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Neutrophil function in chronic liver disease

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SUMMARY

Neutrophil locomotion, phagocytosis and killing of *Candida albicans*, and plasma opsonization of brewer's yeast were studied in 44 out-patients with chronic liver disease. There were four diagnostic groups: alcoholic liver disease (ALD), chronic active hepatitis (CAH), primary biliary cirrhosis (PBC) and cryptogenic cirrhosis (CC). Results were compared with a control group of patients with non-malignant disorders of the upper alimentary tract. Neutrophil locomotion induced by zymosan-activated autologous plasma was significantly depressed in patients with ALD and, to a lesser extent, in cryptogenic cirrhosis. With plasma from healthy donors, the patients' neutrophils showed normal locomotion. Plasma from patients with CAH gave slightly but significantly reduced phagocytosis of both *Candida albicans* and brewer's yeast, but the patients' cells had normal phagocytic and killing activity in the presence of normal plasma. Thus, no intrinsic abnormality in neutrophil function was found in these patients, but plasma defects, which differed in cirrhoses of different underlying aetiology, led to impaired neutrophil locomotion or phagocytosis. No correlations were found between these plasma defects and circulating levels of C3, C4, immune complexes or IgA.

INTRODUCTION

Patients with chronic liver disease have an increased susceptibility to severe bacterial infections. The risk of such infections is difficult to quantify, but liver disease, particularly alcoholic cirrhosis, appears as an important underlying disorder in several published studies of septicaemia (Lufkin *et al.*, 1966; Jones, Crowley & Sherlock, 1967; DuPont & Spink, 1969) and bacterial endocarditis (Uwaydah & Weinberg, 1965), and 'spontaneous' peritonitis is a particular hazard in patients with cirrhosis (Conn & Fessel, 1971). Multiple factors may contribute to the frequency of bacterial infection in such patients. The damaged liver may have impaired capacity to clear or destroy bacteria entering the blood (Rutenberg *et al.*, 1959). Specific antibody responses, despite an overall hyperglobulinaemia, may be impaired (Thomas, 1977) and levels of several complement components are often low (DeMeo & Andersen, 1972; Finlayson *et al.*, 1972; Pagaltsos *et al.*, 1972; Kourilsky, Leroy & Peltier 1973; Thompson *et al.*, 1973). Several reports indicate that neutrophil function is often impaired in patients with liver disease (DeMeo & Andersen, 1972; Maderazo, Ward & Quintiliani, 1975; Van Epps, Strickland & Williams, 1975). Neutrophil chemotaxis has been found to be depressed in the presence of patient's serum, although the chemotactic response of the cells themselves is normal in the presence of normal serum. These reports have related mainly to patients with advanced alcoholic liver disease. We have examined neutrophil phagocytosis,

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Candida killing and locomotion, and plasma opsonizing activity in patients with several different types of chronic liver disease, in whom liver function was relatively well maintained.

PATIENTS

Forty-four patients with chronic liver disease were studied (22 men, 22 women; age range 17–81, mean age 55). All were out-patients without symptoms or signs of hepatic encephalopathy. On the basis of clinical, laboratory and biopsy findings they were categorized as: alcoholic liver disease (13), chronic active hepatitis (10), primary biliary cirrhosis (8) and cryptogenic cirrhosis (13). Thirty-one patients were known to be cirrhotic. Four patients with chronic active hepatitis were taking low doses of prednisolone and one of these was also receiving azathioprine (1 mg kg^{-1} day^{-1}). The others were not on any specific therapy at the time of testing.

A group of 23 out-patients (13 men, 10 women; age range 24–80, mean age 55) attending an endoscopy clinic for investigation of upper gastrointestinal tract symptoms served as controls. These patients were selected as being free of known liver disease or malignant disease. Subsequently, however, one patient was found to have abnormal liver function tests and a biopsy revealed myeloid metaplasia; another was found to have carcinoma of the oesophagus.

MATERIALS AND METHODS

Blood tests. Liver function tests (serum enzymes, bilirubin, albumin and globulin and the one-stage prothrombin time) were done by standard techniques. The following immunological assays were performed on serum samples: levels of IgA, IgG and IgM by turbidimetry in a centrifugal fast analyser (Deverill, 1979), C3 and C4 by radial immunodiffusion, and autoantibodies by indirect immunofluorescence. Immune complexes were detected by a semiquantitative anticomplementary assay (Reeves, Deverill & Wallington, 1979) and by a C1q-binding assay (Zubler *et al.*, 1976; Burden *et al.*, 1980).

For the tests of neutrophil function and plasma opsonizing activity, cells and plasma were separated from 10 ml heparinized blood. After removal of plasma, leucocytes were harvested by sedimentation of the red cells with dextran 110. The cells were washed three times in RPMI 1640 medium and polymorphonuclear cells (PMN) were counted in a haemocytometer.

Neutrophil locomotion. A 'leading front' technique was used (Weksler & Hill, 1969; Zigmond & Hirsch, 1973), the neutrophils migrating into a 3-μm Millipore filter in a 25-mm-diameter Sykes–Moore chamber (obtained from Arnold Horwick Ltd). Chemotactic activity was generated from plasma by the addition of zymosan. A suspension of zymosan particles (Sigma) at a concentration of 12·5 mg/ml in RPMI 1640 was mixed with an equal volume of fresh plasma and the lower compartment of the chamber was filled with this mixture. The upper compartment was filled with PMN in RPMI 1640 (2×10^6 PMN/ml). For each cell sample tested, two chambers were set up containing the patient's cells in the upper compartments, and in the lower compartments (1) zymosan with the patient's own plasma, or (2) zymosan with plasma from a healthy donor. The chambers were incubated for 90 minutes at 37°C after which the filters were fixed in absolute ethanol, stained with Harris' haematoxylin, alcohol-dehydrated, cleared in xylol and mounted under a coverslip on glass slides. The filters were examined at a magnification of 400, and the distance of cell migration (in μm) estimated from the difference in the microscope fine-focus micrometer reading between the position of focus on the upper surface of the filter and the lowest position at which at least two neutrophil nuclei could be seen in focus. Means were taken of readings at 10 different sites on each filter. In this assay, neutrophil migration into the filter is due to a combination of random movement and directional movement in a chemotactic gradient resulting from zymosan activation of complement.

Neutrophil phagocytosis and killing of Candida albicans. The procedure used was that described by Bridges *et al.* (1980) with minor modifications. The assay, which independently estimates phagocytosis and killing of *Candida albicans*, depends upon the fact that viable extracellular

organisms incorporate ^{3}H-uridine present in the medium, whereas leucocytes and phagocytosed or killed organisms do not (Yamamura, Boler & Valdimarsson, 1977). Plasma opsonization of *Candida albicans* may occur through antibody binding or direct activation of the alternative complement pathway. For each PMN sample, phagocytic and killing activity was assayed in the presence of either autologous or healthy donor plasma.

Candida albicans cultures were established in YM broth (DIFCO) by inoculation from YM agar slope cultures and grown for 24 hr at 37°C, after which they were kept at 4°C for use over a period of up to 2 weeks. For each assay, the organisms were washed twice in RPMI 1640, counted in 0·01 M methylene blue and made up to 5×10^6/ml in RPMI 1640.

Triplicate sets of polystyrene tubes (LP3, Luckham) were set up, containing, in 500 μl, 1×10^6 *Candida albicans*, with: (1) 2×10^5 PMN and 5% autologous plasma, or (2) 2×10^5 PMN and 5% plasma from a healthy donor, or (3) 5% plasma without cells. The tubes were rotated at 30 r.p.m. for 40 min at 37°C. One hundred-microlitre aliquots were then transferred from each tube into each of two wells of a Cooke microtitre plate, containing: (1) 0·2 μCi ^{3}H-uridine (TRA 178, Radiochemical Centre, Amersham) in 100 μl RPMI 1640, or (2) 0·2 μCi ^{3}H-uridine, plus sodium deoxycholate 0·6% and DNAase (Sigma), 100 μg/ml in 100 μl RPMI 1640. The first set of wells, in which PMN remain intact, allows assessment of the proportion of the organisms which have been phagocytosed. In the second set, PMN are rapidly disrupted by the deoxycholate and DNAase, liberating phagocytosed organisms. Reduction of ^{3}H-uridine uptake in this case therefore reflects *killing* of the organisms.

The microplates were incubated at 37°C for 1 hr, after which cells and organisms were harvested onto glass-fibre filter discs using a Dynatech Automash harvester. The filters were dried, placed in vials with 5 ml of scintillation fluid (toluene 650 ml, methanol 100 ml, PPO 6 g, POPOP 0·2 g), and β-emission measured in a Philips PW 4540 counter. From the mean counts for triplicate tubes incubated with PMN or with plasma alone, the percentage inhibition of ^{3}H-uridine uptake was estimated by the formula:

$$\frac{\text{c.p.m. with plasma only} - \text{c.p.m. with PMN}}{\text{c.p.m. with plasma only}} \times 100.$$

For phagocytosis, the percentage inhibition was calculated from the counts in wells containing intact PMN. Killing was calculated from the counts in wells containing deoxycholate and DNAase.

Alternative pathway opsonization assay. A modification of the technique of Yamamura & Valdimarsson (1978) was used. The assay was similar to that described above for phagocytosis, except that the organism used was *Saccharomyces cerevisiae* (brewer's yeast), against which antibodies are not normally found in human serum. Opsonization and phagocytosis are dependent upon surface fixation of C3, via direct activation of the alternative complement pathway.

Sera were assessed for their capacity to induce phagocytosis of *Saccharomyces cerevisiae* by PMN from healthy donors. The organisms were cultured and prepared in the same manner as *Candida albicans*. Tubes (LP3) were set up in triplicate containing 5×10^5 *Saccharomyces cerevisiae*, 2·5% test serum, with and without 1×10^5 PMN, in 500 μl RPMI 1640. The tubes were rotated for 30 min at 37°C. Aliquots of 200 μl were then transferred from the tubes into wells of Cooke microtitre plates and 0·2 μCi ^{3}H-uridine was added to each well. After incubation for 60 min at 37°C the well contents were harvested onto filter discs and ^{3}H-uridine uptake estimated by β counting. Phagocytosis was calculated as percentage inhibition of ^{3}H-uridine uptake, using the same formula as for the *Candida albicans* phagocytosis assay.

Statistical methods. The statistical significance of differences between groups of results obtained in liver disease patients and controls was determined by the Wilcoxon rank sum test (Siegal, 1956). For comparison of results of neutrophil function tests in the presence of either autologous or healthy donor plasma, the *t*-test for paired observations was used. Correlations between results of different assays were assessed by Spearman's rank correlation coefficient (*Geigy Scientific Tables*, 1970).

RESULTS

Liver function tests. The patients studied were a group with relatively well-compensated liver disease, on the basis of both their clinical state and results of liver function tests. Thirteen patients

Table 1. Neutrophil locomotion induced by zymosan-treated autologous or normal (healthy donors) plasma

Neutrophil donors (*n*)	Migration distance (mean ± s.d.)		
	Autologous plasma	Normal plasma	Patients *vs* controls*
Alcoholic liver disease (13)	74 ± 25†	95 ± 20†	$P < 0{\cdot}02$§
Chronic active hepatitis (10)	92 ± 19	92 ± 15	n.s.
Primary biliary cirrhosis (8)	85 ± 33	91 ± 22	n.s.
Cryptogenic cirrhosis (13)	82 ± 23‡	94 ± 19‡	$P = 0{\cdot}05$§
Controls (22)	99 ± 26	95 ± 22	—

* *P* value obtained by Wilcoxon rank sum test.

† $P < 0{\cdot}005$; *t*-test for paired observations. Values given only where differences achieved statistical significance ($P < 0{\cdot}05$).

‡ $P < 0{\cdot}05$; *t*-test for paired observations. Values given only where differences achieved statistical significance ($P < 0{\cdot}05$).

§ Autologous plasma.

(30%) had prolonged prothrombin times (patient:control ratio > 1·2); only two had serum albumin levels below 30 g/l. Twenty-one (48%) had raised serum bilirubin levels, although only four had levels above 50 mmol/l. Results of serum enzyme assays varied widely and the patterns observed were characteristic of the several types of liver disease represented.

Blood neutrophil counts. Blood neutrophil counts were within normal limits in all the liver disease patients (range $1{\cdot}95$–$7{\cdot}60 \times 10^9$/l, logarithmic mean $3{\cdot}64 \times 10^9$/l) and did not differ significantly from those in the control group (range $1{\cdot}25$–$7{\cdot}64 \times 10^9$/l, logarithmic mean $3{\cdot}93 \times 10^9$/l). There were no significant differences between the different groups of patients with liver disease.

Neutrophil locomotion. Measurements of the migration distance of the patients' PMN in the presence of zymosan-induced chemotaxin are summarized in Table 1. Polymorphs from patients with alcoholic liver disease showed a significant depression of migration with autologous plasma (mean 74 μm) in comparison to the control group (mean 99 μm; $P < 0{\cdot}02$). In the presence of plasma from healthy donors, however, the migration of these patients' cells was normal (mean 95 μm). There was a similar, but less marked, depression of migration with the autologous combination of cells and plasma from patients with cryptogenic cirrhosis. There was no indication of abnormal PMN migration or plasma chemotaxin generation in patients with chronic active hepatitis or primary biliary cirrhosis.

Phagocytosis and killing of Candida albicans. No group of patients showed gross abnormalities of neutrophil phagocytic (Table 2) or killing (Table 3) activity against *Candida albicans*, or of plasma capacity to induce these functions. Only the chronic active hepatitis group showed a statistically significant, though slight, reduction in phagocytosis (mean 80%) when compared with the controls (mean 88%; $P < 0{\cdot}01$). These slightly low levels of activity were seen only when autologous plasma was the opsonizing agent. There was no significant depression in the presence of plasma from healthy donors.

Alternative pathway opsonization. The antibody-independent opsonin assay did not show any substantial defect in any group of patients (Table 4). As in the Candida phagocytosis assay, plasma from patients with chronic active hepatitis gave slightly low activity (mean 55%), compared with the control group (mean 70%; $P < 0{\cdot}01$).

Serum C3 and C4 levels. Serum C3 and C4 levels are shown in Fig. 1. Results of assays for healthy staff members are also included since C4 levels in the control endoscopy patients were found to be distributed in a somewhat higher range than normal. Only the group of patients with chronic active hepatitis had low levels of these complement components. In the case of C3, the difference

Table 2. Neutrophil phagocytosis of *Candida albicans* opsonized by autologous or normal (healthy donor) plasma

Neutrophil donors (*n*)	Inhibition of ^{3}H-uridine uptake (%) (mean ± s.d.)		Patients *vs* controls*
	Autologous plasma	Normal plasma	
Alcoholic liver disease (13)	87·8 ± 7·2	87·5 ± 9·2	n.s.
Chronic active hepatitis (10)	79·4 ± 9·0	83·9 ± 3·8	$P < 0{\cdot}01$†
Primary biliary cirrhosis (8)	86·9 ± 4·6	84·1 ± 6·4	n.s.
Cryptogenic cirrhosis (13)	84·0 ± 8·1	86·3 ± 7·2	n.s.
Controls (22)	87·4 ± 6·2	86·8 ± 7·9	—

* *P* values obtained by Wilcoxon rank sum test.
† Autologous plasma.

Table 3. Neutrophil killing of *Candida albicans* in the presence of autologous or normal (healthy donor) plasma

Neutrophil donors (*n*)	Inhibition of ^{3}H-uridine uptake (%) (mean ± s.d.)	
	Autologous plasma	Normal plasma
Alcoholic liver disease (13)	64·6 ± 10·4	65·4 ± 8·8
Chronic active hepatitis (10)	56·9 ± 6·7	60·1 ± 4·8
Primary biliary cirrhosis (8)	57·5 ± 9·1	62·3 ± 3·9
Cryptogenic cirrhosis (13)	60·2 ± 9·9	63·0 ± 7·4
Controls (22)	59·7 ± 9·4	59·1 ± 10·9

Table 4. Alternative pathway-mediated opsonization of brewer's yeast (*Saccharomyces cerevisiae*) by patient and control plasma

Plasma donors (*n*)	Inhibition of ^{3}H-uridine uptake (%) (mean ± s.d.)	Patients *vs* controls*
Alcoholic liver disease (13)	66·5 ± 12·9	n.s.
Chronic active hepatitis (10)	55·0 ± 14·2	$P < 0{\cdot}01$
Primary biliary cirrhosis (8)	63·1 ± 17·7	n.s.
Cryptogenic cirrhosis (13)	71·8 ± 12·6	n.s.
Controls (23)	70·2 ± 11·4	n.s.

* *P* values obtained by Wilcoxon rank sum test.

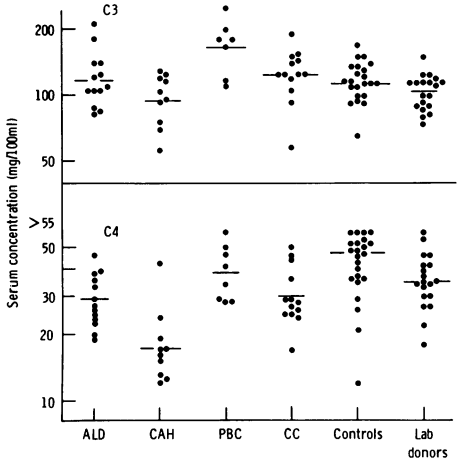

Fig. 1. Serum levels of C4 and C3. The patients are grouped as: alcoholic liver disease (ALD), chronic active hepatitis (CAH), primary biliary cirrhosis (PBC), cryptogenic cirrhosis (CC).

between these patients and each of the two control groups was not statistically significant, but for C4 the depression was much more marked (mean 17 mg/100 ml; $P < 0{\cdot}01$) compared with either the endoscopy control group, or healthy donors (mean 35 mg/100 ml). Patients with primary biliary cirrhosis had C3 levels (mean 166 mg/100 ml) significantly higher than either control group ($P < 0{\cdot}01$). There was a distinct difference in pattern between patients with chronic active hepatitis, who tended towards low C3 and C4 levels, and patients with primary biliary cirrhosis, whose levels tended to be elevated. The levels in patients with alcoholic liver disease and cryptogenic cirrhosis were in a range close to that for healthy adults. These results are similar to those reported by Thompson *et al.* (1973).

Serum immunoglobulins and immune complexes. Serum immunoglobulin levels revealed patterns characteristic for the diagnostic groups. IgG levels were significantly raised and similar in all four groups of liver disease patients ($P < 0{\cdot}01$ for each group, compared with the endoscopy controls). Levels of IgA were raised in the patients with alcoholic and cryptogenic liver disease ($P < 0{\cdot}01$), and IgM levels were high in the chronic active hepatitis and primary biliary cirrhosis patients ($P < 0{\cdot}01$).

Most of the patients with liver disease had detectable anticomplementary activity in the serum (35/44). Weak activity only was found in two of the 23 control patients. Levels of C1q-binding activity above 10% were obtained in 14 of the liver disease patients and in three of the controls. These results suggest that a high proportion of the liver disease patients had circulating immune complexes. The highest levels of activity in both assays were seen in patients with primary biliary cirrhosis.

Correlations between results of different assays. Correlations were sought between the results of the migration, phagocytosis and opsonization assays on the one hand, and several serological factors on the other (using Spearman's rank correlation coefficient). No significant correlations were found, either for the liver disease patients as a whole, or for any single diagnostic group, between (1) neutrophil migration and C3 or C4 levels, (2) Candida phagocytosis and C3 or C4 levels, (3) yeast opsonization and C3 or C4 levels, (4) neutrophil migration and IgA levels. Nor was there any correlation between the results of neutrophil function tests and the immune complex assays.

Both C3 and C4 levels showed a significant correlation with the prothrombin time, low complement levels being associated with prolonged prothrombin time ($r = 0{\cdot}63$ for C3, $0{\cdot}55$ for C4, $P < 0{\cdot}001$ in both cases).

DISCUSSION

This study has revealed minor defects in plasma-dependent functions of neutrophils from patients with chronic liver disease. Neutrophil locomotion induced by zymosan-treated plasma was

significantly reduced in patients with alcoholic cirrhosis and perhaps also in patients with cryptogenic cirrhosis. Phagocytosis of *Candida albicans* and alternative pathway-mediated opsonization of brewer's yeast were slightly reduced in patients with chronic active hepatitis. These defects were all attributable to abnormal plasma activity. In the presence of plasma from healthy donors, the patients' neutrophils showed normal locomotion and phagocytosis.

Our findings for locomotion are consistent with those of De Meo & Andersen (1972) and Maderazo *et al.* (1975), although in these earlier studies the degree of depression of chemotaxis seems to have been much greater than we have found. This may be partly due to differences in methodology. The assay we have used does not distinguish between defective neutrophil chemotactic response and defective random mobility. Our selection of patients also differed, in that all were in relatively good health with well-maintained hepatocellular function as indicated by serum albumin levels and prothrombin times. Previous studies have involved patients with severe alcoholic liver disease, with obvious features of hepatic failure.

Induction of chemotaxis by zymosan requires activation of complement by the alternative pathway. The principal chemotaxin generated is probably C5a (Klebanoff & Clark, 1978). Thus, low levels of alternative pathway components, C3, or C5 could result in reduced chemotactic activity. The liver is the main site of synthesis of many complement components and reduced complement levels have frequently been reported in association with chronic liver disease (DeMeo & Andersen, 1972; Finlayson *et al.*, 1972; Pagaltsos *et al.*, 1972; Kourilsky *et al.*, 1973; Thompson *et al.*, 1973), probably due largely to reduced synthesis (Finlayson *et al.*, 1972; Kourilsky *et al.*, 1973). We have measured levels of C3 and C4 only, and found no correlation between these and plasma capacity to induce neutrophil migration. It is possible that other complement components were more severely depressed in these patients. However, it is clear from a number of reports (DeMeo & Andersen, 1972; Finlayson *et al.*, 1972; Kourilsky *et al.*, 1973) that levels of several different components are closely correlated in patients with liver disease. Thus, it seems unlikely that complement deficiency was responsible for defective chemotaxis in the patients with alcoholic liver disease in whom C3 and C4 levels were normal.

Several published studies have suggested that sera from patients with alcoholic cirrhosis frequently contain factors which block a variety of chemotactic stimuli to normal neutrophils (DeMeo & Andersen, 1972; Maderazo *et al.*, 1975; Van Epps *et al.*, 1975). This inhibitory activity appeared to be associated predominantly with a serum fraction containing IgA (Van Epps *et al.*, 1975).Furthermore, isolated myeloma IgA (Van Epps & Williams, 1976) and normal serum IgA (Kemp, Cripps & Brown, 1980) have been shown to inhibit neutrophil chemotaxis and random mobility. Kemp *et al.* (1980) concluded that polymeric and aggregated forms of IgA, but not monomeric IgA present in normal serum, have inhibitory activity. Patients with alcoholic liver disease generally have raised serum IgA levels, and it is possible that a proportion of this IgA is present in the form of aggregates or complexes. In the patients we have studied, IgA levels were substantially elevated in those with alcoholic liver disease and cryptogenic cirrhosis. However, no correlation between IgA levels and plasma chemotactic activity was found. Further investigation is necessary before more definite conclusions can be drawn regarding the basis of the chemotactic deficiency or migration inhibition shown by the plasma of these patients.

The plasma from patients with chronic active hepatitis showed a slight deficiency in inducing phagocytosis either of Candida, which may be opsonized both by antibody and complement, or of brewer's yeast, whose opsonization by human plasma is dependent entirely on alternative pathway complement fixation. Opsonization and phagocytosis do not appear to have been previously studied in groups of patients comparable to ours. A recent report on serum opsonizing activity in patients with fulminant hepatic failure showed severe deficiency of opsonization of both *E. coli* and brewer's yeast (Wyke *et al.*, 1980). In their patients, levels of factors of both the alternative and the classical complement pathways were very low. Recovery of opsonizing activity occurred in parallel with a return of complement levels to normal.

None of our patients had grossly depressed C3 or C4 levels. The only diagnostic group, however, in whom there was a trend towards low levels was the chronic active hepatitis group. Although no correlation was found between the phagocytosis of either Candida or brewer's yeast induced by patients' plasma, and the C3 and C4 levels, deficiencies in other complement components could have been responsible for the minor reduction in activity.

A further possibility is that soluble immune complexes in the patients' plasma might inhibit phagocytosis by competitive inhibition of attachment of opsonized yeasts to neutrophil Fc and C3 receptors. Anticomplementary activity was detected in the plasma of most of the patients, and significant C1q-binding activity was found in some. There was, however, no correlation between these presumed indicators of circulating immune complexes and depressed levels of plasma-induced phagocytosis. The highest levels of anticomplementary and C1q-binding activity were found in the patients with primary biliary cirrhosis, in whom plasma opsonizing capacity was normal.

It seems unlikely that the slight defects in plasma-induced neutrophil migration and phagocytosis which we have detected in these patients are of major importance in predisposing the patients to bacterial infection. The patients all had well-compensated chronic liver disease when they were studied. Such defects, however, may well become of progressively greater significance with deterioration in liver function. Our results also suggest that impaired neutrophil function *in vivo* may result from quite separate mechanisms in liver diseases of different aetiologies.

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