# Journals A-Z > Critical Care Medicine > 27(7) July 1999 > Apoptotic cell death ...

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Apoptotic cell death in patients with sepsis, shock, and multiple organ dysfunction
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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 tabeling (Tomel) and by bina get 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 "caspase" 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 chemotherapyinduced 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.	Åge (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

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 contro attents 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<sub>2</sub> 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<sup>+</sup>, diaminobenzidine (<sup>+</sup>), 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 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<sup>3</sup> (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 (diałysis)	0.5
3	85/female MVA with intestinal injury and peritonitis,		8	No	Yes	Liver, lung	0.9
4 5	54/male 49/male	54/male Sepsis caused by necrotizing fasciitis, GI bleed 49/male Thigh abscess sepsis, ARDS multiorgan failure		Yes Yes	Yes Yes	Liver, kidney (dialysis) Liver, kidney, lung	0.4 0.9
6	49/female	MI, pneumonia, watershed infarct of brain	3	Yes	Yes	Lung, heart, brain	NA
7 8	74/male 60/female	Pneumonia, sepsis Pneumonia, sepsis	20 5	Yes Yes	Yes Yes	Kidney (dialysis) Lung	11.4 0.3
9	50/male	Cirrhosis, hepatitis C, peritonitis, pancreatitis, acute MI	11	Yes	Yes	Kidney, liver	5.5
10	26/temale	ARDS secondary to MVA, pelvic fractures,	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	1.0
13	45/male	ARDS secondary to aspiration, sepsis	19	Yes	Yes	Liver Liver Kidney	1.9
14	77/female	Pneumonia, pseudomembranous colitis	10	No	Yes	Lung	0.7
15 16	51/female 43/male	Sepsis, peritonitis Ischemic bowel, viral pneumonia	30 10	Yes Yes	Yes Yes	Kidney Kidney (dialysis) Liver	0.3 0.1
17	73/female	Urosepsis, ARDS	2	Yes	Yes	Lung	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

Co-morbidity	Apoptosis Detection by L, G	Necrosis Detection by L	Miscellaneous
None	Liver (L, G)	Centrilobular hepatocytes	Jehovah's witness who refused blood transfusion,
	Spleen (L, G) Adrenal (L)		nematocrit of 10-1376
Emphysema	Colon (L)	Small area of superficial necrosis in kidney	No definitive site of infection at autopsy but treated bronchopneumonia likely; fever, ↑ wbc count, nulmonary 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)	No	
Mana	Heart (G)	Manual Income constant data data	Mathalasadariadaria (60 mathal) and aras of latit
None	Lymph node (L)	necrosis of liver	main coronary artery
Systemic lupus erythematosis	No Spleen (L) Luser (C)	No Patchy hepatocyte necrosis	Hydrocortisone (25 mg tid)
Diabetes	Liver (L)	Liver	
	Colon (L) Lymph node (L) Soleen (L)	Heart	
Muscular dystrophy (mild)	Kidney (G)	No	
Alcoholic with cirrhosis and	Liver (L)	Brain	Blood cultures (+) for S. pneumonia elevated
pancreatitis	Spleen (L) Colon (L) Ileum (L)		troponin I
Chronic renal failure on dialysis	Spleen (L)	Liver	
	Colon (L)	Colon	
Alcoholic liver disease	Lymphocytes in Peyer's patch (L) Colon-lamina propria (L)	No	
Adenocarcinoma of stomach and duodenum	No	No	-777.4
Invasive adrenal carcinoma	Colon (L)	No	Previous splenectomy
Chronic renal failure caused by	Liver (L)	Liver	Hydrocortisone (25 mg q 8)
lupus	Ileum (L) Spleen (L)		Cytoxan
None	Lung (G)	No	Hydrocortisone (20 mg q 8) Blood cultures (+) for enterpressure
Pancreatic cancer, COPD	Spleen (L) Colon (L)	Liver	—
Chronic renal failure	Spleen (L) Kidney lymphoid (L)	No	Pseudomonas in multiple peritoneal fluid culture
Rheumatoid arthritis, emphysema	Colon-lamina propria (L)	No	Chronic steroids

Table 4. Table 1. Continued

Organ	Dysfunction	Necrosis Light Microscopy	Apoptosis Light Microscopy
Brain <sup>a</sup>	2/10	2/10	0/10
Lung	13/20	0/20	0/20
Heart <sup>o</sup>	1/19	2/19	0/20
Liver	11/20	7/20	3/20
Kidney	12/20	1/20	$1/20^{c}$
Spleend	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 <sup>4</sup>	Not evaluated	0/12	2/13
Aorta	Not evaluated	0/9	0/9
Muscle	Not evaluated	0/19	0/19

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



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.



(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 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. 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)/Cender	DX	Brain	Lung	Heart	Liver	Spleen
1	42/male	GI bleed	LTR	Congestion, alveolar hemorrhage	UR	Fibrosis, periportal lympbocyte infiltrates	Expanded germina 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	Eosmophilic infiltrates	Myocyte enlargement. nucleomedaly	UR	UR
6	53/female	Aortic dissection	NA	UR	UR	UR	UR
1	63 male	Cardiac arrest	UR	UR .	Pericarditis edema. fibrosis, ecsinophilic cytoplasm, acute ischemia	Mild congestion	UR
в	60 male	Cardiac arrest	Neuronal necrosis, neuron loss, astrocytosis	Emphysema, ieft	Microscopic scars	UR	UR
9	52/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)

atient	Pancreas	Adrenal	Kidney	Heum	Colon	Lymph Node
1	Fibrosia	UR	Glomerulosclerosis	Autolysis	Autolysis	UR
2	UR	UR	Arteriolar sclerosis	UR	UR	Hyalinizing
3	Fatty infiltration	UR	Arterionephrosclerosis	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,	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	Peroductai fibrosis	UR	Arteriolar sclerosis	NA	Autolysis of epithelial cells but no	NA
9	UR	Fatty infiltration, inflammation	Arterioloscierosis	Autolysis of many epithelial cells, otherwise IIR	Autohysis of many epithelial cells, otherwise UR	UR
10	Fibrusis	UR	Arteriolar sclerosis	NA	NA	UR

No.	(sri/Gender	DX	Dram	Lung	neart		600	Splern
1	81/male	Pneumonia	UR	Acute inflaminiation	n UR	Mar	ked necrosis of	Apoptosis loss of
2	90/male	ARDS, renal fail	lure UR	DAD, interstitial inflammation	lachamic cardiomyopa	P UR Athy	ericentral hepatocytes	white pulp UR
з	85/female	Peritonitis, bro	ncho- NA	DAD	Interstitial fibr	osis Fatt	ty changes,	Inflammation
4	54/male	pneumonia Sepsis, GI bleed	ling UR	UR	Eosinophilic ir	inflaremation nfiltraty Panlobular congestion		NA
5	49/male	Sepsis, ARDS, r hepatic	enal NA	DAD	UR	Congestive changes,		Apoptosis, loss of white pulp
ō.	49/female	dysfunction MI, pneuroonia brain infarct,	Neutrophilic liver infiltration in	Bilateral broncho pneumonia	Changes of act	ate MI Mar	rked acute entrilobular hepatic	Acute congestion.
	Thinnals	disease	infarcted parenchyma	545	Proved instruction	0	ongestion/necrosis	pulp
	/@max	Pheomonia, sep	SIS UK	DAD	fibrosis	ai Pili o b	ongestion, mphocytic	infiltration
8	60-female	Pricumonia, sep	uia. UR	Interstitial fibrosis,	UR	EM	N infiltrate, patchy	Loss of white pulp
9	50/male	Cirrhosis, hepat C, peritonitis	itis UR	DAD UR	Interstitiai hemoerhage	h Cin n	epatocyte necrosis rhosis, coagulative secrosis, apoptosis	Apoptosis in marginal zone
10	26 James	ARDS service	18	DID servers	necrosis	hyocyte c lj Mili	mphocytic infiltrate	NA
10.5	aw Hittah	auro, sepsis	UR .	December	-OR	Phile	s congration	Anise and a
12	53/male	Anoxie injury	Pocal neuron necrosis	Broncho- pneumoraa	UR	Fibr	rosis, hyaline bodies, poptosis, necrosis studated processis	Apoptosis, loss of white pulp
12	ou male	bowel, ARDS	NA	DAD, congestion	UR Bergenstatus	Coa	guates nectors	Apoptosis
	escale	anud, sepus		New organizing	Parkanditis	in in	nflammation, bepatitis	- Andrease
14	rintemale	Pneurtionia, col	nis NA	preumonia	Fibrosis, old in	naret UR		UR
15	51/female	Peritonitis, seps	is NA	Interstitial infiltrat	ie UR	Fat	ly changes, periportal	NA
16	43/male	Pneumonia, isc bowel	bemic NA	DAD acute preumonia	Healed infarct	in Sub fe	nflammation massive necrosis with ocal apoptosis	Loss of white pulp
17	73/temale	Unosensos Alerte	S NA	DAD	üR	phil	d acute inflammation	Loss of white anite
18	50/male	Peritonitis, GI	NA	UR	Interstitui ma	conditis Acu	te inflammation.	Loss of white
19	28/male	bleeding Cardiac arrest,	sepsis NA	UR	NA	UR	ecrosis	pulp, apoptosis Focal apoptosis
20	75/male	Provincial market						20.2
ole T	7. Apper	adix-Table 2.	Detailed convent	ional light micr	roscopic (H&B	E) results i	ty changes, epabocycte dropout n septic patier Aorta	nts (summary Muscle
able 7 Pane R	7. Apper	Adressal	Detailed convent Kidney Mild glomerulosclerosis	ional light micr	Colon Apoptosis in crypts	E) results i	ty changes, epatocycte drupout n septic patier Aorta Athero- sclerosis	Muscle
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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).

# SPLEEN TUNEL



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 10. Immunohistochemical staining for caspase-3. A spleen for a septic patient with large immunoblastlike-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).



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.

# 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 per crypt) and in the lamina propria (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 nistologic 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).

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