In Vivo Neutrophil Dysfunction in Cirrhotic Patients with Advanced Liver Disease

Carmen Fiuza,[1,a] Magdalena Salcedo,[2] Gerardo Clemente,[2] and Jose M. Tellado[1]

[1]*Surgical Infections Unit, Department of Surgery, and* [2]*Hepatology Division, Department of Gastroenterology, Hospital General Universitario Gregorio Marañon, Madrid, Spain*

Bacterial infections are frequent, life-threatening complications in cirrhotic patients. This study investigated in vivo neutrophil migration and phagocytic activity in cirrhotic patients with advanced liver disease, in liver transplant recipients, and in healthy volunteers, by use of the skin window technique. Complement receptor type III (CR3) expression was also measured in blood and elicited neutrophils. Neutrophil migration to skin windows and neutrophil in vivo phagocytosis of heat-killed *Escherichia coli* were significantly decreased in cirrhotic patients compared with healthy controls. Neutrophil migration and phagocytosis were decreased in cirrhotic patients with previous episodes of bacterial infection compared with noninfected patients. Expression of CR3 in circulating neutrophils was significantly higher in cirrhotic patients, whereas it was significantly reduced in elicited neutrophils of cirrhotic patients with previous bacterial infection. These data suggest that deficient neutrophil recruitment to the infection site and impaired phagocytic activity may contribute to bacterial infections in cirrhotic patients with advanced liver disease.

Patients with liver cirrhosis are highly prone to bacterial infection [1–3]. The incidence of bacterial infections in cirrhotic patients is almost 4 times greater than that of the general hospital population (46% vs. 12%), and 42% of these infections are severe [4]. Episodes of bacteremia are 5–7 times more common and have a mortality rate of 33%, despite antibiotic treatment [5]. Furthermore, this increased incidence of bacterial infections represents a higher risk of morbidity and mortality for patients with end-stage liver disease who are candidates for orthotopic liver transplantation (OLT). Cirrhotic patients with infections must be temporarily excluded from an active transplant waiting list and may die from the infection or develop substantial complications leading to liver failure [6].

Spontaneous bacterial peritonitis (SBP) represents 60%–70% of all serious bacterial infections in cirrhotic patients with ascites [7]. SBP occurs in 10%–25% of hospitalized cirrhotic patients, and its mortality rate is 17%–50% [8]. When protein and complement fraction type 3 levels in ascitic fluid are low (<1 g/dL and <15 μg/mL, respectively), there is an increased risk of an episode of SBP (69% during the first year), which also shows a significant recurrence rate [9, 10]. Furthermore, previous episodes of SBP are associated with a greater incidence of infectious complications and a higher mortality rate after liver transplantation [11].

The high incidence of bacterial infections in patients with cirrhosis has prompted an assessment of defects in their immune defenses against microorganisms. They include increased levels in serum and ascitic fluid of cytokines (tumor necrosis factor [TNF]–α, interleukin [IL]–1, IL-6, and interferon [IFN]–γ) [12, 13] and soluble receptors (TNF-α and IFN-γ) [14], as well as reduced serum complement levels [15] and defective serum bactericidal and opsonic activity [16]. In addition, high endotoxin levels have also been found in the systemic circulation of cirrhotic patients without evidence of clinical infection [17]. Abnormalities have also been reported in the cellular components of the inflammatory response, both at the circulatory level (monocytes and neutrophils) [18] and in the reticuloendothelial system of the liver [4, 19, 20]. In vitro functional studies of peripheral blood neutrophils have shown defective chemotaxis [21], phagocytic activity, and intracellular killing capacity [22]; however, neutrophil dysfunction has not been sufficiently investigated in relation to the risk of bacterial infection in cirrhotic patients, and it is not clear whether in vitro behavior of peripheral blood neutrophils adequately correlates with in vivo activity at an infection site [23].

The aim of this study was to assess the in vivo inflammatory response of patients with cirrhosis by evaluating elicited neutrophil function in skin windows. The skin window model provides a means to study neutrophil exudation and phagocytic

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Written informed consent for participation in this study was obtained from all patients and controls. This study was approved by the Hospital General Universitario Gregorio Marañon institutional review board.

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[a] Present affiliation: Critical Care Medicine Department, Clinical Center, National Institutes of Health, Bethesda, Maryland.

Reprints or correspondence: Dr. Jose M. Tellado, Surgical Infections Unit-CGIII, HGU Gregorio Marañon, Dr. Esquerdo 46, 28007 Madrid, Spain (jmtella@iponet.es).

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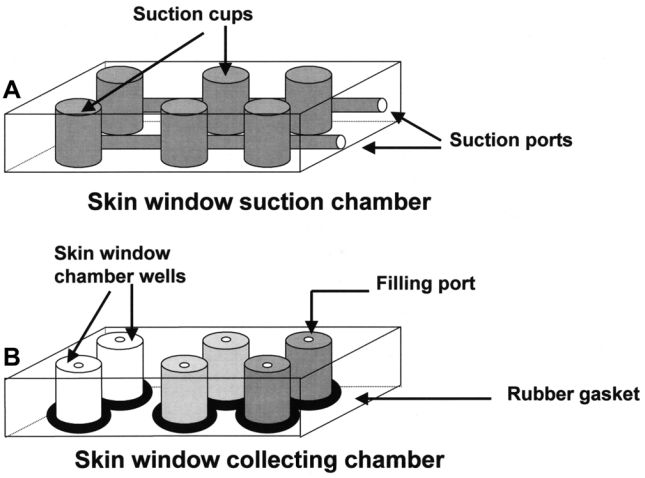

Figure 1. Diagram of a skin chamber device. *A,* Polycarbonate suction chamber with 2 rows of 3 suction cups. Each row can be connected separately or together (via a sterile Y-tube) to a suction pump or wall suction unit through the suction ports. *B,* Collection chamber unit with 6 chamber wells, which is used to cover the blister lesions. After the collection chamber is attached to the skin, each chamber is filled with a suitable attracting medium through the filling port (a small hole in the top of each well). To prevent leakage from the chambers, the lower end of each compartment has a groove that is fitted with a rubber gasket.

activity in vivo in a clinical correlate of the extravascular space [24, 25]. In addition, by maintaining an adequate ratio between the vascular surface and the final skin window volume, this model has been used to reflect the kinetics of deep compartments, such as the peritoneal and pleural cavities [26].

Subjects and Methods

Study subjects and design. The study was performed at the Surgical Infections Unit and Division of Hepatology of the HGU Gregorio Marañon, Madrid. After signing the informed consent, 22 cirrhotic patients with ascites, 6 stable orthotopic liver transplant recipients, and 6 healthy volunteers were included in the study. The diagnosis of liver cirrhosis was based on the medical records and was confirmed by laboratory tests and liver biopsy. All liver transplant recipients received standard immunosuppressive therapy (prednisone, cyclosporine, and azathioprine) and were included if their liver functions were normal. Exclusion criteria were (1) clinical or laboratory evidence of infection within the preceding week, (2) hepatocellular carcinoma; (3) gastrointestinal bleeding within the preceding week; (4) blood transfusions within 10 days before the study. No patient was a consumer of alcohol at the time of the study.

Reagents. Hanks' balanced salt solution with and without Ca^{++} and Mg^{++} ($HBSS^{--}$), 0.4% trypan blue solution, and 0.5% phenol red solution were purchased from Sigma Chemical (St. Louis). The chemoattractants used to fill the skin window chambers included streptokinase 250,000 U (Kabikinase; Kabi Pharmacia, Uppsala, Sweden) and fluorescein-labeled heat-killed *Escherichia coli* K-12 strain (Bodipy BioParticles; Molecular Probes Europe, Leiden, The Netherlands). CellFIX, FACS lysing solution, monoclonal antibody CD11b-PE, its isotopic IgG2a PE control, and Calibrite beads were purchased from Becton Dickinson (San Jose, CA). Quantum 25 and Quantum Simply Cellular microbeads were obtained from Flow Cytometry Standards (Leiden, The Netherlands).

Sample collection and processing. Blood samples were drawn through a butterfly-type catheter into a Vacutainer sterile tube with lithium heparin (15 U/mL, final concentration) or coagulation activator (Venoject; Terumo Europe, Louvain, Belgium). Serum was obtained immediately by centrifugation and stored at −45°C until use. Blood samples for measuring CR3 were placed on ice and processed immediately. Ascitic fluid was aseptically collected by routine paracentesis from each cirrhotic patient. A 10-mL sample was immediately centrifuged, passed through a 0.22-mm filter, and frozen at −45°C until use. All cirrhotic patients had ascitic fluid with a total protein content <1 g/dL, no evidence of spontaneous bacterial peritonitis (<250 polymorphonuclear leukocytes (PMNL)/mm^3 and negative bacterial cultures), and no malignant peritoneal cells.

Skin window technique. Skin blister windows were used to study neutrophil exudation, as described elsewhere [24, 27], with minor modifications to assess in vivo phagocytosis [28]. Skin blisters affecting only the epidermis were raised under sterile conditions on the volar surface of the nondominant forearm by applying negative pressure to intact skin with a suction chamber connected to controlled vacuum. Suction chambers are made of polycarbonate and contained 2 or 3 pairs of suction cups (figure 1*A*). Each suction cup is 8 mm in diameter and generates a 0.5-cm^2 strictly epidermal skin blister.

When blisters were well developed (usually within 2 h), the suction chamber was removed, the roof of each blister was cut off, and the dermal-epidermal interface was exposed. A skin window collecting chamber with wells that match the skin lesions was attached to the forearm using hypoallergenic surgical tape (figure 1*B*). Each well was filled to a total volume of 1 mL with the appropriate chemoattractant solution through the loading port on top of each chamber, as described below. We used skin windows with 2 pairs of wells for healthy controls and OLT recipients and with 3 pairs of wells for cirrhotic patients

Exudate neutrophils and exudative fluid were harvested from the paired collection chambers after 12 h and were immediately placed on ice. Cells were then centrifuged, washed twice in $HBSS^{--}$, and resuspended to a final concentration of 10^6 cells/mL. The elicited cells were 92%–95% neutrophils. The number of exudate neutrophils is expressed as elicited neutrophils/cm^2 skin surface, because paired chambers were filled with identical chemoattractant solution and the surface of 2 skin windows was 1 cm^2.

CR3 receptor expression. Surface expression of CR3 receptors

Table 1. Demographic data of the study population.

Group	No.	Sex (M/F)	Age, years (mean ± SD)
Patients with cirrhosis	22	15/7	55.8 ± 10.4
OLT recipients	6	3/3	51.8 ± 6.5
Healthy volunteers	6	3/3	32.8 ± 5.22

NOTE. OLT, orthotopic liver transplantation.

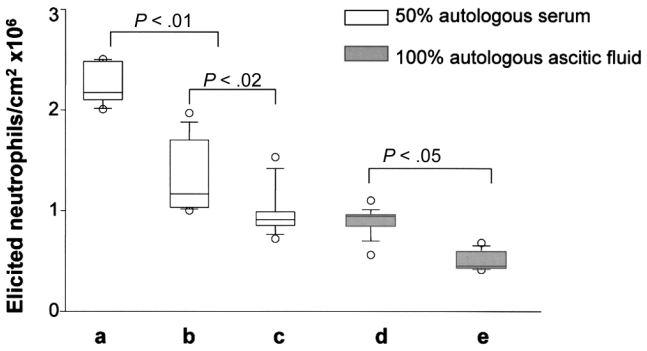

Figure 2. Neutrophil exudation to skin windows. *Open boxes* represent number of neutrophils elicited in skin windows filled with 50% autologous serum + heat-killed intact *Escherichia coli* in healthy controls *(a),* cirrhotic patients without previous bacterial infections *(b),* and cirrhotic patients with previous bacterial infections *(c)*. *Filled boxes* represent number of neutrophils elicited in skin windows filled with heat-killed intact *E. coli* in 100% autologous ascitic fluid in cirrhotic patients without previous spontaneous bacterial peritonitis *(d)* or in patients with previous episodes of spontaneous bacterial peritonitis *(e)*. *P* values reflect differences between groups by Mann-Whitney test. Data are presented as box plots: the extents of the boxes indicate the 25th and the 75th percentiles, and the lines inside the box mark the 50th percentile (median) values. *Capped bars* indicate the 10th and 90th percentiles, and *open circles* mark the 5th and 95th percentiles.

on circulating neutrophils was measured by direct immunostaining in a whole blood assay to minimize up-regulation by neutrophil isolation techniques [29, 30]. Aliquots of 100 mL heparinized blood were incubated with saturating concentrations of monoclonal antibody CD 11b-PE or its isotopic IgG control for 30 min at 4°C in the dark. Erythrocytes were then lysed, using FACSLyse solution; and all samples were washed twice, resuspended in PBS in a final volume of 300 μL, and fixed with 100 μL of CellFix solution. Exudate neutrophils in skin windows (10^5 cells/sample in 100 μL) were processed in the same way but without adding lysing solution.

In vivo phagocytosis assay. In order to simulate the conditions of the extravascular space and peritoneal cavity in cirrhotic patients with ascites, paired skin window chambers were filled to a final volume of 1 mL with serum or ascitic fluid combined with chemoattractants. The chemoattractants used in the 3 study groups were a gram-positive antigen (200 units of streptokinase) in 50% autologous serum and a gram-negative antigen (10^6 particles of heat-killed Bodipy-labeled *E. coli*) in 50% autologous serum. In addition, for patients with cirrhosis a gram-negative antigen (10^6 particles of killed Bodipy-labeled *E. coli*) was added to autologous ascitic fluid to fill an additional pair of chambers.

Our new in vivo phagocytosis assay is based on the uptake of fluorescent *E. coli* particles by exudative neutrophils [31], because they are recruited to the skin chambers in a way similar to recruitment to an infective site. This new dynamic approach to the study of in vivo phagocytosis is much more physiological than the traditional in vitro test. Exudative neutrophils were harvested as previously described, washed twice in cold $HBSS^{--}$ to stop the phagocytic process, counted, and resuspended to a final concentration of 10^6 cells/mL for flow cytometry analysis.

Flow cytometry analysis. In vivo phagocytic activity and CR3 expression were measured in a FACS Vantage Flow cytometer (Becton Dickinson) using Lysis II software. Calibration of the cytometer and 2-color compensation were performed daily with Calibrite beads (Becton Dickinson). Neutrophil populations were identified in both circulating and elicited cells by use of forward and right-angle light scatter, and the fluorescence emission of 10^4 events per sample was recorded as mean channel number (MCN) on a logarithmic scale. In order to calculate the number of CR3 receptors per cell, the MCN of each cell population was transformed into antibody binding sites/cell using the Quantum Simply Cellular Microbeads as described elsewhere [32, 33]. The number of antibody binding sites corresponding to the isotopic immune globulin was subtracted from each cell sample.

In vivo phagocytosis was analyzed by acquiring 10^4 events from the population of elicited neutrophils in skin windows containing Bodipy-labeled *E. coli* after extensive washing to remove bacteria not phagocytosed. Exudative neutrophils in skin windows without bacteria were used as a negative control. Two distinct neutrophil populations were detected in flow cytometry analysis in each sample. One population showed a greater fluorescence than did the negative control, and it represented neutrophils that had ingested $\geq$1 fluorescent bacterium. The second neutrophil population exhibited a similar fluorescence to the negative control. The MCN of the neutrophil populations was transformed into MESF (molecules of equivalent soluble fluorochrome), using Quantum 25 beads [33, 34]. Results are expressed as number of MESF in 10^4 neutrophils.

Statistical analysis. All results are given as mean ± SD and were analyzed using SigmaStat 2.0 software (SPSS, Chicago). The Kolmogorov-Smirnov test was used to test data for a normal distribution. Differences between groups with a normal distribution were analyzed with a *t* test (2 groups) or an ANOVA test (>2 groups). The nonparametric Mann-Whitney rank sum test and the Kruskal-Wallis ANOVA on the ranks test were used for non-normally distributed data. Follow-up pairwise comparisons were based on Dunn's test. A value of $P < .05$ was considered significant for 2-tailed comparison.

Results

Patient demographics. Twenty-two cirrhotic patients, 6 stable liver transplant recipients, and 6 healthy controls were included in the study (table 1). Our healthy control population was substantially younger (mean age ± SD, 32.8 ± 5.2 years)

Table 2. Previous bacterial infections in patients with cirrhosis.

Patient	Child-Pugh class	SBP	Other bacterial infections
1	C	Yes	None
6	C	Yes	None
8	C	Yes	Bacteremia + UTI
11	B	Yes	Bacteremia
12	C	Yes	Bacteremia + pneumonia
13	C	No	Bacteremia + pneumonia
14	B	No	Bacteremia
17	C	Yes	Bacteremia
19	B	No	Erysipelas
20	C	Yes	Bacteremia
21	C	Yes	None
22	C	Yes	None

NOTE. SBP, spontaneous bacterial peritonitis; UTI, urinary tract infection.

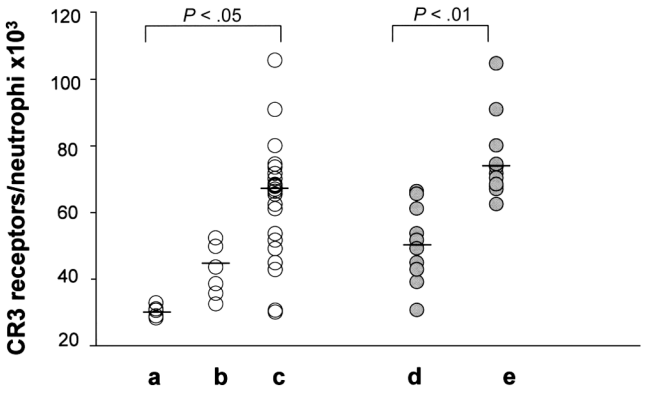

Figure 3. CR3 expression in circulating neutrophils. *Unfilled circles* represent CR3 values in healthy controls *(a),* liver transplant recipients *(b),* and complete cirrhotic population *(c)*. *Filled circles* represent 2 subpopulations of cirrhotic patients: patients without previous bacterial infections *(d)* and cirrhotic patients with previous infections *(e)*. *Horizontal bars* represent mean values for each group. *P* values reflect differences between groups by analysis of variance *(a–c)* or paired *t* test *(d–e)*.

than the patient population of liver transplant recipients (mean age ± SD, 51.5 ± 6.5 years) and cirrhotic patients (mean age ± SD, 55.8 ± 10.4 years). It has been previously shown that neutrophil functions in healthy elderly subjects, including migration, priming, adhesion, activation, and migration in vivo to skin windows do not differ from those in healthy younger people [35–37]. Therefore, we believe the age difference should not affect the experimental results.

The etiology of cirrhosis was viral (11 patients [50%]), alcoholic (5 patients [22%]), both alcoholic and viral (4 patients [18%]), and unknown (2 patients [9%]). On the basis of liver function tests, 10 patients with cirrhosis belonged to Child-Pugh class B, and 12 belonged to class C. Twelve cirrhotic patients had ⩾1 episode of severe bacterial infection in the 6 months before inclusion in this study. Spontaneous bacterial peritonitis was the most common infection, frequently associated with other infections (table 2). Neither OTL recipients nor healthy volunteers had any previous episode of bacterial infection during the preceding months

Neutrophil delivery into skin windows. Cirrhotic patients showed a decreased neutrophil influx into skin windows filled with either gram-negative or gram-positive antigens, compared with healthy controls and liver transplant recipients (table 3). Although the mean ± SD of peripheral blood neutrophils was significantly lower in cirrhotic patients (1315 ± 902) than in healthy controls (5420 ± 892) or in liver transplant recipients (5500 ± 1264), no significant correlation was found between peripheral blood count and neutrophil exudation to skin windows in any of the 3 study groups.

In cirrhotic patients, neutrophil migration into skin windows significantly correlated with the magnitude of clinical liver dysfunction. Neutrophil exudation was more depressed in Child-Pugh class C patients than in Child-Pugh class B patients when challenged with either gram-positive (mean ± SD, 1.01 ± 0.28×10^6 PMNL/cm^2 vs. 1.28 ± 0.28×10^6 PMNL/cm^2; $P < .05$) or gram-negative antigens (mean ± SD, 1.04 ± 0.34×10^6 PMNL/cm^2 vs. 1.27 ± 0.33×10^6 PMNL/cm^2; $P < .05$). Liver transplant recipients showed an improved neutrophil influx into skin windows compared with cirrhotic patients, although a statistically significant difference was found only for skin windows filled with streptokinase. The presence of ascitic fluid, instead of 50% serum, significantly affected recruitment of neutrophils into skin windows in cirrhotic patients. Addition of 100% ascitic fluid markedly reduced the migration of neutrophils to skin windows in the presence of heat-killed *E. coli* (mean ± SD, 0.73 ± 16 PMNL/cm^2 vs. 1.19 ± 0.90 PMNL/cm^2; $P < .05$).

The number of elicited neutrophils in skin windows filled with serum and bacteria was significantly lower in cirrhotic patients with prior bacterial infections than in cirrhotic patients without infections (mean ± SD, 0.98 ± 0.23×10^6 PMNL/cm^2 vs. 1.35 ± 0.37×10^6 PMNL/cm^2, $P < .02$). In cirrhotic patients with previous episodes of SBP, neutrophil influx in response to *E. coli* in ascitic fluid was also significantly lower than in patients without previous SBP (figure 2).

CR3 expression in circulating and elicited neutrophils. Expression of CR3 receptor in circulating neutrophils of patients with cirrhosis was significantly greater than that of healthy controls (mean ± SD, 63,878 ± 16,934 receptors per cell vs. 30,113 ± 16,381 receptors per cell; $P < .05$) but was not greater than that of OLT recipients (42,135 ± 8,039 receptors per cell). Analysis of CR3 expression in subgroups of cirrhotic patients with or without previous bacterial infection showed that CR3 receptor expression is even higher in patients with previous bacterial infections (74,850 ± 11,885 vs. 50,412 ± 11,600 receptors per cell, $P < .01$; figure 3). It is interesting that the differences in the CR3 expression between healthy controls and noninfected cirrhotic patients were also statistically significant ($P < .05$).

There was no significant difference in CR3 expression in elicited neutrophils between the 3 study groups. Elicited neutrophils of cirrhotic patients with previous bacterial infections, however, expressed lower levels of CR3 than did those of patients without previous infection in skin windows filled with gram-negative bacteria (240,714 ± 65,402 vs. 316,760 ± 52,561 receptors per

Table 3. Neutrophil influx into skin windows.

	Chemoattractant	
Study group	Streptokinase in 50% serum	*Escherichia coli* in 50% serum
Patients with cirrhosis	1.13 ± 0.29	1.19 ± 0.90
OLT recipients	1.66 ± 0.58[a]	1.67 ± 1.30
Healthy controls	2.23 ± 0.71[a]	2.24 ± 2.10[b]

NOTE. Data are mean ± SD of elicited polymorphonuclear leukocytes × 10^6/cm^2. OLT, orthotopic liver transplantation.

[a] $P < .05$, compared with patients with cirrhosis challenged with streptokinase.

[b] $P < .05$, compared with patients with cirrhosis challenged with *E. coli*.

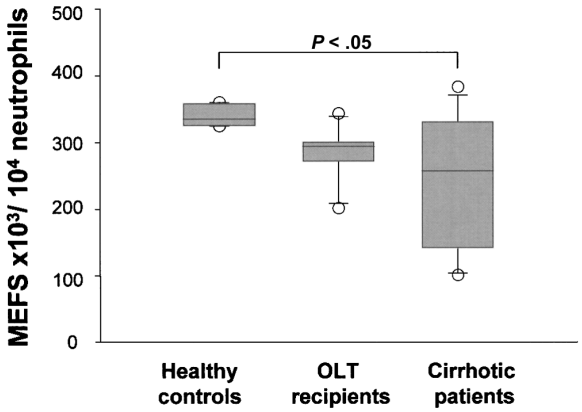

Figure 4. Neutrophil in vivo phagocytosis of heat-killed fluorescein-labeled *Escherichia coli* in skin windows filled with 50% autologous serum was significantly reduced in cirrhotic patients compared with healthy controls ($P < .05$) but not with orthotopic liver transplant (OLT) recipients. Fluorescence intensity is expressed as MESF (molecules of equivalent soluble fluorochrome) $\times 10^3$ in a population of 10^4 polymorphonuclear leukocytes. *P* values reflect differences between groups by Kruskal-Wallis analysis of variance on the rank test and Dunn's test.

cell; $P < .01$) or gram-positive antigen (234,566 ± 66,108 vs. 348,940 ± 72,790 receptors per cell; $P < .01$). This was also true when neutrophils from cirrhotic patients with a previous SBP were compared with neutrophils from cirrhotic patients with no previous episodes of SBP: in skin windows filled with ascitic fluid, patients with a prior SBP had 193,610 ± 80,895 receptors per cell, whereas cirrhotic patients with no previous episodes of SBP 317,220 ± 53,748 receptors per cell ($P < .01$).

In vivo phagocytosis. Neutrophil phagocytosis of heat-killed Bodipy-labeled *E. coli* in skin windows filled with 50% autologous serum was impaired in cirrhotic patients compared with healthy controls (242.5 ± 99.2 × 10^3 MESF vs. 339.4 ± 17.1 × 10^3 MESF, $P < .05$; figure 4). In cirrhotic patients, such impairment was even stronger in the presence of 100% ascitic fluid (185.7 ± 90.2 vs. 242.5 ± 99.2 × 10^3 MESF, $P < .05$). In vivo phagocytosis also correlated with the severity of liver dysfunction, being more depressed in Child-Pugh class C patients than in class B patients (190.07 ± 96.07 × 10^3 vs. 297.98 ± 73.70 × 10^3 MESF, $P < .05$)

In a subgroup analysis, exudate neutrophils of cirrhotic patients with previous bacterial infections showed a decreased ability to phagocytose bacteria in skin windows filled with 50% serum (186.5 ± 87.5 × 10^3 MESF vs. 309.6 ± 66.5 × 10^3MESF; figure 5). In vivo phagocytosis was also depressed in skin windows filled with 100% ascitic fluid in the subgroup of cirrhotic patients having previous episodes of SBP (90.85 ± 1.76 × 10^3 MESF vs. 223.84 ± 1.01 × 10^3 MESF). All cirrhotic patients enrolled in this study had a low total protein level in ascitic fluid (inclusion criteria included a total protein level ⩽1 g/dL). Total protein levels in ascitic fluid and serum did not affect in vivo phagocytosis of *E. coli* in skin windows among cirrhotic patients. Representative flow cytometry dot plots of in vivo *E. coli* phagocytosis are shown in figure 6.

Discussion

Neutrophils represent the first line of host resistance against bacterial infection, as part of the inflammatory-immune defense mechanism or innate immunity. During their migration from the intravascular compartment to the inflammatory focus, neutrophils are exposed to a number of stimuli, including inflammatory mediators, local bacterial products, and chemotactic gradients, that modify and modulate their function [38].

Our data suggest that cirrhotic patients with end-stage liver disease have an impaired ability to deliver neutrophils to an infective focus, and those neutrophils that finally reach the infected sites have a reduced phagocytic activity compared with neutrophils from healthy controls. A similar finding has been reported in a model of pneumococcal pneumonia in rats with carbon tetrachloride–induced cirrhosis, which showed decreased clearance of lung bacteria [39]. Furthermore, deficient neutrophil delivery to skin windows and in vivo phagocytic activity correlated in cirrhotic patients with the severity of liver disease. This phenomenon is even more marked in cirrhotic patients with recent bacterial infections, as demonstrated by decreased mobilization of neutrophils to skin windows, reduced phagocytosis compared with cirrhotic patients without bacterial infections, and decreased expression of CR3 in exudate neutrophils. Because all samples from the skin windows were analyzed at a single time point (12 h), it is possible that this phenomenon

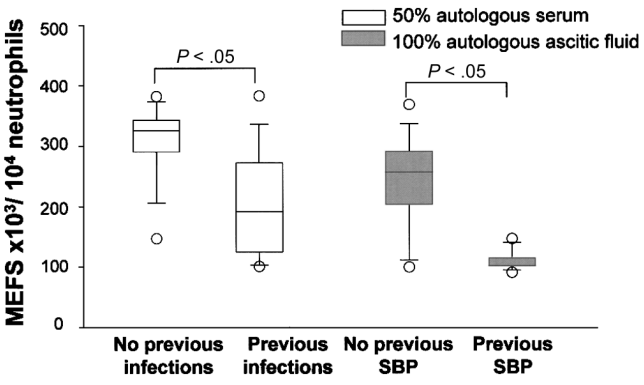

Figure 5. In vivo phagocytosis of fluorescein-labeled *Escherichia coli* particles in subpopulations of cirrhotic patients. *Unfilled rectangles* represent in vivo neutrophil phagocytosis of fluorescent *E. coli* in skin windows filled with 50% autologous serum in cirrhotic patients with or without previous bacterial infections. *Filled rectangles* represent phagocytosis in skin windows filled with 100% autologous ascitic fluid in cirrhotic patients with or without previous spontaneous bacterial peritonitis. Fluorescence intensity is expressed as MESF (molecules of equivalent soluble fluorochrome) $\times 10^3$ in a population of 10^4 polymorphonuclear leukocytes. *P* values reflect differences between groups of cirrhotic patients by Mann-Whitney test.

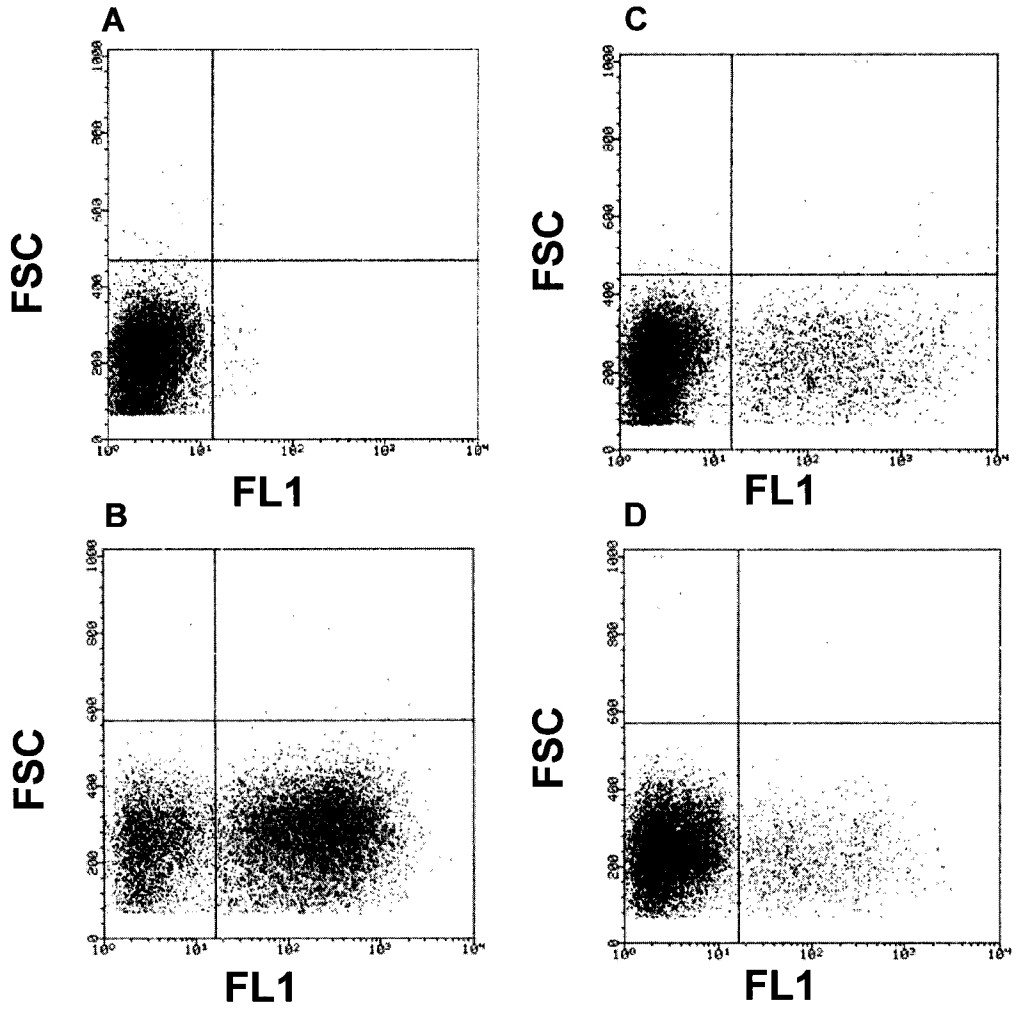

Figure 6. Representative flow cytometry dot-plot diagrams of elicited neutrophils in skin windows, showing in vivo phagocytosis of fluorescein-labeled *Escherichia coli* particles. *A,* Negative control (elicited neutrophils in 50% serum with streptokinase). *B,* In vivo phagocytosis of heat-killed fluorescein-labeled *E. coli* in the presence of 50% serum in a healthy control. *C,* In vivo phagocytosis of *E. coli* in skin windows filled with 50% serum in a cirrhotic patient. *D,* In vivo phagocytosis of *E. coli* particles in the presence of 100% ascitic fluid in the same cirrhotic patient as in *C.* The *x*-axis represents fluorescence intensity in a logarithmic scale (FL1). The *y*-axis represents forward size scatter (FSC).

also reflects a delay in kinetics of neutrophil accumulation in skin windows.

CR3 is a β_2-integrin that mediates firm adhesion of neutrophils to cytokine-activated endothelium. CR3 is well expressed by resting neutrophils and shows a rapid and marked increase after neutrophil activation by inflammatory mediators. This up-regulation is a sensitive marker of cell activation [40, 41]. In the present study, circulating neutrophils of cirrhotic patients exhibited a significantly greater up-regulation of CR3 than did those of healthy controls, which suggests a chronic intravascular activation of peripheral blood neutrophils in these patients, even in cirrhotic patients without previous infections. In addition, high levels of CR3 in peripheral blood neutrophils in cirrhotic patients could increase the neutrophil marginated pool and contribute to the neutropenia observed in these patients.

The CR3 receptor is not only involved in strong adhesion of neutrophils to activated endothelium and subsequent trans-endothelial migration but also in neutrophil binding to C3bi-coated bacteria that is a crucial step in bacterial engulfment and phagocytosis [42]. In the present study, elicited neutrophils of cirrhotic patients with previous bacterial infections showed a significantly lower expression of CR3 receptor compared with patients without previous bacterial infections, particularly in the presence of ascitic fluid. In a microenvironment with reduced C3 levels, failure of phagocytic cells to express CR3 could be directly related to their depressed ability to adhere to and engulf bacteria, as we have demonstrated in skin windows placed on cirrhotic patients.

The liver is a major site for clearance of circulating cytokines. IL-1, IL-3, IL-6, TNF-α, transforming growth factor–β, and

IFN-γ are rapidly cleared from plasma by the liver within minutes after intravenous injection [43]. Several studies suggest that patients with liver cirrhosis have high cytokine levels in both plasma and ascitic fluid even in the absence of clinical infection [44–46]. Increased endotoxin absorption and bacterial translocation from the bowel may play a role in producing a chronic subclinical stimulus of the reticuloendothelial system to produce cytokines [47]. The lack of hepatic clearance of these inflammatory mediators in cirrhotic patients may lead to a chronic activation of peripheral blood neutrophils that is reflected in the higher CR3 receptor expression found in our study. This chronic intravascular neutrophil activation may impair subsequent neutrophil functions, such as transendothelial migration and bacterial phagocytosis, in cirrhotic hosts. It may also be related to the high incidence of the systemic inflammatory response syndrome and subsequent multiple organ failure seen in cirrhotic patients with severe infections [48]. Our data demonstrate that cirrhotic patients with advanced liver disease have defects in the early phases of the inflammatory-immune response against bacteria. The failure of the local inflammatory response may be responsible for the impaired clearance of small inocula of bacteria in the extravascular space that would not result in clinical infections in a healthy subject.

SBP is believed to result from spontaneous bacteremia with secondary seeding into ascitic fluid [2]. Predisposing factors for developing SBP in cirrhotic patients include the severity of the underlying liver disease, gastrointestinal hemorrhage, ascitic fluid total protein $\geq$1 g/dL, low C3 level, and a previous SBP episode [49]. The low numbers of organisms found in the peritoneal fluid are characteristic of SBP. Our data showed that cirrhotic patients exuded fewer neutrophils when skin windows were filled with 100% ascitic fluid instead of 50% serum and that phagocytosis was less efficient in ascitic fluid. Decreases in neutrophil migration and phagocytic activity were significantly greater in cirrhotic patients with a previous episode of SBP. All cirrhotic patients included in this study had a total protein concentration in their ascitic fluid <1 g/dL; therefore, they were at a high risk of developing SBP. The differences found in neutrophil influx and phagocytic activity in cirrhotic patients with previous SBP probably result from patient subgroups with more severe impairment of the immune-inflammatory response to bacteria, because the SBP episodes are probably more a consequence than a cause of this impairment.

It is noteworthy that, in our model, liver transplant recipients receiving chronic immunosuppressive therapy showed a stronger ability to deal with an infective insult than did patients with advanced liver cirrhosis. Neutrophil dysfunction in our study correlated with the severity of liver disease, and this may explain why liver transplant recipients with normal liver function are able to initiate a more efficient immune response against bacterial antigens than are cirrhotic patients.

In conclusion, patients with liver cirrhosis and end-stage liver disease showed an impaired initial immune-inflammatory response characterized by low neutrophil delivery to inflammatory foci in vivo, with a decreased phagocytic activity that is more severe in a microenvironment that resembles the peritoneal cavity. In this group of patients, spontaneous bacterial peritonitis may represent a local failure to overcome a small bacterial inoculum in the peritoneal cavity because of abnormalities in the initiation of the local inflammatory response. Skin windows are a useful tool to measure the resulting inflammatory reaction to intact bacteria and may be appropriate for use in detecting subgroups of cirrhotic patients with an increased susceptibility to bacterial infections and in monitoring their response to new therapies.

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