Opsonic Fibronectin Deficiency and Sepsis

Cause or Effect?

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Opsonic fibronectin is known to modulate macrophage (RE cell) and neutrophil phagocytic function. Its depletion has been documented following trauma, burn, and operation in patients with rapid restoration of normal levels unless bacteremia and/or wound sepsis intervenes. Sepsis is associated with a secondary phase of opsonic fibronectin deficiency. We have observed in burn patients that this secondary phase of opsonic fibronectin depletion following trauma and burn is seen two to three days prior to the onset of clinical sepsis, raising the question of whether this deficiency sensitized the host to the subsequent development of sepsis or whether its depletion was merely an unsuspected sensitive indication of preclinical sepsis. To address the possibility that opsonic fibronectin deficiency might lower resistance to sepsis, Sprague-Dawley rats (200 gm) were partially depleted (35%) of their opsonic fibronectin prior to intraperitoneal inoculation with *Staphylococcus aureus*. Mortality to *S. aureus* peritonitis was significantly ($p < 0.05$) increased in animals with fibronectin deficiency. Furthermore, in control animals, nonsurvival was also associated with significantly ($p < 0.05$) lower initial fibronectin levels than survival. However, peritonitis itself also resulted in an early (within one hour) depletion of opsonic fibronectin followed by a marked "hyperopsonemia" within 12 hours in both groups. Thus, opsonic fibronectin depletion decreases resistance to sepsis, and the development of sepsis itself will initiate opsonic fibronectin deficiency. Host defense against infection may depend on early restoration and maintenance of normal opsonic fibronectin levels following trauma, burn, and operation, as well as the ability of the host to mount an appropriate hyperopsonemic elevation of fibronectin levels in response to infection.

CLINICAL AND EXPERIMENTAL trauma or burn results in a decreased resistance to infection, presumably by depressing numerous host defense parameters.[2,12] The reticuloendothelial system (RES) serves many important host defense functions by clearing bacteria and nonbacterial particles, cellular debris, and products of disseminated intravascular coagulation (DIC).[19] Theoretically, by preventing both systemic dissemination of bacteria and/or microembolization of the products of bacterial sepsis, both overwhelming sepsis and organ failure are prevented.[18,19] Experimental depression of the RES alone by "RE blockade" results in decreased resistance to endotoxin and infection[19,20] emphasizing its pivotal role in host defense. The exact mechanism by which RE blockade results in this decreased resistance is not known. Following RE blockade by gelatin-coated colloids, clearance of a subsequent test dose of colloids or bacteria is delayed, and this is associated with an increase in their lung localization.[9,17] This is evidence implicating inefficient clearance of bacteria and products of sepsis as a possible mechanism underlying this decreased resistance to bacteremia.

Opsonic fibronectin, a 440,000 dalton glycoprotein, modulates RE clearance of nonbacterial test particles, fibrin monomer, and some bacterial species.[18,19] Peritoneal macrophage[6] and neutrophil phagocytosis[10] are also augmented by opsonic fibronectin. This protein is depleted following trauma and burn as well as during RE blockade.[8,11,17] Fibronectin depletion correlates with RE phagocytic clearance depression, and restoration of circulating levels is associated with restoration of RE function. In a recent study in burn patients, opsonic fibronectin depletion preceded the onset of clinical sepsis by two to three days,[11] raising the question of whether opsonic fibronectin deficiency undermines host defense to sepsis or whether its depletion is merely a sensitive indicator of subclinical infection. To answer this question, the present study tested the hypothesis

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that selective depletion of opsonic fibronectin at the time of intraperitoneal inoculation of bacteria would decrease resistance to sepsis. Additionally, the effect of sepsis itself on opsonic fibronectin levels was evaluated.

Materials and Methods

Animals

Sprague-Dawley rats (200 gm) were used in all experiments. New Zealand white rabbits (3–5 kg) were used to prepare antibody to rat opsonic fibronectin.

Bacteria

Staphylococcus aureus isolated from the nasopharynx of an asymptomatic carrier (phage type 96) was maintained in trypticase soy broth. Fresh overnight cultures of the bacteria were grown prior to the experiment. The bacteria were then centrifuged at 3000 × g for 10 min, washed three times with 0.9% saline, and resuspended at a concentration of 1×10^{11} as determined spectrophotometrically and confirmed by standard pour plate technique. Bacteria were injected intraperitoneally at doses ranging from 1×10^{10} to 1×10^{11} in a volume of 2 ml.

Antibody to Opsonic Fibronectin

Rat opsonic fibronectin was isolated and purified by gelatin-Sepharose affinity chromatography as described in detail by Engvall and Ruoslahti.[4] The isolation procedure is based on the high affinity of opsonic fibronectin for gelatin. Pooled rat plasma was pumped through the column at a rate of 26 ml/hr while monitoring the outflow at 280 nm. The absorbed opsonic fibronectin was then eluted with 8M urea.

The isolated protein (approximately 800 μg/ml) was mixed 10:1 (v/v) with 20% sodium dodecyl sulfate and then combined with complete Freund's adjuvant (1:1, v/v). The mixture was injected subcutaneously into the backs of New Zealand white rabbits (3–5 kg) as described.[3] This was followed by three weekly injections using Freund's incomplete adjuvant. Ten days following the final injection antiserum was harvested via an ear vein. The antiserum was absorbed three times with fibronectin-free serum cross-linked to glutaraldehyde.[3] The final antiserum was monospecific when tested against normal rat serum using immunoelectrophoresis. The volume of antiserum to be injected into rats was the amount needed to decrease opsonic fibronectin levels 30% as determined in a pilot study using five rats. The volume thus determined was 0.2 ml/100 gm. Experimental animals received 0.2 ml/100 gm antiserum IV one hour prior to and at the time of intraperitoneal *S. aureus* inoculation.

Immunoassay of Opsonic Fibronectin

Electroimmunoassay was used for determination of opsonic fibronectin using the above monospecific antiserum as previously described.[3] The monospecific antiserum was mixed with 1% agarose gel and layered on a 3 × 10 inch glass plate. Wells (3.0 mm) at 1-cm intervals were cut in the agarose, and 10 μl of serum to be assayed was diluted to 10% and then added to each well. Samples were electrophoresed towards the anode at 7V/cm at 4 C for 20 hours. The plates were then washed and stained as previously described.[3] A double reciprocal standard curve relating rocket height to known standard concentration was constructed and used as a reference to determine the concentration in the unknown samples.

Bacteriologic Studies

Blood cultures were grown from periodic blood samples obtained from a tail vein using sterile technique. Standard dilutions were made and 1-ml samples from each dilution was mixed with warm trypticase soy broth at 37 C in sterile petri dishes and allowed to solidify. Cultures were grown for 24 hours prior to quantification. Lung and peritoneal cultures were obtained from two additional groups of rats killed at 6 and 12 hours for bacteriologic quantification of peritoneal fluid and lung. For peritoneal cultures, 5 ml of sterile saline was injected into the peritoneum, and, after mixing for 30 sec, 0.2 ml of fluid was removed for bacteriologic determination as performed on the blood samples. The lungs were removed through a separate sternal splitting incision, weighed, mixed with 3 ml of sterile saline, and homogenized in a sterilized tissue homogenizer (Beckman). The homogenate was centrifuged at 3000 × g for 10 minutes, and 0.2 ml of the supernatant was removed for bacteriologic determination.

Statistical Analysis

One-tailed Student's t-test, paired t-test and one-tailed Fisher Exact test were used where appropriate. The confidence level was set at 95%.

Results

The effect of intraperitoneal inoculation of *S. aureus* on opsonic fibronectin levels in control animals is shown in Figure 1. Intraperitoneal inoculation of bacteria resulted in a decrease in circulating fibronectin when measured one hour following bacterial injection. This decrease was associated with a significant ($p < 0.05$) increase in hematocrit (from 42.4 to 46.5). When corrected for this hemoconcentration, the decrease is significant ($p < 0.05$) for each dose of *S. aureus* by paired analysis, as presented in Figure 1. While there are dif-

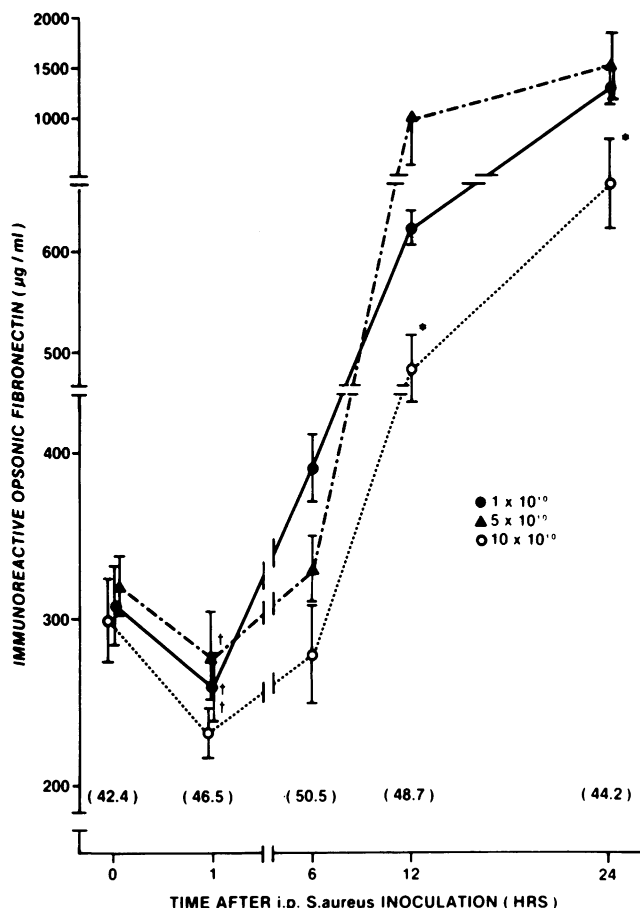

FIG. 1. The effect of *S. aureus* peritonitis on opsonic fibronectin concentration in control rats. Bacteria were inoculated IP at 0 hour. Values shown are corrected for the hematocrit shown in parenthesis at each time period. Values are mean ± SEM; *significantly ($p < 0.05$) different from other groups at the same time (analysis of variance); † = significantly ($p < 0.05$) different from baseline (0 hour) within each group (paired t-test).

ferences in fibronectin levels between each of the three doses, the temporal pattern in each of the three groups is essentially the same. Basically, opsonic fibronectin levels returned to baseline between one and six hours and rose to levels two to three times normal over the 12- to 24-hour period. In rats inoculated with the largest dose of *S. aureus* (10×10^{10}), fibronectin levels remained significantly below those of the other two groups at 12 and 24 hours. The findings presented in Figure 1 suggest that elevation of fibronectin levels during sepsis may represent a host defense response to infection, which if true would indicate that prevention of this response and/or depletion of fibronectin may compromise defense to infection. To test this hypothesis, the effect of administration of antiserum to fibronectin on both fibronectin levels during sepsis as well as overall resistance to sepsis was evaluated.

Injection of two doses of antiserum to fibronectin at one hour prior to and at the time of bacterial inoculation had a profound effect on fibronectin levels (Table 1). In animals receiving antiserum to fibronectin, opsonic fibronectin levels were significantly ($p < 0.01$) lowered at the time of *S. aureus* challenge (0 hour) when compared to either their own baseline levels (34%) or control animals (29% less). They remained lower than controls (30–35%) throughout the 24 hour study period. Elevation of fibronectin levels did occur in the antifibronectin group, although this was delayed and was not of the same magnitude. It should be noted that the fibronectin levels at 12 and 24 hours in the group given antiserum represent the levels attained in only those animals that survived, since mortality was high in this group as will be presented.

Fibronectin deficiency at the time of bacterial challenge was also associated with an increased mortality from *S. aureus* peritonitis (Fig. 2). There was a significant ($p < 0.05$) increase in mortality in the group of animals given antiserum to fibronectin at the 5×10^{10} dose (86% versus 9%) and 10×10^{10} dose (86% versus 36%). Most animals succumbing to sepsis died between 8 and 12 hrs in both groups, indicating that the increased mortality in the antiserum group was not due to a toxic affect of the antiserum itself but was most likely due to an imbalance between host defense and sepsis.

It is known that normal animals typically display a

TABLE 1. *The Effect of IV Administration of Antiserum To Fibronectin on Opsonic Fibronectin Levels Prior to and During* S. aureus *Peritonitis*

Experimental Group	Immunoreactive Opsonic Fibronectin (μg/ml) Baseline (−1 Hr)	0 (Hr)	1 (Hr)	6 (Hr)	12 (Hr)	24 (Hr)
Antialbumin (N = 31)	367 ± 14	309 ± 13	294 ± 15	402 ± 20	935 ± 93	1409 ± 134
Antifibronectin (N = 21)	333 ± 28	220 ± 10	201 ± 7	295 ± 29	599 ± 83	945 ± 140
P (between groups)	NS	<0.01	<0.01	<0.01	<0.01	<0.01
Hematocrit	44.5 ± 0.3	42.4 ± 1.3	46.5 ± 0.6	50.5 ± 0.7	48.7 ± 1.1	44.2 ± 0.5

Anti-fibronectin given IV at −1 and 0 hour at a dose of 0.2 ml/100 gm. Values are uncorrected for the changes in hematocrit. The hematocrit changes were not related to bacterial dose. The three bacterial doses in each group are combined. Values are mean ± SEM.

broad range of endogenous circulating fibronectin levels. This is illustrated by the data shown in Figure 3, which presents the association between fibronectin levels at the time of bacterial challenge and subsequent death. Nonsurviving antialbumin animals had significantly ($p < 0.05$) lower fibronectin levels at the time of *S. aureus* inoculation than did survivors at the bacterial doses (5 and 10×10^{10}) where some mortality was observed (Fig. 2). They also had lower levels when measured one and six hours following *S. aureus* inoculation.

Bacteriologic Studies

In an attempt to determine whether the increased mortality associated with experimentally induced opsonic fibronectin deficiency might be due to increased bacteremia or systemic organ localization of bacteria, blood cultures, peritoneal cultures, and lung cultures were performed on separate groups of animals given a bacterial dose of 5×10^{10} and receiving either antifibronectin or antialbumin. The results of these bacterial studies are shown in Figure 4 and Table 2. Blood cultures were positive throughout the 12-hour study in both the antialbumin and antifibronectin groups, although no significant difference was found between the two

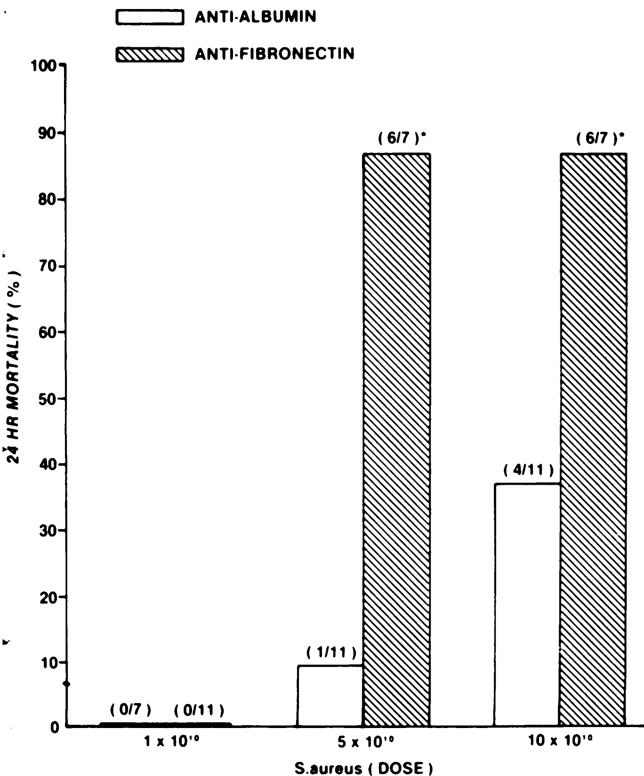

FIG. 2. Twenty-four-hour mortality in rats given either antialbumin (control) or antifibronectin as a function of the *S. aureus* dosage. Numbers in parenthesis are nonsurvivors/total. *Significantly ($p < 0.05$) different from antialbumin (one-tailed Fisher Exact Test).

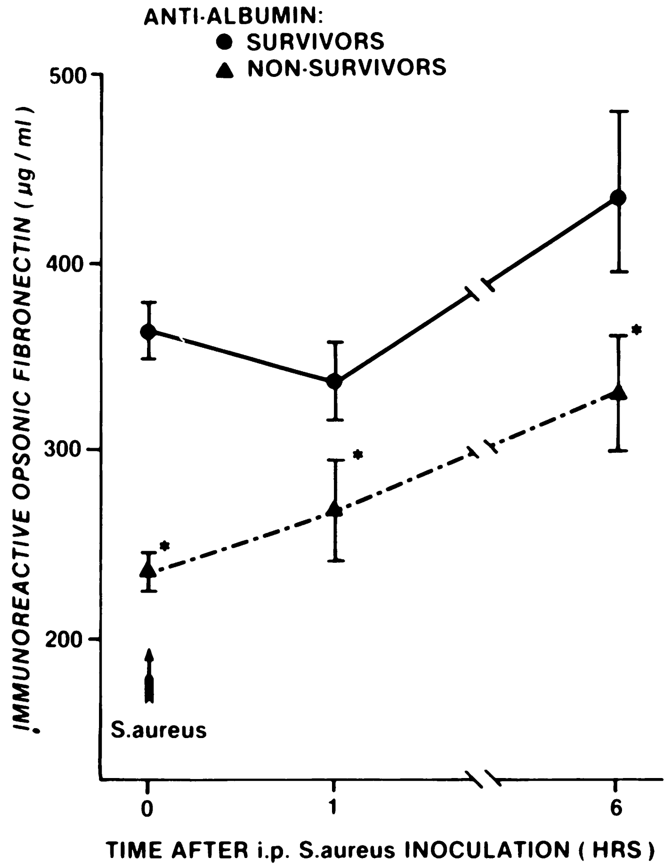

FIG. 3. Immunoreactive opsonic fibronectin levels in surviving (N = 17) and nonsurviving (N = 5) control animals at the time of and following *S. aureus* inoculation. As there was no mortality at the lowest dose of bacteria given, only the animals given the two higher doses of bacteria were used in this analysis. Values are mean ± SEM; *significantly ($p < 0.05$) less than survivors at the same time period (one-tailed t-test).

groups (Fig. 4). In addition, there was no significant difference in quantitative lung cultures between groups (Table 2). However, rats receiving antialbumin had significantly higher ($p < 0.05$) bacterial concentration in the peritoneum at both 6 and 12 hrs than rats receiving antifibronectin, although the amount of bacteria recovered represented less than 0.1% of the original bacterial inoculum.

Discussion

Decreased resistance to infection has been documented acutely after trauma and burn, as determined by either mortality,[1,13] systemic spread,[13] or local proliferation of inoculated organisms.[5] This decreased resistance is a function of the severity of injury and the time postinjury. McRipley and Garrison[13] and Alexander[1] demonstrated that this decreased resistance could be documented within moments following burn. It persisted for approximately 24 hours, followed by an

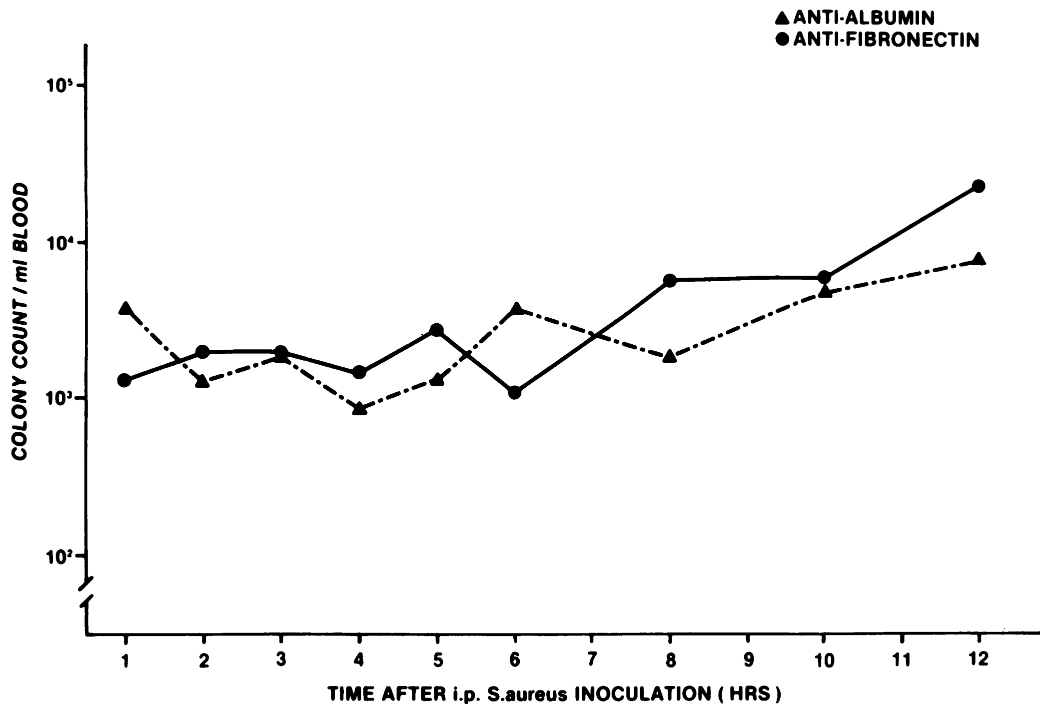

FIG. 4. Blood culture results from rats injected with *S. aureus* IP at a dose of 5 $\times 10^{10}$. There was no significant difference between groups at any time (logarithmic scale).

actual increase in resistance at 48 hours. Reticuloendothelial dysfunction after trauma likewise manifests itself within minutes and lasts up to 24 hours followed by hyperactivity as measured using colloid clearance techniques.[8] RE depression has, therefore, been implicated in the etiology of acute host defense failure following trauma and burn.[19] Indeed "blockade" of the RE system by the injection of exogenous particulates results in a similar decrease in resistance to infection,[19,20] followed by an actual increased resistance. The RE depression following trauma, burn, and blockade appears to be mediated, in part, by depletion of a plasma factor, now known to be opsonic fibronectin.[17,18] This molecule is opsonic for a number of exogenous and endogenous nonbacterial particulates and stimulates neutrophil phagocytosis of some bacteria.[10,18]

The present study demonstrates that selective depletion of opsonic fibronectin by antiserum results in a decrease in resistance to infection comparable to that seen following trauma, burn, or RE blockade. The present study does not address itself to the exact temporal relationship between opsonic fibronectin depletion and sensitivity to bacterial challenge. Thus, whether opsonic fibronectin depletion following bacterial inoculation as opposed to preceding bacterial inoculation would also result in the same increased mortality remains to be evaluated. Depletion was initiated prior to bacterial inoculation in the present study in order to more closely simulate depletion following trauma or burn prior to the onset of sepsis.

We cannot unequivocably state that injection of antiserum to fibronectin is free of effects other than depletion of circulating fibronectin. Antifibronectin antibody against cell-surface fibronectin is capable of lysing fibroblasts in the presence of complement *in vitro.*[16] However, this effect can be inhibited in the presence of small amounts of normal serum due to the binding of the antibody to the opsonic fibronectin present in the

TABLE 2. *Bacteriology of Peritoneum and Lung at 6 and 12 Hrs Following* S. aureus *Inoculation*

	Colony Count ($\times 10^5$)			
	6 Hrs		12 Hrs	
Experimental Group	Lung (per gm)	Peritoneum (per ml)	Lung (per gm)	Peritoneum (per ml)
Antialbumin	3.7 ± 2.1	379.1 ± 199.2	3.7 ± 1.2	308.9 ± 159.1
Antifibronectin	0.6 ± 0.1	3.3 ± 0.7	10.4 ± 4.9	29.9 ± 19.5
P (between groups)	NS	<0.05	NS	<0.05

Two separate groups of animals inoculated with 5 $\times 10^{10}$ *S. aureus* were killed at 6 and 12 hrs for bacterial cultures. To obtain peritoneal cultures, 5 ml of sterile saline was injected ip and agitated for 20 sec. The abdomen was opened sterilly, and 0.2 ml of fluid was removed for culture.

serum. Therefore, at the dose and regimen employed in the present study, we do not feel that this effect has played a role because of the large amount of serum in adult rats (4 ml/100 gm) compared to the volume of antiserum injected. We have injected antiserum into normal rats and have observed no adverse effects other than opsonic fibronectin depletion.

An additional feature of this study is the clarification of the relationship between sepsis and its effect on opsonic fibronectin levels as observed in the control group of rats. Intraperitoneal inoculation of *S. aureus* resulted in a decrease in circulating opsonic fibronectin when measured one hour following the inoculation. This was accompanied by a 10% increase in hematocrit indicative of hemoconcentration. The peritoneum responds to intraperitoneal bacterial challenge with an increase in permeability to fluid as well as large and small proteins.[7,14] Opsonic fibronectin which is 440,000 daltons may readily traverse the peritoneum during such inflammation since fibrinogen, also a large molecule, has been found to traverse the peritoneum during inflammation. The theoretical role of fibronectin in local defense could be to augment bacterial phagocytosis by neutrophils and peritoneal macrophages, aid in clearance of fibrin and tissue debris, and increase bacterial aggregation, thus inhibiting their systemic absorption. Delayed absorption of large amounts of bacteria and/or exotoxin from the peritoneum may enhance immediate survival,[7] while excessively inefficient absorption may result in abcess formation and greater long-term mortality.[7] In the presence of RE depression, such as that induced in the present study, rapid absorption of bacteria may be even more deleterious.[15] Evidence that absorption may have been more rapid in the fibronectin-depleted animals is the larger bacterial counts from the peritoneum in control animals. It is unlikely that greater proliferation in the control animals could account for this difference at 6 and 12 hours following inoculation as counts did not increase between 6 and 12 hours in control animals.

The "hyperopsonemia" observed at 6, 12, and 24 hours in control animals and to a lesser extent in fibronectin-depleted animals has been shown to occur following trauma, burn, and RE blockade.[8,11,17] This may serve to augment local defense as well as RE clearance of circulating bacteria and products of sepsis or tissue injury. The persistently lower opsonic fibronectin concentrations attained by rats injected with the largest bacterial dose suggests ongoing consumption of the protein. Furthermore, as observed in control rats, inability to produce an adequate baseline level of this protein as well as the inability to increase its production following initiation of sepsis may be responsible for decreased host defense. This observation reinforces our concept that the increased mortality in the fibronectin-depleted animals was a direct result of decreased fibronectin levels resulting from the antiserum administration.

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