a time when serum opsonisation and complement factors were severely reduced. Reports of congenital deficiency of serum opsonisation[21] suggest that transfusions of fresh blood or freshly prepared plasma provide the most effective replacement therapy. While opsonisation returned to normal in one patient, the failure of small transfusions of fresh frozen plasma in other cases may have been due to the insufficient volume of the transfusion or to the fact that the plasma had been stored and was not freshly separated. As in other diseases, infection can be overwhelming in the presence of such defects of host defence and attempts to correct these in fulminant hepatic failure should prove worthwhile.

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