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Acute kidney injury

The Seminar on acute kidney injury (AKI) by Rinaldo Bellomo and colleagues (Aug 25, p 756)[1] is excellent. AKI is typically diagnosed by the accumulation of end products of nitrogen metabolism, decreased urine output, or both.[2] In the presence of decreased urine output, however, the diagnosis of AKI should be established only in the absence of increased intra-abdominal pressure.

Intra-abdominal hypertension and abdominal compartment syndrome are severe complications present in about 30–50% of mechanically ventilated patients in intensive-care units.[3] Patients with high intra-abdominal pressure can present with oliguria or anuria owing to direct mechanical pressure and increased renal venous pressure, which could be incorrectly interpreted as AKI. The term AKI implies that a continuum of kidney injury exists that begins long before loss of excretory kidney function can be measured with standard laboratory tests.

Furthermore, in patients with left-ventricular failure and increased intra-abdominal pressure, the elevated haemodynamic variables, together with oliguria, could be incorrectly interpreted as fluid overload, resulting in increased use of diuretics, decreased renal arterial blood flow, and, ultimately, AKI or further deterioration of the already compromised renal function. Additionally, in normovolaemic patients with increased gut permeability and intra-abdominal pressure, oliguria could be attributed to decreased endovascular volume, which might be treated with additional fluids, exacerbating visceral oedema and perhaps leading to decreased glomerular blood flow.[4,5]

Intra-abdominal hypertension must be taken into account during any exploration of the cause of oliguria and should always be excluded before the diagnosis of AKI.

We declare that we have no conflicts of interest.

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Rinaldo Bellomo and colleagues[1] express serious concerns about acute tubular necrosis as an underlying mechanism of acute kidney injury. We would like to endorse this view.

Renal tubular cells require a large supply of energy to accomplish their primary function of electrolyte and fluid reabsorption from glomerular filtrate. However, adequate energy provision can be endangered during sepsis and other states of systemic inflammation when oxygen supply is diminished owing to macrocirculatory and microcirculatory alterations, and ATP generation is compromised by mitochondrial dysfunction.[2] Clinically relevant tubular injury should thus cause massive polyuria if electrolyte and fluid reabsorption were impaired. However, oligoanuria is far commoner. Furthermore, necrosis is rarely found to any significant extent in critically ill patients.[3] In survivors of critical illness who required renal replacement therapy, fewer than 10% required long-term dialysis.[4]

We support the idea that renal dysfunction during sepsis and systemic inflammation is due to functional rather than structural change. The mechanism of oligoanuria is likely to be a drastic decrease in glomerular ultrafiltration from changes in intrarenal blood flow, probably mediated by the neurohumoral response, local vasoactive agents such as nitric oxide, and, crucially, the purinergic molecules ATP and adenosine which are known to be important mediators of tubuloglomerular feedback.[5] Bioenergetic dysfunction could thus play a key role in triggering the reduction in glomerular filtration rate.

Reduced glomerular filtration would spare tubular cells from their main workload and permit a reduction in energy expenditure, allowing the cells to survive in a non-functional yet viable condition. When critical illness resolves and energy production is restored, the cells thus retain the potential for full functional recovery. Hence, renal dysfunction in sepsis and other systemic inflammatory disorders could be viewed as an adaptive and potentially protective mechanism.

We declare that we have no conflicts of interest.

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Authors' reply

We appreciate the concerns expressed by Athanasios Chalkias and Theodoros Xanthos about the role of increased intra-abdominal pressure in the pathogenesis of acute kidney injury (AKI), and indeed mention this possibility in our Seminar.[1] However, as shown in a prospective investigation,[2] although increased intra-abdominal pressure is common in patients in intensive care, its clinical meaning and renal consequences remain unclear in the absence of a full abdominal compartment syndrome. Therefore, we argue that routine measurement of intra-abdominal pressure is not justified unless intra-abdominal compartment syndrome seems clinically likely and renal function is being rapidly lost. Moreover, until randomised trials indicate that patients whose intra-abdominal pressure is routinely monitored achieve or maintain better renal function than do patients assigned standard care, we regard selective measurement of intra-abdominal pressure as a more rational approach.

We fully share Alain Rudiger and Mervyn Singer's view that changes in intrarenal blood flow are most likely to be responsible for AKI, especially in sepsis.[3] Thus, at least in the early phases, AKI seems to be a disease of the microcirculation. If this is correct, as many observations now suggest, the therapeutic implications are substantial. In particular, the administration of large amounts of fluids in a futile attempt to resuscitate the kidney might well aggravate rather than attenuate AKI.[4] Such fluid-based therapy remains the unproven and pathophysiologically unjustified cornerstone of AKI treatment in critically ill patients. Accordingly, in our Seminar, we specifically sought to begin the process of formally challenging yet another unproven dogma in medicine.

We fear that, until physicians are better informed about these developing insights into the pathogenesis of septic AKI, many patients will suffer from iatrogenic disease induced by flawed pathophysiological paradigms.

RB and CR have received consultancy and speaking fees from Alere, Abbott Diagnostics, Gambro, Fresenius, B Braun, and Edwards Lifesciences. JAK has received consultancy and speaking fees from Alere, Abbott Diagnostics, Gambro, Baxter, and Fresenius.

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Prevalence and patterns of tobacco use in Asia

On the basis of data from the Global Adult Tobacco Survey, Gary Giovino and colleagues (Aug 18, p 668)[1] provide some vital smoking prevalence data for planning purposes and for assessing the progress of smoking control interventions nationally and globally.

The number of 301 million smokers in China has been a headline figure in the Chinese media since the paper was published. Although population-based surveys that use self-report are generally regarded as reliable in some western populations,[2-4] they severely underestimate the true numbers of smokers in Asian populations. In a survey in Shanghai, China, we estimated the actual numbers of smokers among 11 104 students (aged 12–20 years) and their parents (aged ≥35 years). Using a capture-recapture method, we found that self-report could capture only 65% of the smokers in adult women, and only 37% in female students (table). Similarly, in a study of Korean adults,[5] self-report could only detect 42% of smokers identified by the biomarker cotinine in urine among adult Korean women. Self-report did reasonably well in adult men, capturing 94% of smokers in Chinese men and 89% in Korean men, but was less satisfactory in young Chinese men, with only 74% of smokers being identified.

	Prevalence		Ratio (95% CI)†
	Self-reported	Estimated actual*	
Young men (n=5482)	748 (13·6%)	1006 (18·4%)	0·74 (0·68–0·81)
Young women (n=5452)	284 (5·2%)	775 (14·2%)	0·37 (0·32–0·42)
Adult men (n=4314)	3386 (78·5%)	3583 (83·1%)	0·94 (0·92–0·96)
Adult women (n=4015)	166 (4·1%)	256 (6·4%)	0·65 (0·54–0·78)

*Estimated using a capture-recapture method. †Ratio=self-reported prevalence/estimated actual prevalence.

Table: **Comparison of self-reported smoking with actual smoking in Chinese adults and students aged 12–20 years**

Therefore, the actual number of smokers in China could be substantially higher than 301 million. Such underestimation could hinder our ability to assess the progress of current and future intervention efforts. Because under-reporting is disproportionately high in women and young Asians, policy makers should be aware of the large number of hidden women and young Asian smokers.

This publication is based on research funded in part by the Bill & Melinda Gates Foundation. The findings and conclusions contained within are those of the authors and do not necessarily reflect positions or policies of the Bill & Melinda Gates Foundation. We declare that we have no conflicts of interest.

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Critical Care Medicine
Issue: Volume 27(7), July 1999, pp 1230-1251

Publication Type: [Feature Articles]
ISSN: 0090-3493
Accession: 00003246-199907000-00002

[Feature Articles]

Apoptotic cell death in patients with sepsis, shock, and multiple organ dysfunction

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Supported, in part, by National Institutes of Health grants GM44118, GM55195 and GM48095 and by the Alan A. and Edith L. Wolff Foundation.

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Abstract

Objectives: The purpose of this study was to determine whether apoptosis is a major mechanism of cell death in patients with sepsis. The activities of caspase-3 and the antiapoptotic protein, BCL-2, were investigated also.

Design: A prospective study of 20 patients who died of sepsis and multiple organ dysfunction was performed. The control group of 16 patients consisted of critically ill, nonseptic patients who were evaluated either prospectively [7] or retrospectively [9]. In addition, normal colon sections from seven patients who had bowel resections were included. Apoptosis was evaluated in hematoxylin and eosin-stained specimens by deoxyuridine triphosphate nick end-labeling (TUNEL) and by DNA gel electrophoresis.

Setting: Two academic medical centers.

Patients: Critically ill patients.

Measurements and Main Results: In septic patients, apoptosis was detected in diverse organs by all three methods with a predominance in lymphocytes and intestinal epithelial cells. Hematoxylin and eosin-stained specimens from septic patients demonstrated at least focal apoptosis in 56.3% of spleens, 47.1% of colons, and 27.7% of ileums. Indirect evidence of lymphocyte apoptosis in septic patients included extensive depletion of lymphocytes in white pulp and a marked lymphocytopenia in 15 of 19 patients. Hematoxylin and eosin from nonseptic patients' tissues revealed a low level of apoptosis in one patient only. The TUNEL method increased in positivity with a delay in tissue fixation and was highly positive in many tissues from both septic and nonseptic patients. Immunohistochemical staining for active caspase-3 showed a marked increase in septic vs. nonseptic patients ($p < .01$), with >25% to 50% of cells being positive focally in the splenic white pulp of six septic but in no nonseptic patients.

Conclusions: We conclude that caspase-3-mediated apoptosis causes extensive lymphocyte apoptosis in sepsis and may contribute to the impaired immune response that characterizes the disorder. (Crit Care Med 1999; 27:1230-1251)

Key Words: multiple organ failure; necrosis; cell death; endotoxin; BCL-2; caspase; autopsy; lymphocyte; spleen; intestine

Sepsis is the leading cause of death in many intensive care units; the Centers for Disease Control and Prevention estimates that >500,000 people develop sepsis and 175,000 die annually in the United States alone [1]. Recently, apoptosis has been shown to be an important mechanism of cell death in animal models of sepsis and endotoxemia [2-5]. Lymphocytes throughout the body are especially predisposed to undergo apoptosis in sepsis and endotoxemia [3,6]. Investigators have postulated that apoptosis-induced loss of lymphocytes may be responsible for the immune depression that typifies the disorder [2,6]. Other types of cells, including hepatocytes [3], intestinal columnar epithelial cells [6], and vascular endothelial cells [7], also may die by apoptosis in sepsis/endotoxemia. A current theory suggests that apoptosis may contribute to the multiple organ dysfunction of sepsis [8]. Although certain viral infections, including human immunodeficiency virus and measles, can cause lymphocyte apoptosis in humans, little information is available on the role of apoptosis in patients with sepsis.

Apoptosis is an evolutionarily conserved and highly regulated program of cell death, which plays an important role in both normal physiologic processes and, when accelerated, in disease states as well [9-11]. Many reports on apoptosis have focused on the role of the executioner cysteine-aspartate proteases termed "caspases" that are triggered in response to proapoptotic signals and that result in disassembly of the cell [12]. Recent studies demonstrated the coexistence of multiple parallel apoptotic pathways; in mammalian cells, at least four distinct pathways exist [13]. Knowledge of caspase regulation may allow manipulation of apoptosis [12,13]. A key caspase involved in the apoptotic pathway is caspase-3 (also known as CPP32, Yama, and apopain) [14,15]. Inhibition of

caspase-3 has been linked to prevention of apoptotic death in vitro [14], although certain stimuli can induce apoptosis by a caspase-3-independent pathway [13]. A second area of apoptosis research focused on the antiapoptotic protein, BCL-2 [16]. Bcl-2 is a member of a new class of oncogenes that possess a remarkable ability to block apoptotic cell death from an array of noxious stimuli, including hypoxia, growth factor withdrawal, chemotherapeutic agents, and oxidative stress, among others [16,17]. The ability of BCL-2 to protect against such diverse causes of cell death suggests that it acts in a final common pathway. Recently, BCL-2 has been shown to play an important role in human disease, as well. The level of BCL-2 correlates with the resistance to chemotherapy in patients with lymphoma and breast and prostate cancer, suggesting that it confers protection against chemotherapy-induced apoptosis [18].

The purpose of this study was to determine whether apoptosis is an important cause of cell death in patients with sepsis and multiple organ dysfunction. We also investigated the role of caspase-3 in the apoptosis program and correlated the expression of BCL-2 with resistance to apoptosis in sepsis.

MATERIALS AND METHODS

Patient Population. A prospective study of 20 critically ill patients in surgical and medical intensive care units at two academic medical centers was performed. The protocol for immediate autopsy allowed for tissue harvesting in the intensive care unit as soon as informed consent could be obtained from next of kin. The protocol was approved by the Human Studies Committee at Washington University School of Medicine. Tissue samples were obtained as follows: 15 to 90 mins post mortem (12 patients), 90 to 180 mins post mortem (seven patients), and 6 hrs post mortem (one patient).

A study was also performed on autopsy tissue samples from ten patients who served as controls. The criteria for inclusion in the control group included the following: a) no evidence of sepsis; b) tissue samples obtained no later than 12 hrs after death (see Table 3 for time of tissue sampling); c) no periods of known protracted shock (>4 hrs duration); and d) no known co-morbidities that could lead to systemic organ compromise. Deaths fulfilling these criteria are unusual in a tertiary hospital because most patients had significant coexisting diseases, shock, and/or had undergone prolonged mechanical ventilation with pulmonary infiltrates. During the time period of the study, only one patient who satisfied the criteria for inclusion in the control group was identified prospectively. Therefore, by necessity, a retrospective study also was undertaken by examining the autopsy files for the past 4 yrs at Barnes Jewish Hospital (BJH). During this time period, nine patients were identified who fit the control criteria. These patients died suddenly of cardiovascular causes and were free from significant coexisting diseases. The initial experimental findings in septic patients showed that the spleen and gastrointestinal tract had a high prevalence of apoptosis. Therefore, to further expand the control population, we included tissues from two additional groups of control patients. Spleens from six critically ill patients who underwent emergent splenectomy for bleeding secondary to trauma were examined. Also, the colons from seven patients who had undergone large-bowel resections for cancer were studied. The area of the intestine not involved with the malignancy (i.e., the histologically normal region) was used for a comparison with the colons of septic patients.

Patient No.	Age (yr)/Gender	DX	Shock	Co-morbidity	Apoptosis	Necrosis	Miscellaneous	Time from expiration to post mortem
1	42/male	GI bleeding	Yes	Cirrhosis	No	No	Died of hemorrhagic shock, required vasopressors (prospective patient)	2 hrs, 42 mins
2	79/male	Presumed cardiac arrest	Unknown	Hypertension	No	No	Found in ventricular fibrillation by EMS	6 hrs, 26 mins
3	87/female	Ruptured aortic aneurysm	Yes	Anemia	No	No	Hypotension in hospital, responsive to iv fluids	8 hrs, 24 mins
4	74/male	Cardiac arrest	Unknown	CASHD	No	No	Witnessed cardiac arrest	6 hrs, 25 mins
5	68/male	Presumed cardiac arrest	Unknown	Heart failure hypertension	No	No	Found in asystole by EMS	6 hrs, 45 mins
6	53/female	Aortic dissection	Unknown	None	Colon epithelial	No	Found unresponsive in bed, with slow ventricular rhythm	10 hrs, 34 mins
7	63/male	Cardiac arrest	Unknown	CASHD, diabetes	No	No	Cardiac arrest while working	4 hrs, 58 mins
8	60/male	Cardiac arrest	Unknown	Emphysema, cancer of larynx	No	Neuronal necrosis	Found in ventricular fibrillation by EMS	12 hrs
9	52/male	Ruptured aortic dissecting aneurysm	Unknown	Chronic pyelonephritis and renal calculi	No	No	Possible compromise of kidney and intestinal blood flow	6 hrs, 10 mins
10	48/male	Cardiac arrest	Unknown	CASHD	No	No	Witnessed cardiac arrest in emergency room	5 hrs, 5 mins

CASHD, coronary atherosclerotic heart disease; EMS, emergency medical service; H&E, hematoxylin and eosin; GI, gastrointestinal; DX, diagnosis. Only one control patient (6) had apoptosis (in the colon) and only one control patient (8) had necrosis (in the brain). No other tissues from control patients demonstrated morphologic evidence of apoptosis or necrosis.

Table 3. Clinical and light microscopic (H&E) findings in nonseptic control patients

In the patients examined prospectively, tissue samples were fixed in 10% buffered form-aldehyde. In selected patients, a second tissue sample was obtained, immediately frozen in liquid nitrogen, and stored at -80[degree sign]C (112[degree sign]F). In patients examined retrospectively, paraffin-embedded slides were obtained from tissue blocks and stained with hematoxylin and eosin (H&E) and by the TUNEL technique.

Criteria for Definition of Sepsis, Shock, and Organ Failure. Nineteen of the 20 patients were classified as septic based on a definitive site of infection at post mortem examination and pre-mortem clinical evidence of sepsis, including persistent fever, hemodynamic instability, and/or positive blood cultures. One patient was classified as septic based on the development of fever, elevated white blood cell count, new pulmonary infiltrates, and progressive multiple organ failure. Autopsy results in this patient revealed histologic evidence of pneumonia, but no pathologic organisms were seen. Patients were classified as suffering from shock if mean arterial pressure was <60 mm Hg and/or vasopressor therapy was needed to maintain a mean arterial pressure of >60 mm Hg.

The following criteria were used to define organ dysfunction: kidney, an increase in serum creatinine to >2 mg/dL or urine output of <20 mL/kg/hr for 6 hrs and not responsive to fluids and diuretics; liver, a prothrombin time elevated above the upper limit of laboratory normal for BJH or post mortem histologic findings of moderately extensive hepatocyte necrosis, apoptosis, or dropout; lung, respiratory failure requiring mechanical ventilation with an inspired FIO_2 of >or=to60% with 5 cm of positive end-expiratory pressure to maintain an arterial oxygen saturation of 90% and a chest radiograph demonstrating bilateral infiltrates. The diagnosis of the acute respiratory distress syndrome (ARDS) was assigned if the patient had the preceding criteria and also exhibited post mortem histologic changes typical of the disorder.

Two septic patients were classified as having cardiac dysfunction based on post mortem pathologic findings of acute myocardial infarction. Two septic patients were classified as having brain dysfunction based on a diagnosis of acute ischemic injury on post mortem examination.

Detection of Apoptosis

Three independent methods that vary in sensitivity and specificity were used to detect apoptosis. No one method has both the sensitivity and specificity that would enable it to be used as a single criterion. By using the three overlapping methods, an accurate assessment of apoptosis was possible.

Light Microscopy of H&E-stained Specimens. Conventional light microscopy of H&E-stained specimens is one of the least sensitive methods for detection of apoptosis but is highly specific if characteristic morphologic changes are observed. Light microscopy reveals cell shrinkage with condensed nuclei (pyknosis) and nuclear fragmentation (karyorrhexis). Apoptosis is readily distinguished from a second type of cell death (which is commonly referred to as necrosis) in which there is apparent destruction of the nucleus and other organelles by swelling [11,19,20]. Some pathologists prefer to term this type of cell death caused by swelling as oncosis (derived from onkos, meaning swelling) because the term necrosis technically is not a form of cell death but rather a set of changes that occur after cell death [11,20]. Stained specimens were examined in a blinded fashion by an experienced pathologist (PES) who identified apoptosis based on characteristic morphologic changes of apoptosis (vide supra). Rare, scattered apoptotic nuclei (less than one per high powered field) were considered physiologic and scored as negative for apoptosis. A minimum of five random fields were evaluated at x400 magnification in each sample.

Fluorescent TUNEL. Despite its limitations in specificity (failure to distinguish apoptosis vs. necrosis) [21,22] and high false-positive rate in certain cases [23], the TUNEL method is useful in detecting apoptosis if used in conjunction with other techniques. The TUNEL method has increased sensitivity compared with conventional light microscopy of H&E slides; therefore, tissues that are TUNEL-negative are unlikely to have cells that are undergoing apoptosis. The fluorescent TUNEL protocol was conducted as previously described [6]. The paraffin tissue sections (4 to 6 [micro sign]M) were deparaffinized and rehydrated with graded dilutions of ethanol in water. By using an apoptosis detection kit and protocol (Boehringer, Indianapolis, IN), cells were permeabilized by microwave treatment in citrate buffer and DNA labeled with deoxyuridine triphosphate fluorescein via action of terminal deoxynucleotidyl transferase. Tissue sections were examined at x400 magnification in a blinded fashion.

DNA Agarose Gel Electrophoresis. Although DNA agarose gel electrophoresis is highly specific, it lacks sensitivity, and numerous studies have shown that this method may be falsely negative for apoptosis, despite unequivocal evidence of apoptotic cell death [21,24,25]. This failure of the DNA agarose gel method to detect apoptosis when it is clearly present is owing to the fact that apoptotic cells may be phagocytized before degradation of the large DNA fragments into the smaller DNA fragments ([similar]180 to 200 base pairs), which are detected by the agarose gel method [21,24,25]. Therefore, a negative DNA agarose gel does not eliminate apoptosis, but a positive gel is highly diagnostic. DNA agarose gel electrophoresis was performed as described previously [6], with minor modifications. Tissue was homogenized in digestion buffer (DNAzol; Molecular Research, Cincinnati, OH). After centrifugation, the top layer was transferred to a second tube and absolute ethanol was added. After a second centrifugation, the precipitate was washed twice with 95% ethanol and DNA was dissolved in Tris EDTA buffer (pH 8.0). DNA was pipetted onto a 2% agarose gel and electrophoresis was performed. After electrophoresis, the gel was stained for 1 hr in Tris borate EDTA buffer containing SYBER Gold nucleic acid gel stain (Molecular Probes, Eugene, OR).

Immunohistochemical Staining for Active Caspase-3. A polyclonal rabbit antibody specific for active caspase-3 [13] was generously provided by Dr. Don Nicholson (Merck Frosst, Quebec, Canada). Active caspase-3 was evaluated in spleens because it was the organ in septic patients with the highest prevalence of apoptosis by both light microscopy and TUNEL. Spleens obtained at post mortem from 16 septic patients were compared with spleens obtained at post mortem from nine critically ill nonseptic patients and with spleens obtained intraoperatively from six critically ill patients who underwent emergent splenectomy for bleeding. Paraffin-embedded tissue slides were dewaxed by immersion in three changes of xylene and dehydrated in absolute alcohol. Blocking of endogenous peroxidase activity was performed with 2% (vol/vol) hydrogen peroxide in methanol at room temperature for 30 mins. Slides were rehydrated in phosphate-buffered saline (pH 7.4). Antigen retrieval was performed by microwaving in 0.1 M citrate buffer (pH 6.0) to boiling. After rinsing in buffer, slides were blocked with 5% fetal calf serum plus 10% goat

serum in phosphate-buffered saline. The anti-caspase-3 antibody (specific for active caspase-3) was added at a dilution of 1:1000 at 37[degree sign]C (98.6[degree sign]F) for 2 hrs. A biotinylated secondary goat anti-rabbit antibody was added, followed with a streptavidin-biotin peroxidase complex (Vector, Burlingame, CA,). Slides were rinsed and developed with metalenhanced diaminobenzidine (Pierce, Rockford, IL). Hematoxylin was used as a counterstain. Slides were evaluated at x400 in a blinded fashion. A minimum of five random fields in both white and red pulp of the spleen were evaluated for the percentage of cells with positive staining for active caspase-3.

BCL-2 Immunohistochemical Staining in the Spleen. Paraffin-embedded tissue slides were dewaxed, endogenous peroxidase was quenched, and citrate buffer antigen retrieval was performed as described above. A commercially available BCL-2 murine monoclonal antibody (clone 124; DAKO, Carpinteria, CA; diluted 1:40) was then applied, and a visible reaction product was observed using an automated staining protocol (Autostainer; DAKO) and a labeled streptavidin technique ($LSAB^+$, diaminobenzidine ($^+$), DAKO). Slides were examined in a blinded fashion.

Statistical Analysis. Differences in extent of apoptosis in septic vs. nonseptic patients were analyzed by chi-square or one-tailed Student's t-test assuming unequal variances. Statistical significance was accepted at the $p < .05$ level.

RESULTS

Patient Profiles, Septic. Twenty septic patients were included in the study (Table 1 and Table 4). The causes of sepsis were multifactorial with a predominance of nosocomial pneumonia. Most patients had multiple organ dysfunction syndrome with an almost equal prevalence of lung (65%), liver (55%), and kidney (60%) dysfunction (Table 1 and Table 4 and Table 2). In addition to sepsis, 17 of 20 patients also experienced a period of shock. A significant and, in some instances, a profound decrease in the absolute lymphocyte count to <1.2 K/mm^3 (the lower limit of normal for the absolute lymphocyte count at BJH) occurred in 15 of the 19 patients in whom laboratory data were available (Table 1 and Table 4). One of the 20 patients had chronic lymphocytic leukemia, with a markedly elevated lymphocyte count. It is important to note that four patients were receiving corticosteroids, although in three of these four patients, only low physiologic doses of hydrocortisone (25 mg three times daily) were being administered.

Patient No.	Age (yr)/ Gender	DX	Days in ICU	Shock	Sepsis	Organ dysfunction	Absolute Lymph
1	81/male	Pneumonia, MVA with fractures, severe anemia	7	Yes	Yes	Lung, liver	0.6
2	92/male	ARDS and renal failure after aortic aneurysm repair	12	No	Yes	Lung, kidney (dialysis)	0.5
3	85/female	MVA with intestinal injury and peritonitis, bronchopneumonia	8	No	Yes	Liver, lung	0.9
4	54/male	Sepsis caused by necrotizing fasciitis, GI bleed	7	Yes	Yes	Liver, kidney (dialysis)	0.4
5	49/male	Thigh abscess sepsis, ARDS multiorgan failure	16	Yes	Yes	Liver, kidney, lung	0.9
6	49/female	MI, pneumonia, watershed infarct of brain	3	Yes	Yes	Lung, heart, brain	NA
7	74/male	Pneumonia, sepsis	20	Yes	Yes	Kidney (dialysis)	11.4
8	60/female	Pneumonia, sepsis	5	Yes	Yes	Lung	0.3
9	50/male	Cirrhosis, hepatitis C, peritonitis, pancreatitis, acute MI	11	Yes	Yes	Kidney, liver	5.5
10	26/female	ARDS secondary to MVA, pelvic fractures, pneumonia	15	Yes	Yes	Lung	0.8
11	53/male	Cardiac arrest, pneumonia, sepsis	1	Yes	Yes	Kidney, liver	0.4
12	50/male	Peritonitis, ischemic bowel, ARDS	7	Yes	Yes	Kidney Lung Liver	1.0
13	45/male	ARDS secondary to aspiration, sepsis	19	Yes	Yes	Liver Kidney Lung	1.9
14	77/female	Pneumonia, pseudomembranous colitis	10	No	Yes	Lung	0.7
15	51/female	Sepsis, peritonitis	30	Yes	Yes	Kidney	0.3
16	43/male	Ischemic bowel, viral pneumonia	10	Yes	Yes	Kidney (dialysis) Liver Lung	0.1
17	73/female	Urosepsis, ARDS	2	Yes	Yes	Lung Kidney	0.3
18	80/male	Upper GI bleeding, peritonitis	4	Yes	Yes	Liver	0.6
19	28/male	Cardiac arrest with anoxic injury, sepsis, pancreatitis	5	Yes	Yes	Kidney	1.5 (count was 1.0 24 hrs before)
20	75/male	Pneumonia, ARDS mediastinitis	14	Yes	Yes	Lung	0.4

Table 1. Clinical and light microscopic findings (H&E) in septic patients (summary)

Co-morbidity	Apoptosis Detection by L, G	Necrosis Detection by L	Miscellaneous
None	Liver (L, G) Colon (L, G) Spleen (L, G) Adrenal (L)	Centrilobular hepatocytes	Jehovah's witness who refused blood transfusion, hematocrit of 10–15%
Emphysema	Colon (L)	Small area of superficial necrosis in kidney	No definitive site of infection at autopsy but treated bronchopneumonia likely; fever, ↑ wbc count, pulmonary infiltrate
None	Spleen (L)	No	Heart contusion and ↑ troponin I
Pancreatitis with diabetes	Spleen (L)	No	*C. perfringens* cause of fasciitis
None	Spleen (G) Colon (L) Ileum (G) Heart (G)	No	
None	Spleen (L) Lymph node (L)	Heart, brain centrilobular necrosis of liver	Methylprednisolone (60 mg tid), occlusion of left main coronary artery
Chronic lymphocytic leukemia	No	No	
Systemic lupus erythematosis	Spleen (L) Liver (G)	Patchy hepatocyte necrosis	Hydrocortisone (25 mg tid)
Diabetes	Liver (L) Colon (L) Lymph node (L) Spleen (L)	Liver Heart	—
Muscular dystrophy (mild)	Kidney (G)	No	—
Alcoholic with cirrhosis and pancreatitis	Liver (L) Spleen (L) Colon (L) Ileum (L)	Brain	Blood cultures (+) for *S. pneumonia* elevated troponin I
Chronic renal failure on dialysis	Spleen (L) Ileum (L) Colon (L)	Liver Ileum Colon	—
Alcoholic liver disease	Lymphocytes in Peyer's patch (L) Colon-lamina propria (L)	No	—
Adenocarcinoma of stomach and duodenum	No	No	—
Invasive adrenal carcinoma	Colon (L)	No	Previous splenectomy
Chronic renal failure caused by lupus	Liver (L) Ileum (L) Spleen (L)	Liver	Hydrocortisone (25 mg q 8) Cytoxan
None	Lung (G)	No	Hydrocortisone (20 mg q 8) Blood cultures (+) for enterococcus
Pancreatic cancer, COPD	Spleen (L) Colon (L)	Liver	—
Chronic renal failure	Spleen (L) Kidney lymphoid (L) Lymph node (L, G)	No	*Pseudomonas* in multiple peritoneal fluid cultures
Rheumatoid arthritis, emphysema	Colon-lamina propria (L)	No	Chronic steroids

MVA, motor vehicle accident; ARDS, adult respiratory distress syndrome; GI, gastrointestinal; MI, myocardial infarction; COPD, chronic obstructive pulmonary disease; apoptosis detection by L, light microscopy, and G, DNA agarose gel electrophoresis; H&E, hematoxylin and eosin; DX, diagnosis; ICU, intensive care unit.

Table 4. Table 1. Continued

Organ	Dysfunction	Necrosis Light Microscopy	Apoptosis Light Microscopy
Brain[a]	2/10	2/10	0/10
Lung	13/20	0/20	0/20
Heart[b]	1/19	2/19	0/20
Liver	11/20	7/20	3/20
Kidney	12/20	1/20	1/20[c]
Spleen[d]	Not evaluated	0/16	9/16
Pancreas	Not evaluated	0/16	1/16
Adrenal	Not evaluated	0/11	1/11
Ileum	Not evaluated	1/19	5/18[e]
Colon	Not evaluated	1/18	8/17[e]
Lymph node[f]	Not evaluated	0/12	2/13
Aorta	Not evaluated	0/9	0/9
Muscle	Not evaluated	0/19	0/19

[a]Both patients had recent cerebrovascular accidents. [b]Patient had refractory shock and acute thrombosis of the left coronary artery at post mortem. [c]Apoptosis in the kidney was in a lymphoid collection (see Fig. 3*C*). [d]In addition to apoptosis, eight of 16 spleens also had depletion of cells in the white pulp region (see Fig. 3, *A* and *B*). [e]Apoptosis in the ileum and colon occurred in epithelial cells in villi or crypt. [f]Five of 13 lymph nodes had depletion of cells in the cortical region.

Table 2. Clinical and light microscopic (H&E) results of septic patients (no. of patients positive/total no. of patients)

Patient Profiles, Control. Ten control patients who died abruptly were included in the study (Table 3). One patient was enrolled prospectively, whereas the other nine patients were evaluated retrospectively from cases in the autopsy files of BJH. Six of these ten patients had presumed cardiac arrest based on detection of ventricular fibrillation by emergency medical services, known cardiac history, and/or autopsy findings consistent with acute myocardial injury. Two patients died of dissecting aneurysms; one patient died of a ruptured aortic aneurysm; and one patient died of uncontrollable upper gastrointestinal bleeding.

The six critically ill patients who underwent emergent splenectomy had blunt chest and/or abdominal trauma with splenic injury and bleeding. Splenic tissue sections were evaluated in a blinded fashion by conventional light microscopy (H&E) and caspase-3 staining.

For comparison of apoptosis in the colon, tissues from eight patients with large-bowel malignancy were evaluated in addition to tissues from two prospective patients. The distal margin of the colon resection (which was not involved with the malignancy) was evaluated in a blinded fashion by light microscopy (H&E).

Conventional Light Microscopy (H&E)

Spleens and colons from septic and nonseptic patients were evaluated in a blinded fashion by a pathologist (PES) who graded the tissues on the following scale: <1% of cells apoptotic = 0; 1% to 5% of cells apoptotic = 1; 6% to 25% of cells apoptotic = 2; 26% to 50% of cells apoptotic = 3; >50% of cells apoptotic = 4.

Spleen. Examination of spleens from septic patients showed that nine of 16 spleens (56.3%) had histologic changes characteristic of apoptosis, i.e., nuclear condensation and nuclear fragmentation (Figure 1A and Figure 2). Splenic apoptosis was present focally at a high degree in three septic patients, i.e., 6% to 25% of splenocytes appeared apoptotic in the field of view (Figure 2C, and Figure 2D). In six septic patients, apoptosis was present focally in 1% to 5% of splenocytes. Of the 15 spleens from nonseptic patients (seven prospective, eight retrospective), only one spleen demonstrated apoptotic cells (1% to 5% of cells had apoptotic changes), and this was statistically different from septic patients ($p < .01$). An additional finding in eight of 16 spleens from septic patients (but not in spleens from nonseptic patients) was a decreased density of lymphocytes in white pulp (Figure 3A, and Figure 3B). A similar lymphocyte depletion was observed in five of 13 lymph nodes from septic patients, each of whom had cell loss primarily in the cortical zone. Although not quantitatively evaluated, areas of markedly increased lymphocyte apoptosis were observed also in lymphoid aggregates in kidney (Figure 3C, and Figure 3D) and bowel in several septic patients but not in control patients.

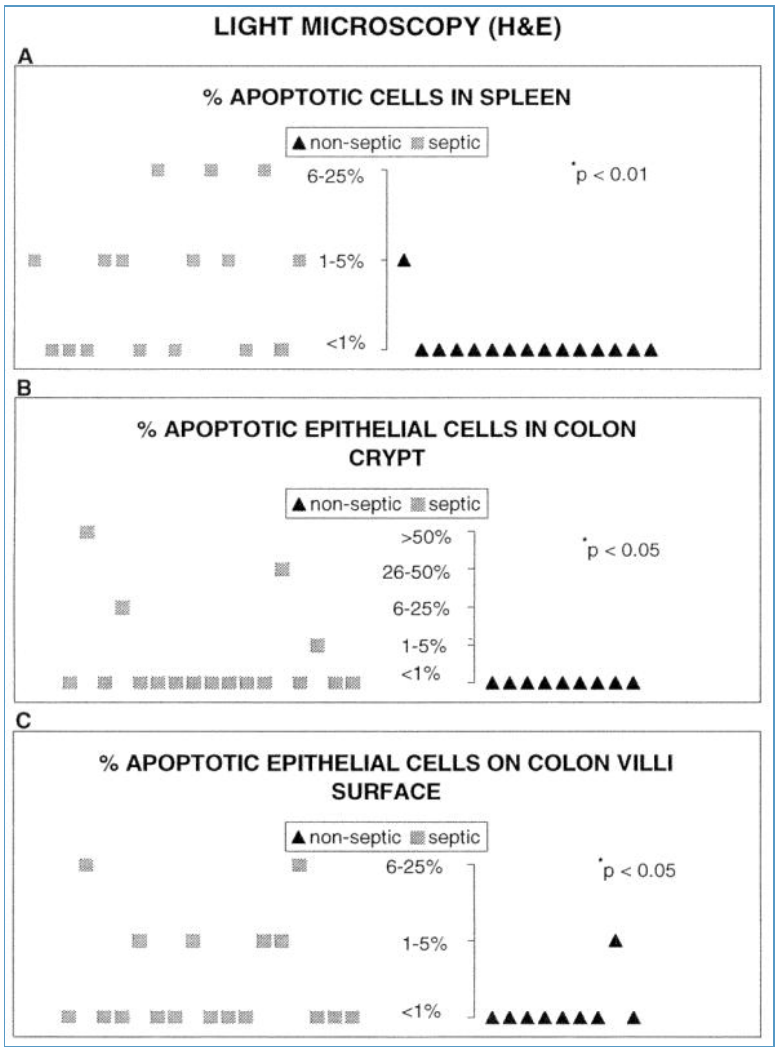

Figure 1. Percentage (%) of apoptotic cells determined by light microscopy of hematoxylin and eosin (H&E)-stained specimens. Patient samples examined at x400 by a pathologist who was blinded as to the sample identity. Cells were judged to be apoptotic if they exhibited nuclear condensation and/or nuclear fragmentation. A minimum of five random fields were evaluated, and the % of apoptotic cells were expressed as <1%, 1% to 5%, 6% to 25%, 26% to 50%, or >50%. The % of apoptotic cells was greater in spleens ($p < .01$), colon crypts ($p < .05$), and colon villi surface ($p < .05$) in septic than in nonseptic patients.

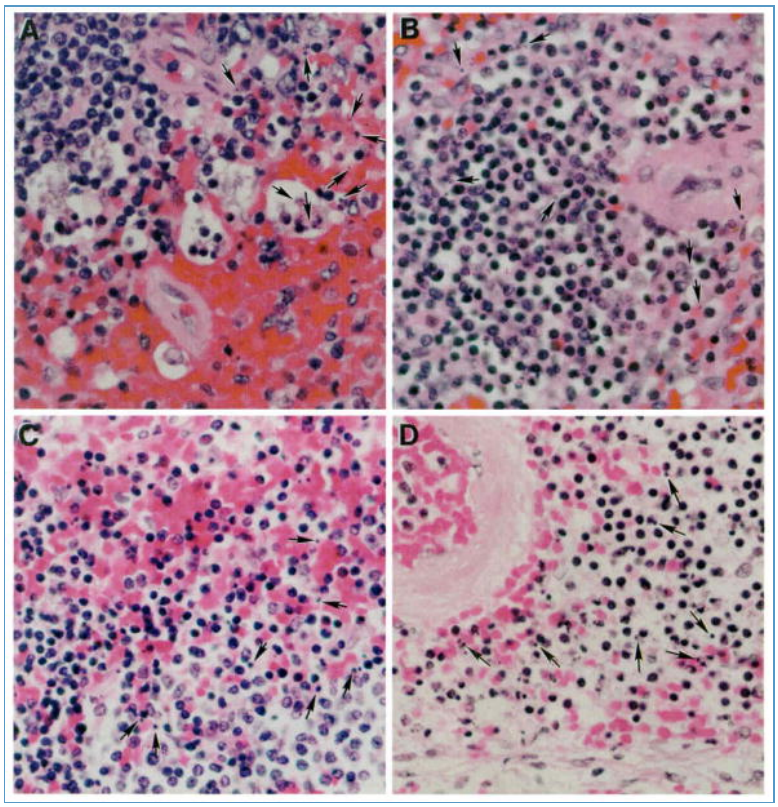

Figure 2. A-D, hematoxylin and eosin (H&E) stains of tissues from patients with sepsis examined at 400x magnification, except where stated otherwise. Many cells exhibit the hallmark changes of apoptosis, i.e., nuclear condensation and nuclear fragmentation. The percentage of cells with changes typical of apoptosis was calculated by using a magnifying lens and a transparent grid divided into 1-cm squares to count the number of apoptotic cells and dividing by the total number of cells. Arrows denote only a few of the apoptotic cells. A, spleen; patient 9; 41 of 295 splenocytes were apoptotic (13.9%); B, spleen; patient 12; 29 of 372 splenocytes were apoptotic (7.8%); C, spleen; patient 1; 48 of 434 splenocytes were apoptotic (11.1%); D, spleen; patient 18; 59 of 261 splenocytes were apoptotic (22.6%).

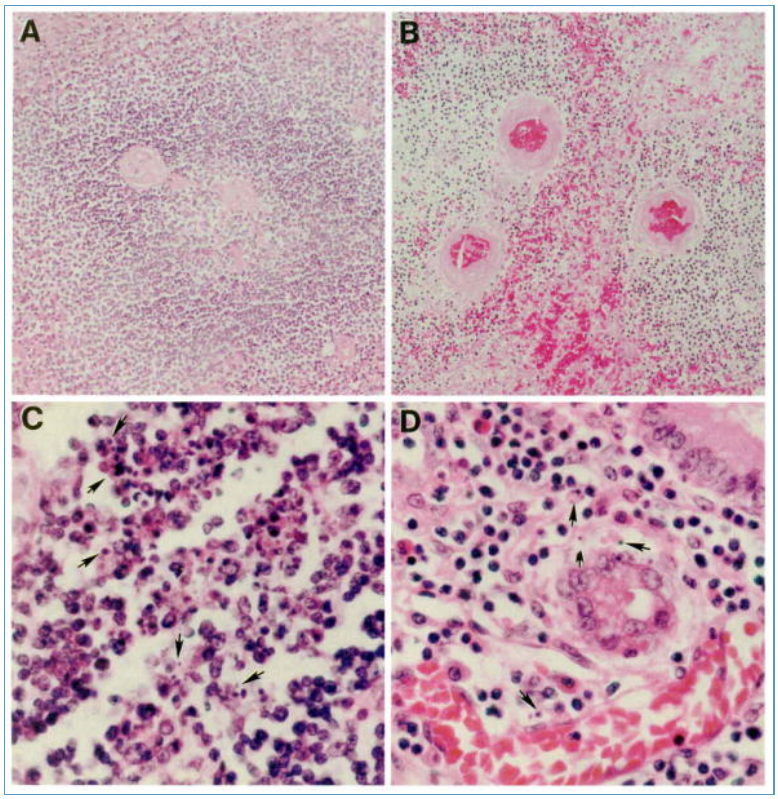

Figure 3. A, spleen from control patient 4 demonstrating normal lymphoid follicle with robust periarteriolar and B-cell zones (x200 magnification). B, spleen from septic patient 18 demonstrating lymphocyte depletion in lymphoid follicle. C, lymphoid aggregate in kidney from septic patient 19. Forty-nine of 282 lymphocytes were apoptotic

(17.4%). D, ileum from patient 13 demonstrating apoptotic lymphocytes and plasma cells in lamina propria. Seven of 181 cells were apoptotic (3.9%).

Colonic Epithelial Cells. There was a generalized low level of baseline apoptosis in colons from septic and nonseptic patients that was less than one apoptotic cell per villus tip or crypt. In addition to the low baseline level of apoptosis, there was increased apoptosis in colons from four of 17 septic patients in whom focal regions of columnar epithelial apoptosis occurred in crypt or villus involving 6% to 25% of cells in the field of view (Figure 1B, and Figure 1C, and Figure 4A, and Figure 4C). In addition, four septic patients had a lesser increase in apoptosis (1% to 5% of cells in villus or crypt with apoptotic changes). The control colon samples were well preserved and consisted of nine samples: one sample obtained from a bowel resection of a trauma patient; one sample obtained from a rapid post mortem examination; and seven samples obtained from bowel resections for cancer. Only one of nine control colons had increased apoptosis, and this occurred on the villus surface with 1% to 5% of cells focally apoptotic. The degree of apoptosis in the colonic villi and colonic crypts was greater in septic than in nonseptic patients (Figure 1B, and Figure 1C) ($p < .05$).

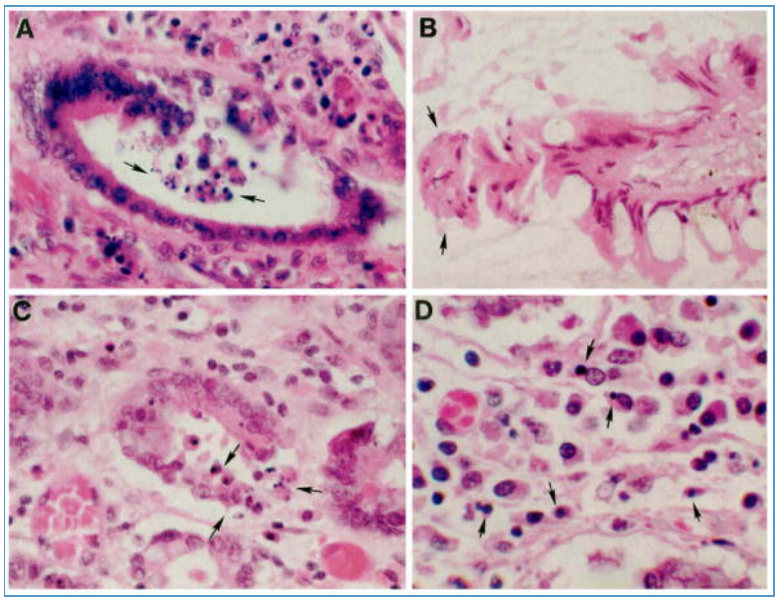

Figure 4. A, colon from patient 1 demonstrating a dying intestinal crypt with many apoptotic epithelial cells shed into the lumen (note apoptotic bodies). B, ileum from patient 16 demonstrating many apoptotic columnar epithelial cells at the villus tip. C, colon from patient 12 demonstrating apoptosis with nuclear fragmentation in intestinal crypt cells. D, ileum from patient 14 demonstrating apoptotic lymphoid cells within the lamina propria. The two arrows in the upper left of the Figure depictmacrophages that have ingested apoptotic cell fragments.

Ileum. Examination of ileums of septic patients showed that four of 18 patients had increased focal apoptosis in epithelial cells (Figure 4B) and one patient had marked apoptosis (>6% to 25% of lymphocytes) in a Peyer's patch. Mononuclear cells in the lamina propria also exhibited apoptosis to varying degrees. At least some of these cells were recognizable as lymphocytes and plasma cells (Figure 4D). Of note, a readily apparent increase in the number of sloughed apoptotic cells was observed in the intestinal lumen of septic patients compared with nonseptic patients. Ileums from ten control patients were obtained at post mortem examination and, in general, were not as well preserved as comparable specimens from septic patients because of the longer delay between patient death and sample preparation. As a result, a larger number of bowel epithelial cells had undergone autolysis in the control patients vs. septic patients. However, no apoptosis was observed in the attached intestinal epithelial cells.

Liver. Liver specimens from the control patients revealed no evidence of apoptosis or necrosis (Table 3 and Appendix (Table 5 and Table 6)). In contrast, foci of hepatocyte necrosis were observed in seven of 20 livers from patients with sepsis (Table 2; Figure 5A, and Figure 5B). In three of the seven septic patients who had hepatocyte necrosis, apoptotic cell death was also present and usually was located in close proximity to the necrosis (Figure 5B; Appendix (Table 7 and Table 8)).

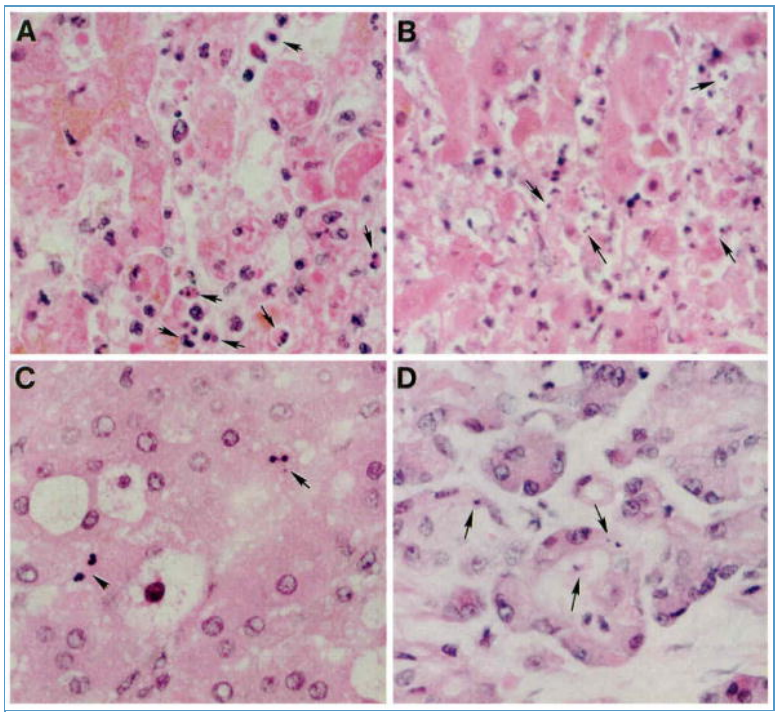

Figure 5. A, liver from septic patient 1 demonstrating numerous apoptotic cells, including both hepatocytes and inflammatory cells. Sixteen of 84 cells were apoptotic (19%). B, liver from septic patient 11 demonstrating hepatocyte apoptosis and necrosis. Seventeen of 104 cells were apoptotic (16.3%). C, adrenal from septic patient 1 demonstrating apoptotic cortical cells. Three of 53 cells were apoptotic (5.7%). D, pancreas from septic patient 16 demonstrating acinar cell apoptosis. Eight of 119 cells were apoptotic (6.8%).

Patient No.	Age (yr)/Gender	DX	Brain	Lung	Heart	Liver	Spleen
1	42/male	GI bleed	UR	Congestion, alveolar hemorrhage	UR	Fibrosis, periportal lymphocyte infiltrates	Expanded germinal center
2	79/male	Cardiac arrest	UR	Emphysema	Fibrosis	Periportal fibrosis	UR
3	87/female	Ruptured aneurysm	NA	UR	Subendocardial infarct, fibrosis	Focal periportal inflammation	UR
4	74/male	Cardiac arrest	UR	UR	Fibrosis	Inflammatory infiltrate and hepatocyte dropout	UR
5	68/male	Presumed cardiac arrest	NA	Eosinophilic infiltrates	Myocyte enlargement, nucleomegaly	UR	UR
6	53/female	Aortic dissection	NA	UR	UR	UR	UR
7	63/male	Cardiac arrest	UR	UR	Pericarditis edema, fibrosis, eosinophilic cytoplasm, acute ischemia	Mild congestion	UR
8	69/male	Cardiac arrest	Neuronal necrosis, neuron loss, astrocytosis	Emphysema, left	Microscopic scars	UR	UR
9	32/male	Ruptured aneurysm	NA	Pulmonary emboli, congestion	Subendocardial thickening	Fatty changes, mild chronic inflammation	UR
10	48/male	Cardiac arrest	UR	Congestion	Acute thrombus, left anterior descending artery	Macrovesicular steatosis	UR

Table 5. Appendix-Table 1. Clinical and microscopic findings (H&E) in control patients (summary)

Patient	Pancreas	Adrenal	Kidney	Ileum	Colon	Lymph Node
1	Fibrosis	UR	Glomerulosclerosis	Autolysis	Autolysis	UR
2	UR	UR	Arteriolar sclerosis	UR	UR	Hyalinizing fibrosis
3	Fatty infiltration	UR	Arteriosclerosis	Inflammation	NA	NA
4	UR	UR	Glomerulosclerosis, interstitial inflammation	NA	NA	UR
5	Chronic pancreatitis	UR	Nephrosclerosis	Autolysis of epithelial cells, otherwise UR	Autolysis of epithelial cells, otherwise UR	NA
6	UR	UR	Nephrosclerosis, inflammation	Intact epithelium	Apoptosis of epithelium	UR
7	UR	Mild chronic inflammation	UR	Mild autolysis with normal epithelial cells in crypts	Mild autolysis with normal epithelial cells in crypts	NA
8	Periductal fibrosis	UR	Arteriolar sclerosis	NA	Autolysis of epithelial cells but no apoptosis	NA
9	UR	Fatty infiltration, inflammation	Arteriolosclerosis	Autolysis of many epithelial cells, otherwise UR	Autolysis of many epithelial cells, otherwise UR	UR
10	Fibrosis	UR	Arteriolar sclerosis	NA	NA	UR

GI, gastrointestinal; UR, unremarkable; H&E, hematoxylin and eosin; DX, diagnosis; NA, not available.
A minimum of 15 fields was examined per specimen with a magnification of ×400.

Table 6. Appendix-Table 1. Continued

Patient No.	Age (yr)/Gender	DX	Brain	Lung	Heart	Liver	Spleen
1	81/male	Pneumonia	UR	Acute inflammation	UR	Marked necrosis of pericentral hepatocytes	Apoptosis loss of white pulp
2	92/male	ARDS, renal failure	UR	DAD, interstitial inflammation	Ischemic cardiomyopathy	UR	UR
3	85/female	Peritonitis, broncho-pneumonia	NA	DAD	Interstitial fibrosis	Fatty changes, inflammation	Inflammation
4	54/male	Sepsis, GI bleeding	UR	UR	Eosinophilic infiltrate	Panlobular congestion	NA
5	49/male	Sepsis, ARDS, renal hepatic dysfunction	NA	DAD	UR	Congestive changes, cirrhosis, hepatitis	Apoptosis, loss of white pulp
6	49/female	MI, pneumonia, brain infarct, liver disease	Neutrophilic infiltration in infarcted parenchyma	Bilateral broncho pneumonia	Changes of acute MI	Marked acute centrilobular hepatic congestion/necrosis	Acute congestion, loss of white pulp
7	74/male	Pneumonia, sepsis	UR	DAD	Focal interstitial fibrosis	Mild sinusoidal congestion, lymphocytic infiltration	Lymphocytic infiltration
8	60/female	Pneumonia, sepsis, lupus	UR	Interstitial fibrosis, DAD	UR	PMN infiltrate, patchy hepatocyte necrosis	Loss of white pulp
9	50/male	Cirrhosis, hepatitis C, peritonitis	UR	UR	Interstitial hemorrhage coagulative myocyte necrosis	Cirrhosis, coagulative necrosis, apoptosis cholestasis pmn and lymphocytic infiltrate	Apoptosis in marginal zone
10	26/female	ARDS, sepsis	UR	DAD, severe	UR	Mild congestion	NA
11	53/male	Anoxic injury	Focal neuron necrosis	Broncho-pneumonia	UR	Fibrosis, hyaline bodies, apoptosis, necrosis	Apoptosis, loss of white pulp
12	50/male	Peritonitis, ischemic bowel, ARDS	UR	DAD, congestion	UR	Coagulated necrosis	Apoptosis
13	45/male	ARDS, sepsis	NA	DAD, organizing	Pericarditis	Cirrhosis, acute inflammation, hepatitis	Apoptosis
14	77/female	Pneumonia, colitis	NA	Broncho-pneumonia	Fibrosis, old infarct	UR	UR
15	51/female	Peritonitis, sepsis	NA	Interstitial infiltrate	UR	Fatty changes, periportal inflammation	NA
16	43/male	Pneumonia, ischemic bowel	NA	DAD acute pneumonia	Healed infarct	Submassive necrosis with focal apoptosis	Loss of white pulp
17	73/female	Urosepsis, ARDS	NA	DAD	UR	Mild acute inflammation	Loss of white pulp
18	80/male	Peritonitis, GI bleeding	NA	UR	Interstitial myocarditis	Acute inflammation, necrosis	Loss of white pulp, apoptosis
19	28/male	Cardiac arrest, sepsis	NA	UR	NA	UR	Focal apoptosis
20	75/male	Pneumonia, mediastinitis	NA	Fibrosing alveolitis	UR	Fatty changes, hepatocyte dropout	NA

Table 7. Appendix-Table 2. Detailed conventional light microscopic (H&E) results in septic patients (summary)

Pancreas	Adrenal	Kidney	Ileum	Colon	Lymph Node	Aorta	Muscle
UR	Focal apoptosis	Mild glomerulosclerosis	UR	Apoptosis in crypts	UR	Athero-sclerosis	UR
UR	UR	Arteriolosclerosis, focal necrosis, cholesterol emboli	UR	UR	UR	UR	UR
UR	NA	Dilated tubules	UR	UR	NA	NA	Fiber atrophy
NA	UR	Focal segmental sclerosis, proximal tubules with vacuolar changes	Many apoptotic sloughed cells	Apoptotic sloughed cells	UR	Loss of epithelial cells	UR
UR	NA	Tubular injury	UR	3–4+ apoptosis of epithelial cells	NA	NA	Fiber atrophy
UR	UR	UR	UR	UR	UR	UR	UR
UR	Lymphocytic infiltration	Lymphocytic infiltration	UR	UR	↑ Lymphs	UR	UR
UR	UR	Mild calcification in tubules	UR	UR	UR	UR	Fiber atrophy
Hemorrhagic pancreatitis with pmn infiltrate	UR	UR	Regenerative changes	UR	Apoptosis, cortical depletion	UR	UR
UR	Left cortical infarction	Left focal infarction	UR	UR	Cortical depletion	UR	UR
Fibrosis chronic inflammation	UR	Arteriosclerosis	Apoptotic crypt cells	Apoptotic crypt cells	Cortical depletion	UR	Fiber dropout
UR	Mild hemorrhage	End-stage polycystic calcification	Necrosis, apoptosis	Necrosis, apoptosis	Cortical depletion	Severe athero sclerosis	UR
UR	NA	Glomerulosclerosis interstitial fibrosis	Apoptosis in lymphocytes in Peyer's patch	Mononuclear infiltrate	NA	NA	Fiber atrophy
UR	Inflammatory infiltrate	Arteriolar sclerosis	Apoptosis in stromal cells	Pseudo-membranous colitis, apoptosis epithelial cells	UR	NA	Fiber atrophy
NA	NA	Mild changes, tubular injury	UR	Apoptotic epithelial cells	NA	NA	Fiber atrophy
Acute pancreatitis with focal acinar apoptosis	NA	Glomerulo-sclerosis, chronic	Apoptosis, extensive in villi	NA	—	—	—
Fat necrosis	NA	Focal calcification of tubules	Sloughing, autolysis	Sloughing autolysis	NA	NA	Single-fiber atrophy
NA	NA	Inflammation, arteriosclerosis	Decreased epithelial cells	Decreased epithelial cells	Cortical depletion	NA	Fiber atrophy
NA	NA	Glomerulosclerosis, apoptosis in lymphoid aggregate	NA	NA	Apoptosis	NA	NA
Hyalinized islets	NA	Dilated proximal tubules	NA	UR	UR	NA	Interstitial infiltration, fiber atrophy

NA, not available; Neg., negative; DAD, diffuse alveolar disease; UR, unremarkable; ARDS, adult respiratory distress syndrome; MI, myocardial infarct; PMN, polymorphonuclear leukocyte; H&E, hematoxylin and eosin; DX, diagnosis; GI, gastrointestinal.

Table 8. Appendix-Table 2. Continued

Kidney. Histologic findings in kidneys from control patients revealed no acute changes (Table 3). Surprisingly, despite the high prevalence of clinical renal dysfunction (65%) in patients with sepsis, only one septic patient had evidence of kidney necrosis. No renal tubular nor glomerular cell apoptosis was seen in any septic patients. Hence, in patients without preexisting renal disease, renal histology did not reflect the severity of renal injury indicated by the decrease in kidney function.

Lung. Histologic findings in lungs from control patients revealed pulmonary congestion but no apoptosis or necrosis (Table 3). Although light microscopic examination of lungs from patients with sepsis did not demonstrate either necrosis or apoptosis, extensive inflammation, fibrosis, and/or hyaline membrane formation were observed in specimens from several patients (Table 2 and Appendix (Table 7 and Table 8)). Seven septic patients had a clinical diagnosis of ARDS, and ten septic patients had autopsy findings demonstrative of acute and/or organizing bronchopneumonia.

Miscellaneous Findings in Septic Patients. Light microscopic examination of skeletal muscle from septic patients also failed to reveal necrosis or apoptosis; however, a majority of specimens demonstrated at least focal group fiber atrophy. Apoptosis was noted in scattered adrenal cortical cells (Figure 5C) and pancreatic acinar cells (Figure 5D) in one patient each.

Fluorescent TUNEL-Failure to Discriminate Between Septic and Control Tissues

TUNEL staining was highly positive in the majority of tissues from both septic and control patients, and the TUNEL method did not discriminate between the two patient populations. The high percentage of TUNEL-positive specimens may have been partially attributable to the delay in tissue fixation (vide infra) as well as a high degree of false-positive staining (see "Discussion" section on TUNEL). In septic patients, the TUNEL method demonstrated a higher prevalence of apoptosis than did the light microscopy method. Spleen and lymph nodes had the highest prevalence (100%) and severity of apoptosis. Liver had an 80% prevalence of apoptosis, followed by colon (50%) and ileum (47%). The organ with the lowest prevalence of apoptosis was the aorta, in which only one of ten specimens was TUNEL-positive. TUNEL labeling occasionally demonstrated cell morphologic features characteristic of apoptosis, including nuclear condensation and nuclear fragmentation (apoptotic bodies) (Figure 6).

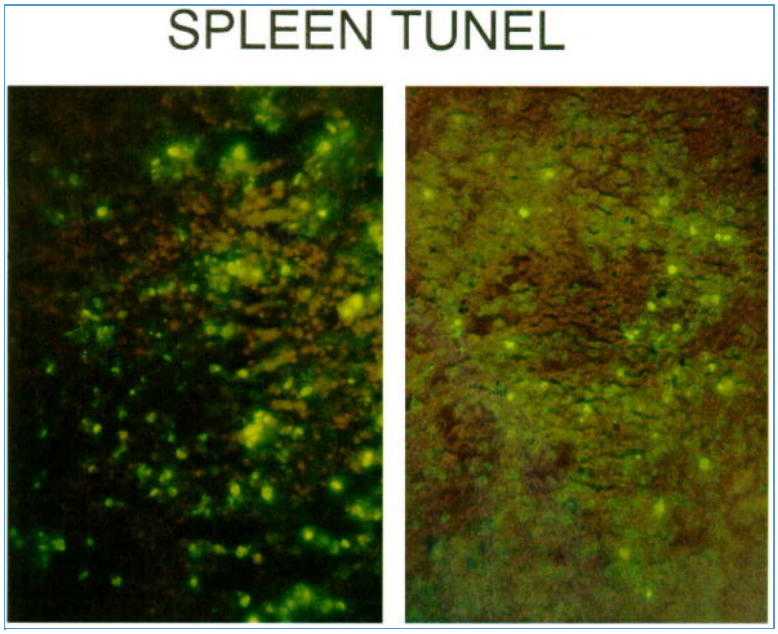

Figure 6. Fluorescent labeling of apoptotic nuclei using fluorescein deoxyuridine triphosphate and terminal deoxynucleotidyl transferase (TUNEL). Tissues were examined at x400 magnification using fluorescence microscopy. Nuclei that appear bright green are labeled by deoxyuridine triphosphate and are positive for apoptosis. TUNEL-positive spleens: patient 3, left; patient 8, right.

Tissue specimens from the group of control patients also demonstrated a high prevalence of TUNEL-positive labeling: lung, nine of ten (90%); heart, nine of ten (90%); liver, seven of ten (70%); spleen, seven of ten (70%); pancreas, three of nine (33.3%); adrenal, six of nine (66.7%); kidney, nine of ten (90%); ileum, four of six (66.7%); colon, seven of seven (100%); lymph nodes, one of three (33.3%); and brain, one of two (50%).

Fixation Time Course Study-Effects on H&E and TUNEL. To investigate the effect of delay in tissue fixation on the evaluation of apoptosis by H&E staining and TUNEL, sections from three patients were formalin-fixed immediately after obtaining the specimen (15 mins to 1 hr post mortem) and at 3 hrs and 6 hrs later. In one of the three patients, a 6-hr time point was not available. Histologic examination of H&E specimens demonstrated a significant increase in columnar epithelial cell autolysis in ileum and colon beginning at 3 hrs and progressing at 6 hrs Other tissues showed a much reduced degree of autolytic changes over time. Importantly, conventional light microscopy of H&E specimens did not detect any effect of delay in fixation on the percentage of cells exhibiting apoptotic findings. On the other hand, the TUNEL method did demonstrate that a delay in tissue fixation caused a marked increase in the number of positive (but presumably not apoptotic) cells at 3 and 6 hrs compared with immediate fixation in almost all organs. In

several fields of view (x400 magnification), every cell was labeled.

DNA Agarose Gel Electrophoresis

DNA agarose gel electrophoresis was performed on 117 specimens from patients with sepsis including colon (n = 10), aorta (n = 5), pancreas (n = 8), brain (n = 5), heart (n = 6), spleen (n = 10), kidney (n = 11), lymph node (n = 11), lung (n = 12), ileum (n = 12), thymus (n = 1), liver (n = 11), adrenal (n = 7), and muscle (n = 9). DNA agarose gels were positive for apoptotic laddering in eight of the 117 specimens (6.8%) including spleen (n = 1), kidney (n = 1), lymph node (n = 1), lung (n = 1), ileum (n = 1), and liver (n = 3) (Figure 7). DNA agarose gel electrophoresis was performed on three tissues obtained from the one prospective control patient (aorta, lymph node, adrenal) and was negative for apoptosis.

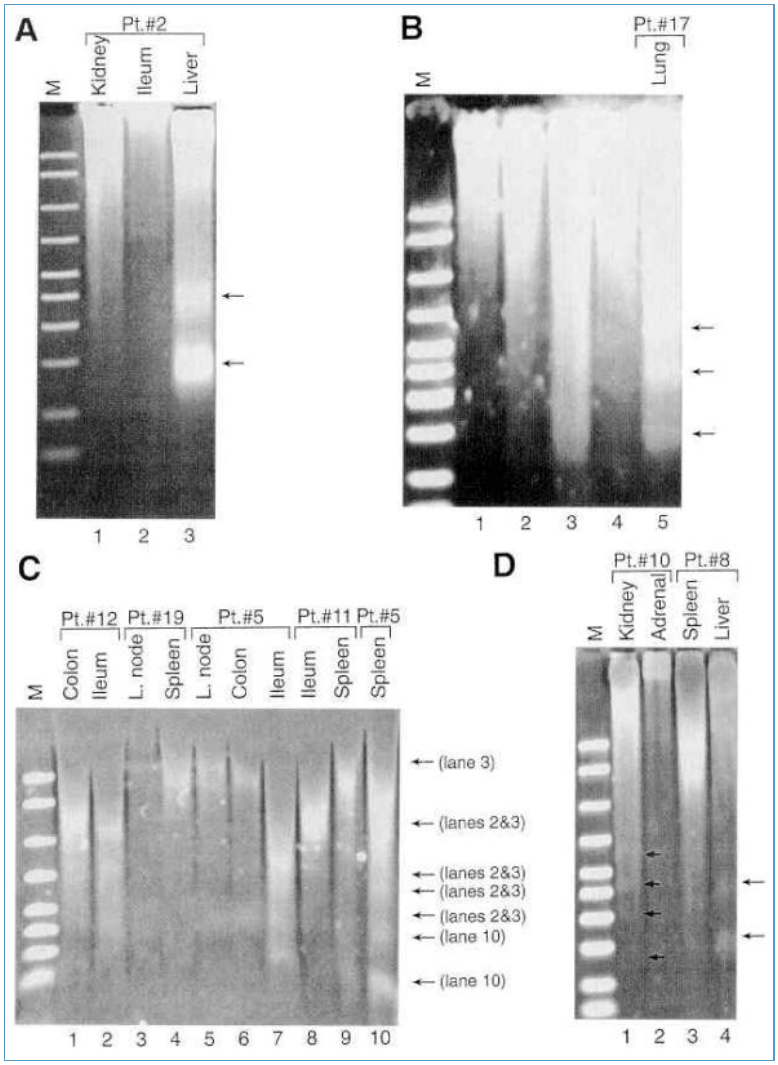

Figure 7. DNA agarose gels of tissues from septic patients. Tissue was homogenized in digestion buffer, DNA was extracted, and electrophoresis was performed. M, commercial molecular markers, 50 to 2000 base pairs. Arrows depict the DNA "ladder formation," which is a hallmark of apoptosis. L, lymph.

Caspase-3 Immunohistochemistry

Immunohistochemical staining for active caspase-3 demonstrated a marked increase in reactivity in spleens from septic vs. nonseptic patients (Figure 8) ($p < .02$). In spleens from septic patients, white pulp had the greatest percentage of cells that were caspase-3-positive. In six septic patients >25% to 50% of cells were focally positive for caspase-3 (Figure 8 and Figure 9). In addition to mature lymphocytes, larger immunoblast-like cells were also positive (Figure 10). Examination of the spleens in septic patients was similar to conventional light microscopy (H&E) in that there was a noticeable decrease in lymphocyte density in the white pulp of septic patients.

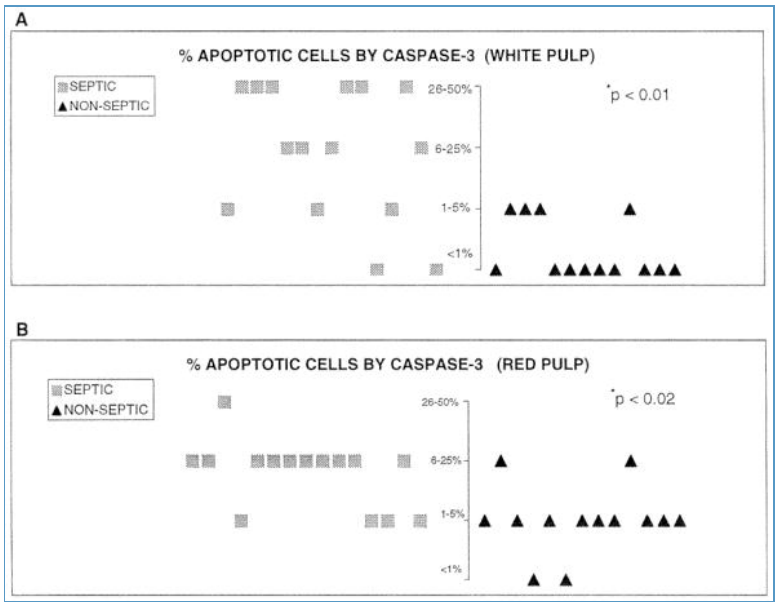

Figure 8. Percentage of caspase-3-positive cells in spleen white pulp (A) or spleen red pulp (B) of septic vs. nonseptic patients. Spleens from septic and nonseptic patients were examined at x400 magnification by a pathologist who was blinded as to the sample identity. The percentage of cells that had brown staining (active caspase-3-positive) were graded using the following scale: <1%, 1% to 5%, 6% to 25%, 26% to 50%, >50%. Spleens from septic patients had more caspase-3-positive cells in white pulp ($p < .01$) and in red pulp ($p < .02$).

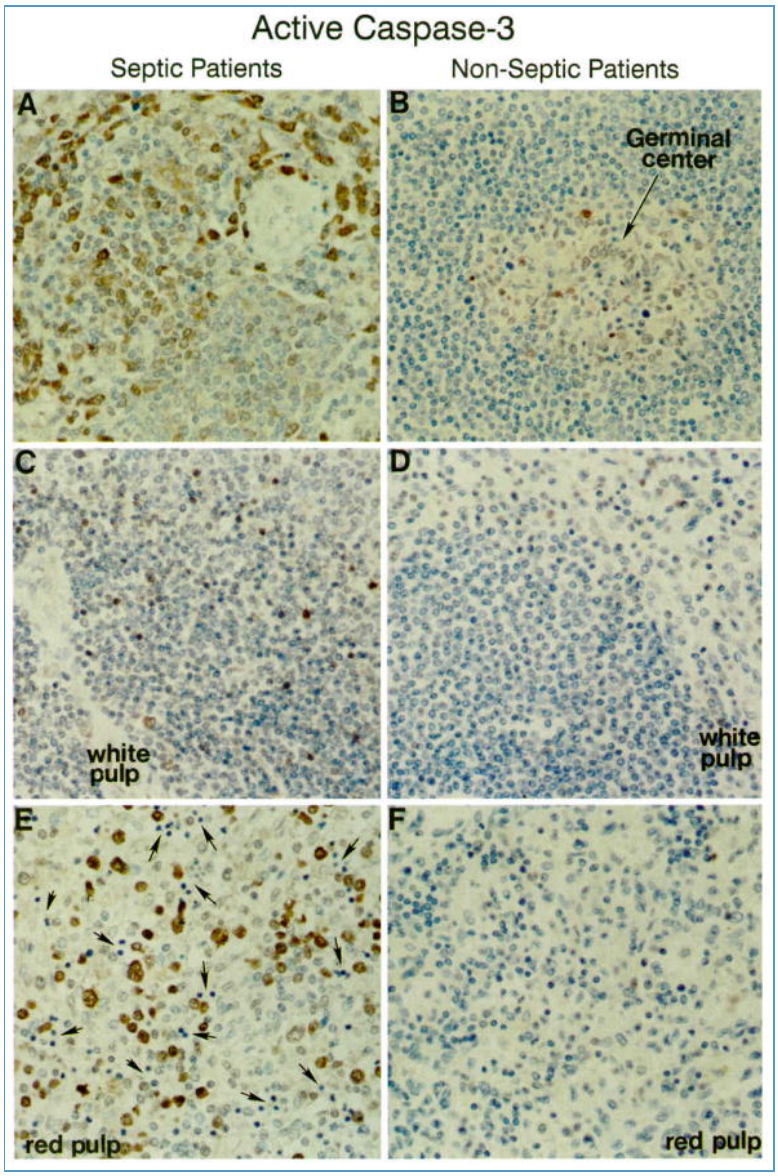

Figure 9. Immunohistochemical staining for active caspase-3 in spleens from septic and nonseptic patients. Deparaffinized slides underwent antigen retrieval by microwaving in 0.1 M citrate buffer (see "Materials and Methods"). The anticaspase-3 antibody (specific for active caspase-3) was added, and after rinsing, a biotinylated secondary antibody was applied. Slides were rinsed and developed with metal-enhanced diaminobenzidine. Slides were evaluated in a blinded fashion by a pathologist who scored the percentage of caspase-3-positive cells in white and red pulp. Note the markedly increased number of caspase-3-positive cells in white and red pulp of septic vs. nonseptic patients. Germinal centers were much more common in nonseptic patients, and these areas were frequently caspase-3-positive.

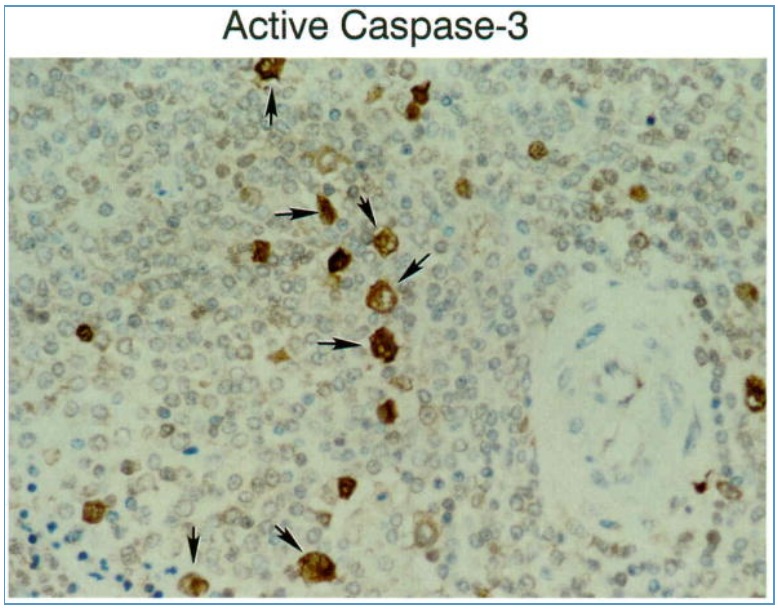

Figure 10. Immunohistochemical staining for caspase-3. A spleen for a septic patient with large immunoblast-like-appearing cells (identified by arrows) that are caspase-3-positive. Note also the pyknotic lymphocytes (dark blue small cells) in the lower left corner.

BCL-2 Immunohistochemistry

Spleens were uniformly positive for BCL-2 in lymphoid elements of mantle and marginal zones in both septic and nonseptic patients; no differences in the BCL-2 staining pattern were detected in the two groups of patients. An obvious decrease in the number of lymphocytes in white pulp (both BCL-2-positive and BCL-2-negative lymphocytes) was observed by immunohistochemistry (Figure 11). Similar to conventional light microscopy (H&E), apoptotic nuclei were readily detected in spleens from septic patients (Figure 11). Some of the apoptotic nuclei stained positive for BCL-2 (Figure 11).

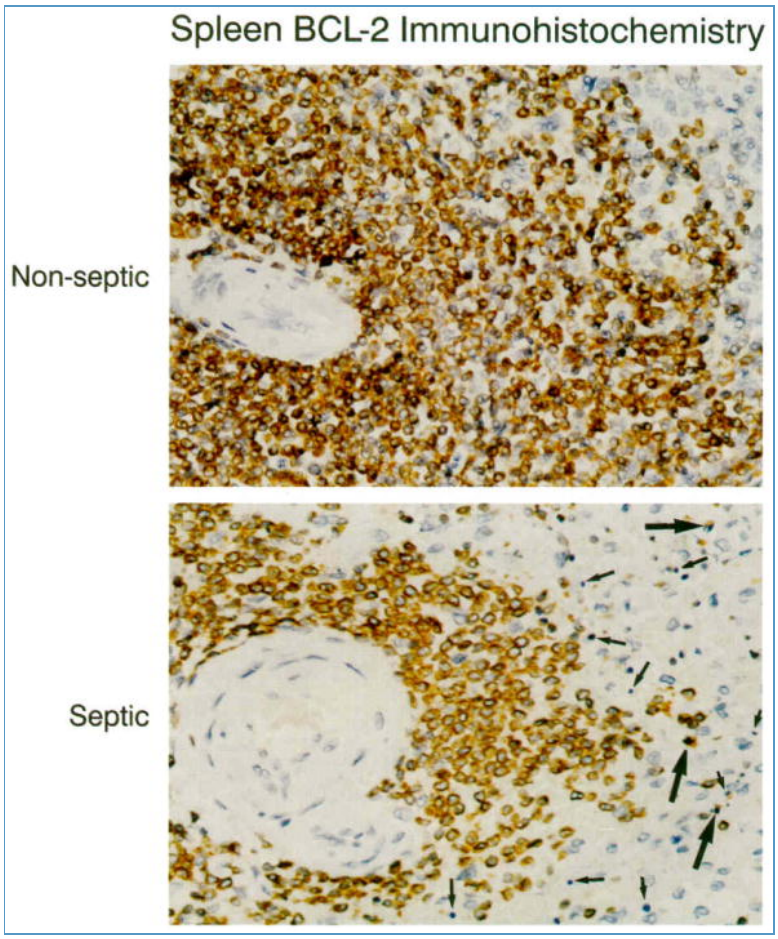

Figure 11. Immunohistochemical staining for BCL-2. Spleens were uniformly positive for BCL-2 in lymphoid elements

of mantle and marginal zones in both septic and nonseptic patients. Spleens from septic patients had an obvious decrease in the number of lymphocytes in white pulp (both BCL-2-positive and BCL-2-negative). Large arrows identify BCL-2-positive apoptotic cells. Small arrows identify BCL-2-negative apoptotic cells.

DISCUSSION

The present study represents the first examination of the prevalence of apoptosis in patients dying of sepsis and multiple organ failure. Findings in the clinical study closely paralleled results from animal models of sepsis [2,4,6] and showed that lymphocytes and gastrointestinal columnar epithelial cells undergo accelerated apoptosis in sepsis. Two points should be considered when evaluating the significance and extent of lymphocyte apoptosis in sepsis that were demonstrated in the present study. First, apoptotic cells undergo rapid phagocytosis (Figure 4D), with the entire process lasting <2 hrs in some cells [21]. Therefore, even if apoptosis is occurring, it may be missed in random histologic sections. That conventional light microscopy of H&E-stained specimens (a relatively insensitive method of detecting apoptosis) [21] disclosed a prevalence of apoptosis of [similar]56.3% in spleens from septic patients is remarkable. Lymphocyte apoptosis was not confined to the spleen but occurred in lymph nodes and lymphoid aggregates in the kidney and intestine as well (Figure 3C, and Figure 3D; Table 2). Lymphocyte apoptosis was detected by H&E in the spleen from only one nonseptic patient and this was at a low level (1% to 5% cells positive for apoptosis focally). Although the degree of apoptosis was variable in tissues of septic patients, in some fields of view (x400 magnification) as many as 10% to 20% of cells were apoptotic (Figure 2). This high percentage of apoptotic cells detected by H&E staining in spleens of septic patients is supported by immunohistochemical staining for active caspase-3, which showed that 25% to 50% of splenocytes in white pulp were undergoing apoptosis in six septic patients (Figure 9). The anti-caspase antibody is specific for the active form of caspase-3 and has been shown to colocalize with apoptotic myocytes after in vivo myocardial ischemia and reperfusion [15]. A second important point is that apoptosis was evaluated at only one point during each patient's illness. Most patients had a prolonged septic phase, and it is probable that the apoptosis occurred throughout much of their hospital course. Therefore, even a modest degree of apoptosis detected at one point in time should be considered highly significant.

Demonstration of apoptosis in septic tissues by DNA agarose gel electrophoresis is another important confirmatory finding because, although this method lacks sensitivity and may be falsely negative despite unequivocal apoptosis [21,24,25], it is highly specific. The low sensitivity of the DNA agarose gel method is attributable to two factors. First, the method may fail to detect apoptosis in tissues because it requires a considerable number of apoptotic cells to be positive [21,24,25]. Second, and most important, apoptotic cells may undergo phagocytosis before breakdown of the DNA into the small base pair fragments that are detected by electrophoresis [21,24]. Therefore, the 6.8% occurrence rate of DNA ladder formation in tissues from septic patients most likely reflects a significantly higher prevalence of apoptosis.

Lymphocyte Apoptosis in Sepsis-A Pre- or Post Mortem Event? Although it is possible that lymphocyte apoptosis may be attributable to post mortem changes, there are a number of reasons why we believe that the lymphocyte apoptosis detected in this study represents a premortem and clinically relevant observation. a) Fifteen of the patients with sepsis had a profound decrease in their circulating lymphocyte count, often to levels less than half of the reference value. Our finding of sepsis-induced lymphopenia agrees with work by Castelino et al. [26], who reported that lymphopenia in hospitalized patients is most frequently caused by acute illness, notably sepsis and trauma. Similarly, Jahangiri and Wyllie [27] reported that patients with sepsis resulting from gangrenous appendicitis had a significant decrease in the absolute lymphocyte count (53% lower compared with controls). b) Histologic examination of H&E-stained specimens of spleen showed a remarkable decrease in lymphocyte density in splenic white pulp and in the cortical zone of lymph nodes (Figure 3A, and Figure 3B). c) The fixation time course study showed that the delay in fixation of tissues did not cause histologic evidence of apoptosis (as evaluated by light microscopy of H&E samples) in any specimens. Also, these histologic findings of apoptosis are not typical of normal post mortem findings. d) The present documentation of extensive lymphocyte apoptosis in patients with sepsis closely reflects the results in certain animal models of sepsis and endotoxemia. For example, a mouse peritonitis model of sepsis caused extensive lymphocyte apoptosis in thymus, spleen, gastrointestinal-associated lymphoid tissue, and lymphoid aggregates in lung [6]. e) Our findings of plasma cell and mononuclear cell apoptosis in the intestinal lamina propria are consistent with those of Coutinho and colleagues [28] who identified a significant loss of B lymphocytes in the intestines of patients with sepsis. f) Lymphocyte apoptosis was present in tissues that were obtained almost immediately after the patient's death. Patient 19 was a heart transplant donor. In this case, tissues were obtained in the operating room within 15 mins after expiration, thereby avoiding significant post mortem changes. Apoptosis was detected by light microscopy (Figure 3C), DNA agarose gel (Figure 7A), and caspase-3 immunohistochemical staining in spleen, lymph nodes, and a lymphoid aggregate in the kidney. In short, there is over-whelming direct and indirect evidence that patients with sepsis have widespread lymphocyte cell death.

Caspase-3-Mediated Apoptosis in Sepsis. A major component of the cell death program is a proteolytic system involving a family of cysteine proteases that have been termed "caspases." Of these proteases, caspase-3 is one of the most widely studied; its activation has been linked to apoptotic death [14]. Activated caspase-3 is known to cleave a number of important enzymes, including the DNA repair enzyme poly(ADP-ribose) polymerase and DNA-dependent protein kinase [14]. Although it is known that apoptosis occurs in sepsis, it is not known which caspase family members are involved. Hakem et al. [13] determined that multiple parallel apoptotic pathways exist in mammalian cells, some of which are independent of caspase-3 activation. The present work is important because it shows that activated caspase-3 is involved in lymphocyte apoptotic cell death in septic patients. Caspase-3-mediated lymphocyte death also occurred in the control patients, but it was restricted primarily to the germinal centers where accelerated apoptosis may be a normal physiologic means of eliminating unwanted lymphocytes [29]. The percentage of caspase-3-positive cells in the septic patients was greater in white vs. red pulp, and this may be related to the different cell types that populate the two anatomical regions. The white pulp consists of the lymphocyte-rich regions, including the periarteriolar lymphoid sheaths and follicles. In contrast, the red pulp has relatively fewer lymphocytes but also contains erythrocytes, macrophages, dendritic cells, and plasma cells. An interesting observation in the septic spleens was the number of large immunoblast-like cells that stained positive for active caspase-3 (Figure 10). Caspase inhibitors decrease the extent of brain injury in recent stroke models [30] and may be

effective in myocardial ischemia-reperfusion injury, as well [12]. If lymphocyte apoptosis is ultimately determined to be detrimental to host survival in sepsis, capase-3 inhibitors may be useful therapeutically. Finally, it is important to emphasize that the results of the caspase-3 immunohistochemical staining not only provide important mechanistic information but also serve as a strong confirmatory method for the H&E findings, which showed apoptosis in lymphocytes from patients with sepsis.

Failure of the TUNEL Method to Discriminate Between Septic and Nonseptic Patients. Although the TUNEL technique did indicate that tissues from septic patients had a large percentage of apoptotic cells, the method was equally positive in tissues from many control patients. This inability of the TUNEL method to discriminate between septic and control tissues is likely related to a number of factors, including oversensitivity of the method and a high rate of false-positive results [21-23]. Investigators also have demonstrated that the TUNEL method lacks specificity and may not discriminate between apoptotic and necrotic forms of cell death [23]. Another important limitation of the TUNEL method, as emphasized by this study, is that delay in tissue fixation may cause an increase in the number of TUNEL-positive cells. Delay in tissue fixation has also been shown to cause an increase in the percentage of apoptotic cells identified by the in situ end-labeling method. The time between death and tissue fixation was significantly longer in control patients who underwent post mortem examination (often exceeding 6 hrs) than septic patients, and this delay may have been a factor in the high percentage of TUNEL-positive cells in control patients. We believe that delay in tissue fixation was a major cause of TUNEL-positive cells, because a previous study from our laboratory using the TUNEL method found a lower percentage of TUNEL-positive cells in tissues from sham-operated mice than septic mice. In this study, there was no delay in tissue fixation after the animals were killed. The increase in TUNEL-positive cells with delay in tissue fixation may be organ specific because other investigators claim that no increase in TUNEL-positive staining occurred in tissues processed up to 12 hrs post mortem [31].

Lymphocyte Apoptosis in Sepsis-Beneficial or Adverse? A key question remains: What is the effect of lymphocyte apoptosis on host survival? A current paradigm proposed by Bone [8] is that host response to sepsis represents a balance between proinflammatory and compensatory anti-inflammatory factors. The loss of the proper balance between these two systems can result in organ dysfunction or death. Undoubtedly, apoptosis through its effects on lymphocyte survival is an important factor regulating these two opposing processes. One potential effect of widespread lymphocyte apoptosis could be to compromise the host's defense mechanisms.

An effective host response to sepsis must involve a coordinated response among the various immunologic cells, including B and T cells, cytotoxic cells, and macrophages. For example, lymphocytes produce a variety of cytokines that activate macrophages and vice versa. In addition, macrophages and B lymphocytes have an important role as antigen presenting cells to T cells [32]. A significant decrease in the number of lymphocytes will impact on multiple facets of the immunologic response. Evidence for an abnormality in lymphocyte response in sepsis has been known for many years. The measurement of delayed skin-test response to skin-test antigens is an effective test of cell-mediated immunity and is attributable in large part to TH1-type lymphocytes [33,34]. Patients with sepsis become anergic, i.e., have no response to skin testing with antigens derived from microbes to which exposure would be expected (positive controls) [35]. Thus, the absence of a delayed skin-test response in septic patients is highly suggestive of a loss in T cell-mediated function. Therefore, lymphocyte apoptosis could hamper the ability of the septic patient to eradicate the infection and to predispose the individual to a secondary infection.

An alternative hypothesis is that apoptosis is beneficial to host survival by down-regulating the inflammatory response that accompanies sepsis. Much of the tissue injury occurring in lungs and other organs during sepsis may be caused by uncontrolled inflammation [36]. In this context, deletion of proinflammatory lymphocytes may improve organ function and survival. In addition, apoptosis may be an essential component of lung parenchymal recovery after pneumonia and ARDS, without which severe lung fibrosis could result.

Although it is not possible to know whether apoptosis is beneficial or detrimental to host survival in sepsis, there are some clues. Rajan and Sleigh [37] indicated that intensive care patients who developed a decreased lymphocyte count of 3 days duration had a greatly increased risk of nosocomial sepsis. A realistic and relevant picture of the impact of the loss of lymphocytes in patients with sepsis is provided by a genetic defect of nature. Patients with congenital thymic hypoplasia (DiGeorge's syndrome) have decreased T lymphocytes and are much more susceptible to bacterial, fungal, viral, and mycobacterial infections. Significantly, patients with decreased lymphocytes have infections with many of the same organisms, including Gram-positive bacteria (streptococci and staphylococci) and fungi [38], which together account for > 50% of all infections in many intensive care units [39]. Although patients with lymphopenia are not especially susceptible to Gram-negative bacteria, they are at an increased risk of infection from any organism because of their impaired defenses. Recent laboratory work supports the essential role of the lymphocyte in survival from sepsis. Results in a murine peritonitis model of sepsis show that transgenic mice that overexpress the antiapoptotic protein, BCL-2, in T lymphocytes have almost complete protection against sepsis-induced lymphocyte apoptosis in T cells and improved survival compared with wild-type mice with sepsis [40]. In this regard, that a small number of the lymphocytes that were undergoing apoptosis were BCL-2-positive (Figure 11) does not signify that overexpression of BCL-2 will fail to prevent lymphocyte apoptosis in sepsis.

Sepsis-Induced Apoptosis in the Intestine. Similar to the results in animal models of sepsis, the gastrointestinal tract was another major site of apoptosis in septic patients [6]. Apoptosis was observed by conventional light microscopy of H&E-stained specimens of the ileums and colons of septic patients (27.7% and 47.1%, respectively) (Figure 4A, Figure 4B and Figure 4C). Apoptotic bodies are normally present at a low level in both the small- and large-intestinal epithelium [41-43]. Examination by conventional light microscopy of H&E-stained specimens has been reported to reveal a prevalence of visible apoptotic bodies in approximately one cell per small intestinal villus. In the normal colon, apoptotic bodies were found toward the top of many (but not all) crypts, both in the epithelium (overall mean of 0.74 apoptotic bodies per crypt) and in the lamina propria (overall mean of 0.245 apoptotic bodies per villi) [24]. In contrast to the low physiologic level of apoptosis in control colons, apoptosis in the colons and small

bowels from septic patients was frequently extensive and involved the entire crypt or intestinal villus (Figure 4A, and Figure 4B). A current theory is that the gut is the "motor" of the systemic inflammatory response syndrome [44]. An impairment in the barrier function of the gut is postulated to result in translocation of bacteria and endotoxin into the bloodstream [44]. The anatomical findings of this study provide some support for this concept.

Sepsis and/or Shock as a Cause of Apoptosis. It is not possible to say definitely that sepsis is the cause of the extensive apoptosis in septic patients. Almost all septic patients had concomitant shock at one point in their hospital course. Animal studies and some human data indicate that ischemia/reperfusion injury can induce apoptosis in the gastro-intestinal tract [45], heart [46], kidney [47], and brain [48]. However, lymphocyte and gastrointestinal epithelial cell apoptosis in septic patients correlates closely with animal studies that demonstrate sepsis-induced apoptosis in these same target organs. Another possibility is that shock acted synergistically with sepsis to cause more severe apoptotic cell death than would have occurred with either insult alone.

Absence of Cause of Death in Septic Patients. A fundamental question that remains unresolved despite these extensive histologic studies is the primary mechanism responsible for patient death in sepsis. To our knowledge, this study represents the most exhaustive histopathologic study of patients dying of sepsis. Furthermore, the tissues were obtained relatively soon after the patients' demise, thereby avoiding the autolytic changes that occur after death. Although apoptosis and necrosis were present in many organs, the extent of cell death was not, in general, sufficient to cause organ failure. It can be argued that only a small fraction of the respective organ was examined and, therefore, the severity of cell death may have been underestimated. However, other evidence supports the concept that cell death is not overwhelming in patients with sepsis. Cardiac output is usually maintained at a normal to elevated range, even in the final stages of sepsis [49]. Despite the high prevalence of ARDS in sepsis, very few patients succumb because of hypoxemia or hypercarbia. Hepatic dysfunction frequently occurs in septic patients but rarely is severe enough to cause fulminant hepatic encephalopathy. Despite a high prevalence of renal failure, kidney dialysis is used routinely and, consequently, patients do not die of uremia. The absence of histologic evidence of cell death sufficient to explain the morbidity/mortality of sepsis suggests that other as yet unrecognized mechanisms may be involved in the pathogenesis of the disorder.

An alternative theory for organ dysfunction in sepsis is a cellular stress response involving down-regulation or "hibernation" of the cell. An analogy is found in the setting of myocardial infarction and "stunned" myocardium. The response of many cells including cardiac myocytes to stress includes a shift to fetal gene expression [50] and may be an attempt to revert to a lower energy-using state and avoid death. The cell does only enough to keep alive and does not perform other whole-organ functions. In any event, the lack of a clear-cut histologic explanation for much of the morbidity and mortality of sepsis suggests that a new paradigm is needed.

Limitations. This study can be criticized because of the lack of a control population of nonseptic patients who were identically matched to the patients with sepsis. Despite inclusion of three groups of control patients, including both prospective and retrospective control patients, the patient populations were not exactly identical. Nevertheless, the important point is that the light microscopic findings of apoptosis in the tissues from the septic patients, which are demonstrated in the numerous color photomicrographs, are pathognomonic of apoptosis and are never a normal finding in post mortem tissue. Therefore, this study demonstrates irrefutably that apoptotic cell death is an important component of sepsis.

In summary, apoptosis occurs systemically in many types of cells in patients with sepsis and shock. The lymphoid organs and columnar epithelial cells of the gastrointestinal tract are particularly vulnerable. It cannot be determined from this study whether apoptosis is beneficial or detrimental to the host. If apoptosis is determined to be detrimental to host survival, drugs that inhibit caspase-3 may be useful therapeutically. Histologic examination of tissues from septic patients did not reflect the severity of organ dysfunction in patients, and an alternative as yet unidentified mechanism may be responsible for the multiorgan failure.

Note Added in Proof: Since submission of this manuscript, we have documented extensive rapidly occurring colonic intestinal epithelial and lymphocyte apoptosis in the colon of a trauma patient in shock (unpublished data).

ACKNOWLEDGMENT

The authors gratefully acknowledge the nurses and staff of the 8400 Surgical Intensive Care Unit at Barnes Jewish Hospital.

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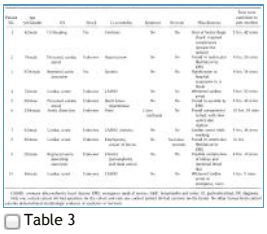

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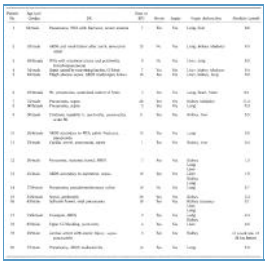

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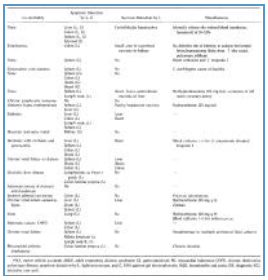

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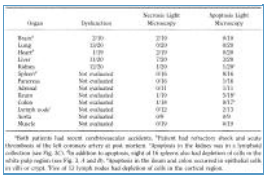

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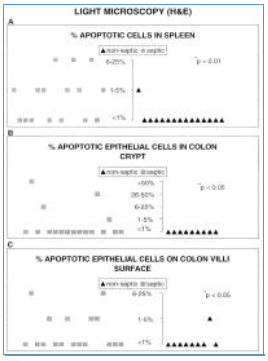
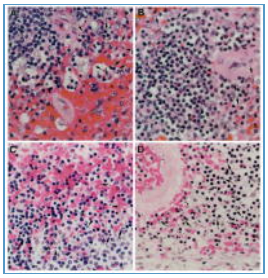


Figure 1



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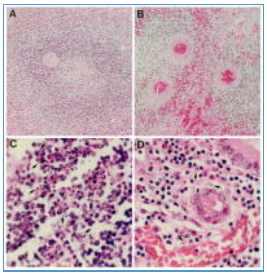

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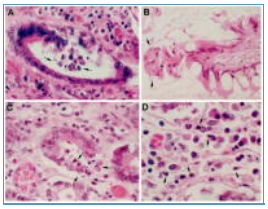

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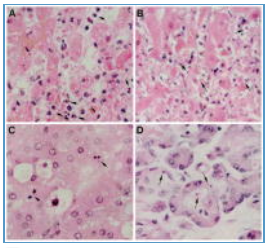

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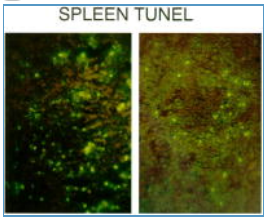

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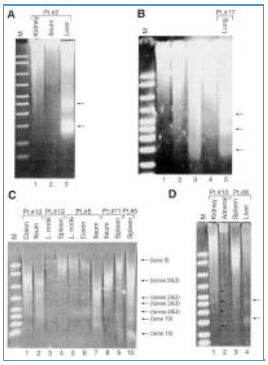

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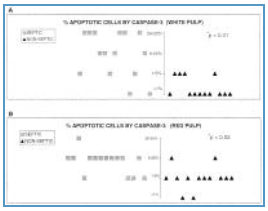

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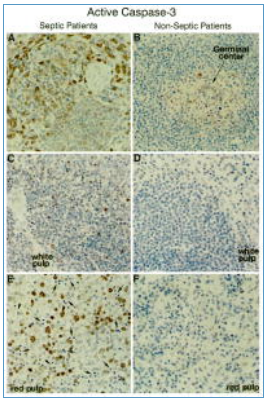

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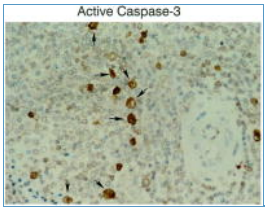

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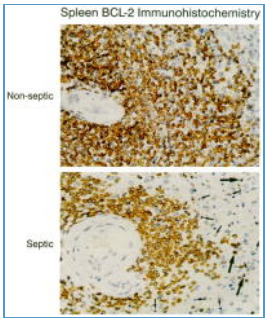

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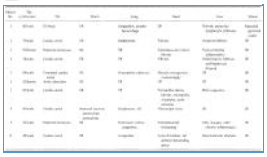

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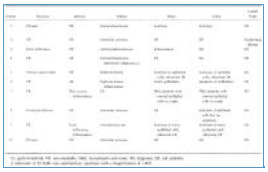

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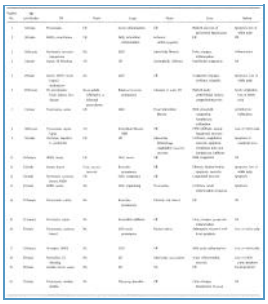

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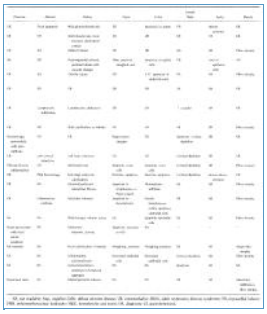

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Pathophysiology of septic acute kidney injury: What do we really know?

Li Wan, MD; Sean M. Bagshaw, MD, MSc; Christoph Langenberg, MD; Takao Saotome, MD; Clive May, PhD; Rinaldo Bellomo, MD, FRACP

Septic acute kidney injury accounts for close to 50% of all cases of acute kidney injury in the intensive care unit and, in its various forms, affects between 15% and 20% of intensive care unit patients. However, there is little we really know about its pathophysiology. Although hemodynamic factors might play a role in the loss of glomerular filtration rate, they may not act through the induction of renal ischemia. Septic acute renal failure may, at least in patients with a hyperdynamic circulation, represent a unique form of acute renal failure: hyperemic acute renal failure. Measurements of renal blood flow in septic humans are now needed to resolve this pivotal pathophysiological question. Whatever may happen to renal blood flow during septic acute kidney injury in humans, the evidence available suggests that urinalysis fails to provide useful diagnostic or prognostic information in this setting. In addition, nonhemodynamic mechanisms of cell injury are likely to be at work. These mechanisms are likely due to a combination of immunologic, toxic, and inflammatory factors that may affect the microvasculature and the tubular cells. Among these mechanisms, apoptosis may turn out to be important. It is possible that, as evidence accumulates, the paradigms currently used to explain acute renal failure in sepsis will shift from ischemia and vasoconstriction to hyperemia and vasodilation and from acute tubular necrosis to acute tubular apoptosis or simply tubular cell dysfunction or exfoliation. If this were to happen, our therapeutic approaches would also be profoundly altered. (Crit Care Med 2008; 36[Suppl.]:S198–S203)

KEY WORDS: sepsis; septic shock; acute renal failure; acute kidney injury; renal blood flow; renal vascular resistance; mean arterial pressure; apoptosis; glomerular filtration rate; cardiac output; cytokines

Acute renal failure (ARF) affects approximately 35% of intensive care unit (ICU) patients (1). Sepsis and septic shock remain the most important cause of ARF in critically ill patients and account for >50% of cases of ARF in the ICU (2).

Despite our increasing ability to support vital organs and resuscitate patients, the incidence and mortality of septic ARF remain high (2). A possible explanation of why mortality has remained high might relate to our limited understanding of septic ARF and its pathogenesis. It is therefore very important for critical care physicians to have an appreciation of what is known and not known about this condition to implement rational therapies. In this article, we review what is known about the pathophysiology of this condition, present the limitations and strengths of the evidence behind our knowledge, and discuss areas that require further investigation.

Definition

Before discussing any condition, it is imperative that there should be a common understanding of the topic. To do this, consensus definitions are needed. Until recently, there was no agreed way to define, identify, and classify septic acute kidney injury (AKI). However, more recently, the Acute Dialysis Quality Initiative developed a consensus definition of AKI that goes under the acronym of *RIFLE* (3). This definition and classification system is described in detail elsewhere in this issue of *Critical Care Medicine*. Its relevance here lies in the fact that together with the widely established consensus definition for sepsis (4), severe sepsis, and septic shock, which has been in use for >15 yrs, it provides a standardized ability to define the presence of septic AKI. Thus, septic AKI is defined by the simultaneous presence both of the RIFLE criteria for AKI and the consensus criteria for sepsis and by the absence of other clear and established, non–sepsis-related (e.g., radiocontrast, other nephrotoxins) causes of AKI. In this regard, a recent study of 41,972 admissions (1) shows that AKI occurs in 35.8% of patients when the RIFLE criteria are applied. A further study of AKI in 54 hospitals from 23 countries shows that close to 50% of AKI is secondary to sepsis (2). Thus, septic AKI probably occurs in somewhere between 15% and 20% of all ICU admissions. Its mortality varies with the severity of AKI from 20.9% to 56.8% (1). The obvious conclusion is that septic AKI is a major problem in ICU patients that requires investigation and a clearer understanding of its pathogenesis.

Pathogenesis

Our understanding of the pathogenesis of human AKI in general and septic AKI in particular is markedly affected by the lack of histopathologic information. This lack of information stems from the risks associated with renal biopsy (especially repeated renal biopsy), which make

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The authors have not disclosed any potential conflicts of interest.

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DOI: 10.1097/CCM.0b013e318168ccd5

it ethically unjustifiable to obtain tissue from patients who do not have suspected parenchymal disorders such as vasculitis or primary glomerulonephritis. In the absence of such information, we rely on indirect assessments of what might be happening to the kidney. Such assessments are based on blood tests and urine tests and force us to "guess" what might be happening to kidney cells by using indirect forms of assessment, such urine output, urinary sodium concentration, fractional excretion of sodium, fractional excretion of urea, and the like. It is not surprising, therefore, that our understanding of septic human ARF has advanced very little in the last 50 yrs.

To overcome such limitations, animal models of AKI have been developed that enable more sophisticated and invasive measurements to be made. Unfortunately, as recently highlighted (5), these animal models have been mostly based on ischemia–reperfusion injury or drug-induced injury. These models *are not relevant to septic AKI*, and information obtained from such models may be misleading when applied by clinicians to interpret what might be happening to a septic patient who is developing AKI in the ICU.

Renal Blood Flow in Septic AKI. A major paradigm that has been derived from observations in animals and humans with hypodynamic shock (hemorrhagic, cardiogenic, or even septic) is that AKI is due to renal ischemia. This construct implies that restoration of adequate renal blood flow (RBF) should therefore be the primary means of renal protection in critically ill patients (septic or not). Whether RBF in septic patients, in the presence of a normal or increased cardiac output, actually decreases significantly, remains stable, or even increases, however, remains unknown. This is because RBF cannot be measured continuously in humans and even its intermittent assessment requires a high level of invasiveness.

In several experimental studies of septic ARF, global RBF declines after induction of sepsis or endotoxemia (6, 7). This may result not only in a reduction in glomerular filtration but also, if hypoperfusion is severe and prolonged, in metabolic deterioration and diminished contents of high-energy phosphates, possibly causing cell death, acute tubular necrosis, and severe AKI.

On the other hand, other studies show that the renal circulation participates in the systemic vasodilation observed during severe sepsis/septic shock, so RBF does not diminish, and the development of septic ARF occurs not in the setting of renal hypoperfusion but in the setting of adequate and even increased renal perfusion. Ravikant and Lucas (8), for example, studied a pig model of sepsis and showed that during hyperdynamic sepsis, there was an *increase* in global RBF and an increase in medullary blood flow. Brenner et al. (9) developed and studied a percutaneously placed thermodilution RBF catheter in eight critically ill patients with AKI. They demonstrated that sepsis-induced AKI occurred despite normal values of total RBF (10). During human sepsis, patients in the ICU typically show a hyperdynamic circulation. Observations in hyperdynamic models of sepsis may, therefore, be much more relevant to human septic shock. Indeed, the reason why the results of experimental studies are so different in terms of RBF may be entirely related to the animal models (including animal type and type of insult), different methods of measurement, the time and frequency of measurements, and more importantly, the state of the systemic circulation (hypodynamic or hyperdynamic state) (9). In fact, the consistent observation is that once a hyperdynamic state exists, global renal hypoperfusion/ischemia is *not* the norm (11).

A comprehensive review of electronic reference libraries, focusing on experimental models of sepsis and ARF, has been recently published (12). This systematic review found that 160 experimental studies had been conducted that induced sepsis and focused on aspects of renal function or dysfunction and that measured RBF by one of several available techniques. In such studies, close to a third showed that RBF was either preserved or increased in experimental sepsis. To further investigate what factors might influence RBF in experimental sepsis, the authors assessed which experimental variables were associated with preserved or increased RBF. They found that several aspects of the model (awake animal, time from surgery, use of endotoxin, cardiac output) predicted RBF during the experiment. When multivariable logistic regression analysis was used, cardiac output alone remained as the predictor of RBF: high cardiac output sepsis was associated with preserved or increased RBF. Conversely, low cardiac output sepsis (mixed septic and cardiogenic shock) was associated with a low RBF. As noted above, most patients seen in the ICU with sepsis have a high cardiac output state. In recent experimental studies in sheep, in which both cardiac output and RBF were measured continuously and high cardiac output septic state was induced by the infusion of *Escherichia coli*, investigators were able to simulate the typical clinical and hemodynamic state seen in severe sepsis or septic shock (12). Using this model, the investigators were able to show that in hyperdynamic sepsis in a conscious large mammal, RBF is markedly increased and renal vascular resistance in markedly decreased (Fig. 1). In this setting, glomerular filtration rate (GFR) is markedly diminished, with a three-fold increase in serum creatinine concentration and an equivalent decrease in creatinine clearance. In accordance with these findings, renal recovery from this form of septic AKI has been found to be associated with a decrease in cardiac output, an increase in renal vascular resistance, and a decrease in RBF (13). These observations suggest that changes in renal vascular activity (vasodilation) may be important in the loss of glomerular filtration pressure during the first 24–48 hrs of sepsis. They also provide "proof of concept" that glomerular filtration pressure can be lost in septic AKI in the setting of markedly increased RBF. Put another away, septic AKI may represent a unique form of AKI: hyperemic AKI. Such understanding requires a further logical step: an appreciation that GFR is determined by glomerular filtration pressure. Glomerular filtration pressure, in turn, is determined by the relationship between the afferent and efferent arterioles. If the afferent arteriole constricts, glomerular filtration pressure will fall and urine output and GFR will also decrease. However, if the afferent arteriole dilates and the efferent arteriole dilates even more, RBF will markedly increase, yet pressure within the glomerulus will fall. In this setting, GFR will also decrease. This may be the case in human sepsis. To know whether this is indeed the case, one would need to measure RBF in humans during the development of septic AKI.

Unfortunately, little is known about what happens to RBF in humans during severe sepsis or septic shock. This is because measurement of RBF requires invasive approaches. Nonetheless, RBF was measured in a small cohort of patients with sepsis. In these patients, RBF was either preserved or increased (14). To put it

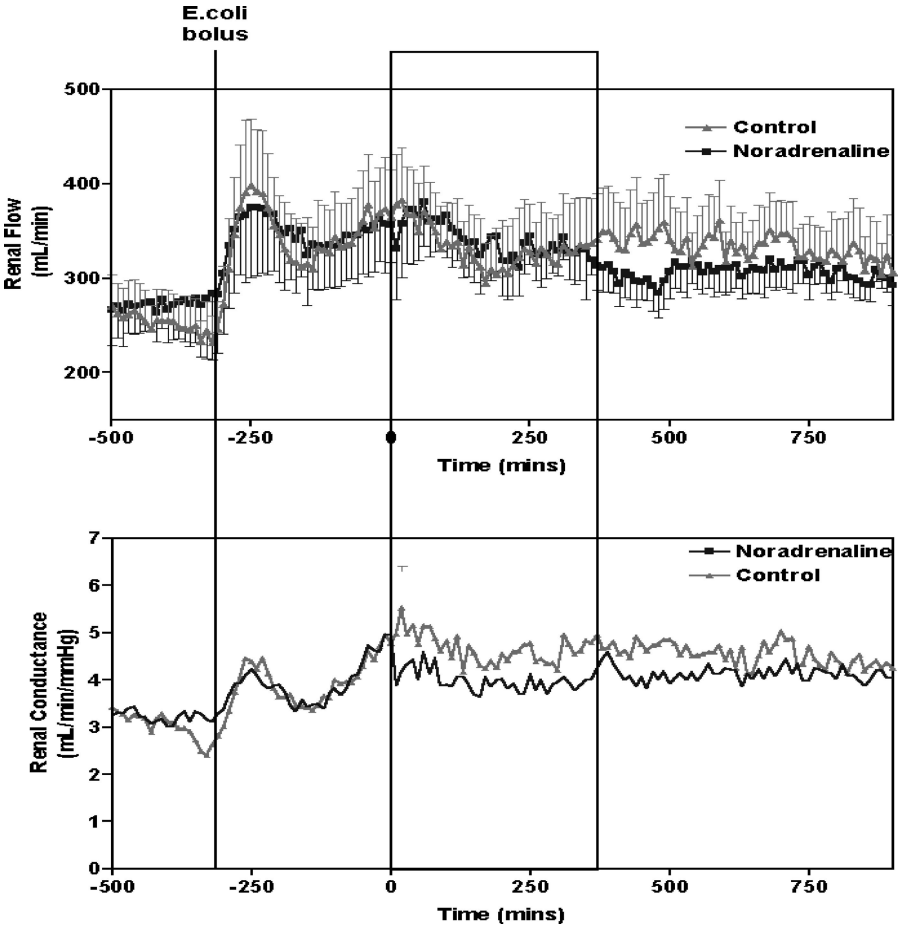

Figure 1. Changes in mean renal blood flow and renal vascular conductance (inverse of resistance) during experimental hyperdynamic hypotensive sepsis in sheep. The timing of *Escherichia coli* (*E. coli*) injection is marked. Renal blood flow and renal vascular conductance in control animals treated with placebo are marked as *triangles*. The timing of noradrenaline (norepinephrine) infusion (*squares*) is framed between 0 and 360 mins. In both groups, there is marked hyperemia and renal vascular vasodilation, which is not altered by noradrenaline infusion (0.4 $\mu g \cdot kg^{-1} \cdot min^{-1}$).

bluntly, we simply do not yet know what happens to RBF in human septic AKI. As this is a crucial issue for our understanding of the pathogenesis of septic AKI, this issue must be the focus of future investigations of the pathogenesis of septic ARF.

In conclusion, renal hypoperfusion might be important in septic AKI associated with hypodynamic states (a relatively uncommon finding in the ICU) but may not play a key role in the development of AKI during hyperdynamic sepsis (the state seen in the vast majority of critically ill, septic patients with severe ARF). Further work is needed in humans to better understand the changes in RBF that occur during septic AKI.

Intrarenal Hemodynamics and Bioenergetics in Septic AKI. It is possible that although there is preserved or increased global RBF in hyperdynamic sepsis, internal redistribution of blood flow favoring the cortex may occur. Unfortunately, no studies have looked at medullary and cortical blood flow in hyperdynamic sepsis with technology that allows continued measurement over time. A recent investigation by our group used laser Doppler flowmetry to continuously monitor medullary and cortical flow in hyperdynamic septic sheep (15). We found that both flows remain unchanged and that the administration of vasopressor (vasoconstrictor) therapy in the form of norepinephrine induced a significant increase in such flows. These observations challenge the view that the medulla is ischemic during hyperdynamic sepsis and simultaneously highlight that hemodynamic factors are indeed at work, which can be modified by interventions capable of affecting systemic blood pressure and cardiac output. In additional work applying a magnetic resonance spectroscopy technique with simultaneous measurement of RBF, we were also able to show that adenosine triphosphate is preserved during septic shock in the sheep (Fig. 2), further supporting the notion that ischemia or bioenergetic failure may not be the primary cause of loss of GFR in sepsis (16, 17). Thus, intrarenal hemodynamic events do occur, which might affect function. However, their favorable modification by vasoconstrictor therapy challenges the widely held view of what is optimal renal resuscitation in septic AKI. Furthermore, although hemodynamic changes might be important, they are likely to represent only part of the mechanisms responsible for loss of function. Other mechanisms may be at work.

Urine Changes in Septic ARF

A variety of textbooks suggest that it is possible to use urinary tests to distinguish acute tubular necrosis (structural injury) from so-called *prerenal ARF* (functional injury). Can this be done in septic ARF? What is the evidence? Recently, we completed a systematic review of the urinary findings seen in experimental models of sepsis and assessed their diagnostic and prognostic value. We found that all tests that are widely promoted as useful did not have sufficient data to support diagnostic accuracy, prognostic value, or clinical utility (18). Similarly, in a systematic review of the value of such tests in humans, we found significant lack of data and a wide variety of findings in septic ARF (19). All of these observations strongly support the concept that, in septic ARF, biochemical analysis of urine using standard measurements of sodium, urea, and creatinine and calculating various indices of tubular function is not diagnostically accurate, prognostically valuable, or clinically useful. More research is needed in this field to better understand the role of urinalysis in sepsis. In this regard, emerging biomarkers of kidney injury may prove more valuable (20).

Nonhemodynamic Injury. From the above discussion, we know that neither global renal hemodynamic changes nor intrarenal hemodynamic changes can be consistently shown to be the sole contributor to septic AKI. There must, therefore, be other mechanisms at work that are not hemodynamic in nature. These factors that contribute to AKI in sepsis might be immunologic or toxic in nature.

Sepsis is characterized by the release of a vast array of inflammatory cytokines,

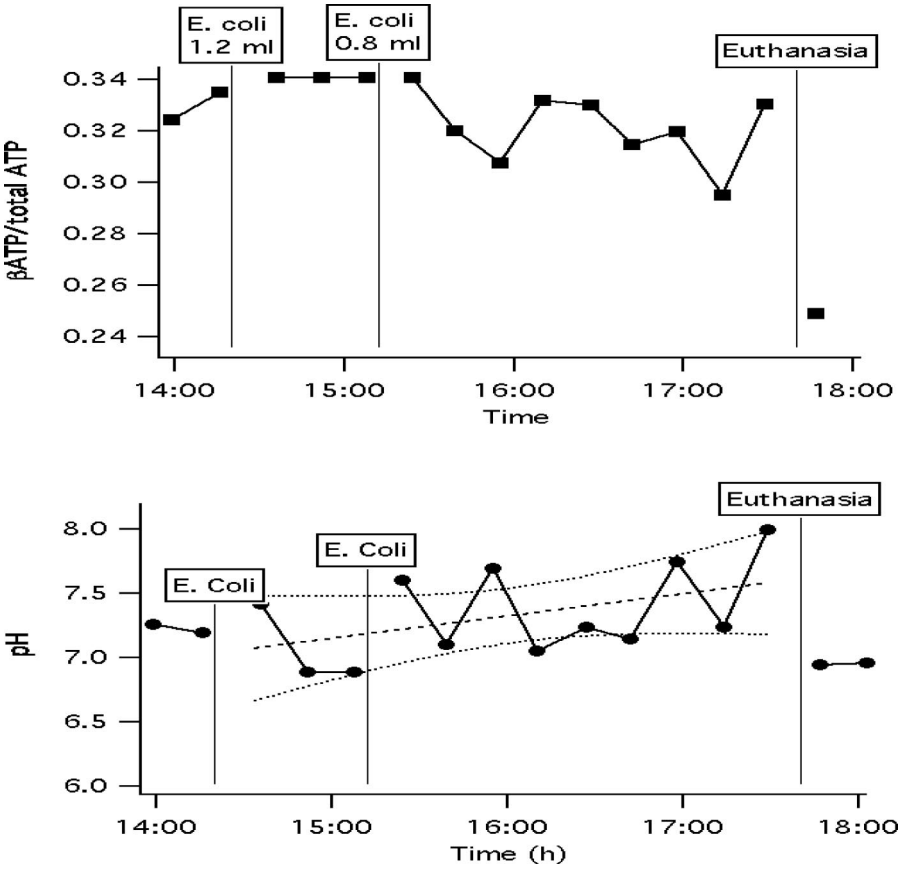

Figure 2. Changes in renal bioenergetics and pH in a model of hyperdynamic hypotensive sepsis in sheep. The timing of *Escherichia coli* (*E. coli*) injection is marked. There is no significant change in β-adenosine triphosphate (*ATP*)/total ATP ratio during sepsis. However, once the circulation stops after the animals are killed, the value plummets to near zero. There is also no evidence of intracellular acidosis until the circulation is stopped.

arachidonate metabolites, vasoactive substances, thrombogenic agents, and other biologically active mediators. A large body of experimental data suggests that these various mediators and neuroendocrine mechanisms might be involved in the pathogenesis of organ dysfunction in sepsis (21).

For example, tumor necrosis factor-α (TNF) has been demonstrated to play a major role in the pathogenesis of Gram-negative septic shock, mediating a broad spectrum of host responses to endotoxemia. In the kidney, endotoxin stimulates release of TNF from glomerular mesangial cells (22). More recently, the direct toxic role of TNF to the kidney has been become clear. Knotek et al. (23), using TNFsRp55-based neutralization of TNF, achieved protection against lipopolysaccharide (LPS)-induced renal failure in wild-type mice. With pretreatment using TNFsRp55, GFR decreased only by 30%, as compared with a 75% decrease without TNF neutralization, suggesting that TNF plays an important role in septic ARF. Cunningham et al. (24) used an intraperitoneal injection of *E. coli* LPS to establish a mice model of sepsis and showed that LPS-induced ARF can be attributed to TNF acting directly on its receptor, TNFR1, in the kidney. Mice deficient in TNF receptor were resistant to LPS-induced renal failure, had less tubular apoptosis, and fewer infiltrating neutrophils. Whereas TNF-receptor–positive kidneys transplanted in TNF-receptor–negative mice developed LPS-induced renal failure, TNF-receptor–negative kidney implanted in TNF-positive mice did not. Thus, TNF seems to be an important direct mediator of endotoxin's effects during septic AKI. These observations suggest that toxic/immunologic mechanisms are important in mediating renal injury during sepsis and that hemodynamic factors do not operate in isolation and may not even be of major importance.

Is Septic AKI Caused by Renal Cell Apoptosis? Apoptosis is a form of cell death that is mediated by a genetically determined biochemical pathway and characterized morphologically by cell shrinkage, plasma membrane blebbing, chromatin condensation, and nuclear fragmentation (25–29). Cells can die by one of two pathways: necrosis or apoptosis. Necrosis results from severe adenosine triphosphate depletion. Such depletion leads to rapid uncoordinated collapse of cellular homeostasis. Apoptosis is an energy-requiring and genetically directed process.

There is now good evidence to show that human renal tubular cells die by apoptosis and necrosis in experimental models of acute ischemic and toxic renal injury (26–29). The endothelial cells can undergo apoptosis in response to a variety of stimuli, especially immune-mediated cell injury via TNF and Fas ligand.

Schumer et al. (29) demonstrated that after a very brief period of ischemia (5 mins), apoptosis bodies could be found at 24 and 48 hrs after reperfusion, without any evidence of necrosis. After more prolonged periods of ischemia, areas of necrosis became evident, but substantial numbers of apoptotic bodies were still seen after 24–48 hrs of reflow. The evidence of whether apoptosis plays an important role in tubular injury *in vivo* remains controversial. It is particularly controversial whether renal cell apoptosis occurs during septic AKI. However, Jo et al. (30) have recently shown that apoptosis of tubular cells by inflammatory cytokines and LPS is a possible mechanism of renal dysfunction in endotoxemia. They found that if high-dose TNF was added to cultured kidney proximal tubular cells, there was increased expression of Fas messenger RNA, the Fas-associated death domain protein, and increased DNA fragmentation. Messmer et al. (31) have also shown that TNF and LPS elicit apoptotic cell death of cultured bovine glomerular endothelial cells that is time and concentration dependent. Their effect was characterized by an increase in pro-apoptotic proteins and a decrease in anti-apoptotic proteins such as Bcl-xL. Unfortunately, TNF blockade with monoclonal antibodies fails to protect animal or kidney during endotoxemia (32, 33). Observations in a preliminary experiment in septic sheep by our group also show that after only 3 hrs of sepsis induced by intravenous injection of *E. coli*, there was expression of early phase pro-apoptotic proteins such as BAX and of counterbalancing anti-apoptotic proteins such as Bcl-xL in the tubular cells, indicating that there is early activation of the apoptotic cascade in septic kidneys.

Organ Cross-talk and Septic AKI. Ventilation of patients with the acute respiratory distress syndrome by means of a low-tidal volume strategy has been shown to reduce mortality (34). The mechanisms for such reduced mortality, however, remain unknown. It is possible that protective ventilatory strategies might affect the well-being of other organs. In a fascinating series of studies, Imai et al. (35) demonstrated that low tidal volume ventilation might protect the kidney from injury in the setting of experimental and clinical acute respiratory distress syndrome. Using a rabbit model of acute respiratory distress syndrome, these investigators found that animals randomized to an injurious ventilatory strategy had increased epithelial cell apoptosis in the kidney and the small intestine. Furthermore, such animals had evidence of renal dysfunction. When renal cells were incubated *in vitro* with plasma from rabbits exposed to an injurious ventilatory strategy, apoptosis of such cells was induced and was markedly greater than seen with exposure to control plasma. These investigators hypothesized that Fas ligand might be responsible for these changes and used FasIg (a fusion protein that blocks soluble Fas ligand) to test this hypothesis. They found that Fas-ligand blockade attenuated *in vitro* apoptosis of renal cells. To further confirm such association, they obtained plasma from patients enrolled in a previous acute respiratory distress syndrome study comparing low-tidal volume ventilation with traditional tidal volume ventilation and found that there was a significant correlation between Fas-ligand levels in plasma and serum creatinine. Given that the vast majority of patients with acute respiratory distress syndrome have sepsis, these observations are highly relevant to septic AKI and highlight yet another pathway potentially responsible for AKI in the setting of sepsis.

CONCLUSIONS

Our understanding of the pathogenesis of septic AKI is limited. Although hemodynamic factors might play a role in the loss of GFR during sepsis, they may not act through the induction of renal ischemia. Septic ARF may represent a unique form of ARF: hyperemic ARF. Measurements in humans are now needed to resolve this pivotal pathophysiological question. Whatever may happen to RBF during septic AKI in humans, the evidence available suggests that urinalysis fails to provide useful diagnostic or prognostic information in this setting. In addition, nonhemodynamic mechanisms of cell injury are likely to be at work, which are immunologic/toxic/inflammatory in nature and may affect the vasculature and the tubular cells. Among these mechanisms, apoptosis may turn out to be important. It is possible that, as evidence accumulates, the paradigms currently used to explain ARF in sepsis will shift from ischemia and vasoconstriction to hyperemia and vasodilation and from acute tubular necrosis to either acute tubular apoptosis or simply tubular cell dysfunction. If this were to happen, our therapeutic approaches would also be profoundly altered. The journey of understanding the pathophysiology of septic AKI has barely started and is likely to be long indeed.

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