Fas/Fas Ligand System Mediates Epithelial Injury, but Not Pulmonary Host Defenses, in Response to Inhaled Bacteria

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Received 15 February 2001/Returned for modification 26 March 2001/Accepted 21 May 2001

The Fas/Fas ligand (FasL) system has been implicated in alveolar epithelial cell apoptosis during pulmonary fibrosis and acute respiratory distress syndrome. However, Fas ligation can also lead to cell activation and cytokine production. The goal of this study was to determine the role of the Fas/FasL system in host defenses against Escherichia coli, Staphylococcus aureus, and Streptococcus pneumoniae. We administered bacteria by aerosolization into the lungs of Fas-deficient (lpr) mice and wild-type (C57BL/6) mice and measured bacterial clearance at 6 and 12 h. One hour prior to euthanasia, the mice received an intraperitoneal injection of human serum albumin (HSA) for alveolar permeability determinations. At all times after bacterial challenges, the lungs of the lpr mice contained similar or lower numbers of bacteria than those of the C57BL/6 mice. Alveolar permeability changes, as determined by bronchoalveolar lavage fluid HSA concentrations, were less severe in the lpr mice 6 h after the challenges. In response to E. coli, the lpr mice had significantly more polymorphonuclear leukocytes (PMN) and macrophage inflammatory protein 2 in the lungs, whereas histopathologic changes were less severe. In contrast, in response to the gram-positive cocci, the lpr animals had similar or lower numbers of PMN. We conclude that the Fas/FasL system contributes to the development of permeability changes and tissue injury during gram-negative bacterial pneumonia. The Fas/FasL system did not have a major role in the clearance of aerosolized bacteria from the lungs at the bacterial doses tested.

Fas-mediated apoptosis of alveolar pneumocytes is an important mechanism of alveolar epithelial injury in humans and animals with lung diseases (8, 11, 12, 26, 27). Fas (CD95) is a 45-kDa membrane surface receptor that belongs to the tumor necrosis factor alpha (TNF-α) family of proteins (9, 23). Binding of Fas to its natural ligand, Fas ligand (FasL), can trigger apoptosis via activation of caspase 8 (24, 25). FasL exists in a membrane-bound form and in a soluble form (sFasL), both of which are capable of activating Fas. Monoocyte-derived macrophages contain preformed sFasL, which can be rapidly released upon cell activation (10a). In addition to triggering apoptotic pathways and independently of its apoptotic function, the activation of Fas can also lead to NF-kB translocation and cytokine production (22). The relative contributions of the proapoptotic and proinflammatory functions of the Fas/FasL system to the pathogenesis of lung disease and the effects of inhibiting this pathway remain poorly understood.

A growing body of evidence suggests that the proapoptotic function of Fas is involved in the pathogenesis of alveolar epithelial injury during inflammatory lung diseases (19). In humans, the concentration of sFasL in lung fluids from patients with early acute respiratory distress syndrome (ARDS) is associated with mortality (19). Lung fluids from patients with ARDS induce apoptosis of distal lung epithelial cells by a Fas-mediated mechanism. Fas-mediated apoptosis of alveolar epithelial cells has also been reported for rodents and humans with pulmonary fibrosis (8, 11, 12, 26, 27). Activation of Fas in the lungs of mice results in apoptosis of alveolar epithelial cells and permeability changes (20). Furthermore, Kawasaki et al. have shown that the blockade of apoptosis with the broad caspase inhibitor Z-VAD.fmk improves survival in lipopolysaccharide (LPS)-treated mice (10). These findings suggest that the proapoptotic function of Fas is involved in tissue injury during lung disease. Therefore, specific anti-Fas agents could provide a useful therapeutic strategy to protect the lungs from inflammatory injury.

The potential role of the proinflammatory function of Fas in lung disease is less clear than that of the proapoptotic function of Fas. Alveolar macrophages and lung epithelial cells, which produce proinflammatory cytokines, express Fas on their surface membranes (2). Mice deficient in Fas fail to sustain a neutrophilic response following intranasal LPS administration (G. Matute-Bello, R. K. Winn, T. R. Martin, and W. C. Liles, Abstract, Am. J. Respir. Crit. Care Med. 161:A900, 2000). A recent study suggests that a Fas deficiency renders the host more susceptible to sepsis following the instillation of high doses of Pseudomonas aeruginosa (6). Thus, blockade of the Fas/FasL system to prevent alveolar epithelial cell apoptosis may also impair the inflammatory response and increase the susceptibility of the host to bacterial and other infections in the lungs.

The main goal of this study was to determine the role of the Fas/FasL system in lung clearance of gram-positive and gram-negative bacteria. Wild-type mice and mutant mice deficient in Fas (lpr) were exposed to aerosols of Escherichia coli, Staphy-
lococcus aureus, or Streptococcus pneumoniae and then studied over 12 h. The main findings were, first, that bacterial clearance was similar in lpr and wild-type mice, and second, that tissue damage and epithelial permeability changes were less severe in Fas-deficient, lpr animals.

MATERIALS AND METHODS

Bacteria. E. coli serotype K-1 was originally isolated from the blood of a patient with biliary sepsis. The methods used to passage and store the bacteria have been described elsewhere (17). The pathogenicity of these bacteria in C57BL/6 mice was verified in pilot experiments. For each experiment, a separate aliquot of the bacterial stock was thawed, inoculated into 2.0 liters of Luria broth (Gibco-BRL Laboratories, Gaithersburg, Md.), and incubated overnight at 37°C.

For 20 min, washed twice in phosphate-buffered saline (PBS), resuspended in 20 ml of PBS, and applied as an aerosol as described below. The bacterial concentration in the slurry and the clot was confirmed in each experiment by performing quantitative culturing with the pour plate method.

S. aureus was isolated from a patient with catheter-related sepsis. S. aureus from frozen stock was used to seed a blood agar plate and was incubated overnight at 37°C. Following incubation, 5 to 10 colonies were removed from the plate and used to seed 50 ml of Trypticase soy broth (Difco, Detroit, Mich.). The broth was incubated for 6 h at 37°C in a shaking incubator, transferred to a flask containing 1.0 liter of Trypticase soy broth, and incubated for 4 h at 37°C in a shaking incubator. Following incubation, the bacteria were washed three times in sterile PBS and resuspended in 20 ml of PBS for aerosolization.

S. pneumoniae serotype 3, ATCC 6303, was purchased from the American Type Culture Collection (ATCC). For preparation and storage of frozen aliquots, material from the original stock of S. pneumoniae obtained from the ATCC was used to seed a 5% sheep blood agar plate and incubated for 18 h at 37°C in 5% CO2. Following incubation, 5 to 10 colonies were transferred to 9.6 ml of Todd-Hewitt broth containing 16.7% fetal calf serum (FCS) (Difco). The mixture was incubated for 18 h at 37°C in 5% CO2, and then 3 ml was transferred to a flask containing 108 ml of Todd-Hewitt broth–16.7% FCS. The broth was incubated at 37°C in 5% CO2 until it reached an optical density at 620 nm of 0.25 (approximately 4 h). Aliquots (50 ml) of the broth were flash-frozen in liquid nitrogen and then stored at −70°C until used (1). The day before an experiment, an aliquot was thawed, used to seed a 5% sheep blood agar plate, and incubated for 18 h at 37°C in 5% CO2. Ten to 20 colonies were used to seed 100 ml of brain heart infusion containing 10% FCS and incubated for 18 h at 37°C in 5% CO2. Following incubation, 3-ml aliquots were transferred to 10-100 ml flasks containing brain heart infusion–10% FCS. After incubation for 6 h at 37°C in 5% CO2, the broth was pooled and spun at 10,000 × g for 30 min. The bacterial pellet was washed once in PBS, spun again, resuspended in 20 ml of PBS, and applied as an aerosol as described below.

Animal protocols. The animal protocol was approved by the Animal Care Committee of the Seattle Veterans Affairs Medical Center. Two strains of mice were used: C57BL/6 and B6.MRL-Fas−/− (lpr mice). All mice were male, weighed 20 to 30 g, and were obtained from Jackson Laboratories (Bar Harbor, Maine). The lpr mice, which have a C57BL6 background, carry a naturally occurring mutation which renders them deficient in cell membrane Fas.

The mice were placed in individual wire-mesh cages, which were fit into a closed aerosolization chamber (16). The bacterial suspension was placed in twin 10-ml nebulizers and applied as an aerosol at 15 B/min for 30 min. Thereafter, some mice were studied immediately (time-zero group) to determine the initial bacterial deposition and lung responses. The remainder were euthanatized at either 6 or 12 h for measurement of bacterial clearance. One hour before euthanasia, each mouse received an intraperitoneal injection of 1.0 mg of pyronegen-free human serum albumin (HSA) (Baxter, Deerfield, Ill.) in order to measure lung permeability. Euthanasia was performed with ketamine-xylazine given intraperitoneally. Following euthanasia, the thorax was rapidly opened and the animal was exanguinated by direct cardiac puncture. The trachea was cannulated with a 20-gauge catheter, the left hilum was clamped, and the left lung was removed and homogenized in 1.0 ml of PBS. The right lung was lavaged with five separate 0.5-ml aliquots of 0.9% NaCl–0.6 M EDTA at 37°C and then fixed by intratracheal instillation of 4% paraformaldehyde at 15 cm of H2O.

Samples were stored in 4°C for 30 min. The bacterial pellet was washed twice in phosphate-buffered saline (PBS), resuspended in 20 ml of PBS, and stored in separate aliquots at −70°C.

The left lung was homogenized as described below. An aliquot of the lung homogenate was cultured quantitatively. The remainder was stored at −70°C for cytokine, total protein, HSA, and myeloperoxidase (MPO) determinations. The right lung was stained with hematoxylin-eosin and analyzed by light microscopy.

The lungs were weighed and then homogenized in 1.0 ml of sterile distilled H2O using a hand-held homogenizer. An aliquot was used for quantitative bacterial culturing. The remainder of the homogenate was divided into aliquots for later cytokine and MPO measurements. For cytokine measurements, the homogenate aliquot was vigorously mixed with a buffer containing 0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, 1 mM CaCl2, and 1 mM MgCl2 (pH 7.4), incubated for 30 min at 4°C, and then spun at 10,000 × g for 20 min. The supernatants were stored at −70°C. For MPO measurements, the homogenate aliquot was vigorously mixed with 50 mM potassium phosphate (pH 6.0)–5% hexadecyltrimethyl ammonium bromide (Sigma Chemical Co., St. Louis, Mo.)–5 mM EDTA. The mixture was sonicated and spun at 12,000 × g for 15 min at 25°C. The supernatants were stored at −70°C.

Measurements. (i) Bacterial clearance. In experiments with E. coli, lung homogenates were cultured using 10-fold serial dilutions and the pour plate method with lung broth agar. In experiments with S. aureus and S. pneumoniae, quantitative culturing was performed by spreading serial 10-fold dilutions on 5% sheep blood agar plates.

(ii) Lung albumin permeability. Lung albumin permeability in lung homogenates was measured by an immunoenzyme assay using rabbit anti-human albumin immunoglobulin G (IgG) (Dako, Carpinteria, Calif.) as the capture antibody, horseradish peroxidase (HRP)-labeled anti-human albumin IgG (Dako) as the detecting antibody, and HSA (Albuminar-25; Centenex LLC, Kankakee, Ill.) as the standard. Briefly, a 96-well, flat-bottom, high-binding plate (Costar Corp, Cambridge, Mass.) was incubated overnight at 4°C with 0.14 μg of rabbit anti-human albumin IgG/ml in 25 mM Na2CO3 buffer (pH 9.5). After incubation, the plate was washed once with PBS containing 0.05% Tween 20 (Sigma) (PBS-T) and blocked for 1 h at 37°C with 0.2% BSA (Sigma) and HSA (Albuminar-25; Centenex LLC, Kankakee, Ill.) for 1 h at 37°C. The plate was washed three times with PBS-T, and the standards and samples were added to the plate and incubated for 1 h at 37°C. The plate was washed three times with PBS-T, and HRP-labeled rabbit anti-human albumin IgG was added to 0.26 μg/ml. Following incubation for 1 h at 37°C, the plate was washed three times with PBS-T and developed with 3.3’5’-tetramethylbenzidine-peroxide substrate solution (Kirkegaard & Perry Laboratories, Inc, Gaithersburg, Md.). After 20 min at 37°C, the reaction was stopped with 1.0 M phosphoric acid and read on a spectrophotometer at 450 nm. The antibodies were diluted in 0.2% BSA. This assay is insensitive to murine albumin, and no reactivity was detected with mouse serum.

(iii) Cytokine assays. MCP-1, MIP-2, and TNF-α levels in lung homogenates were measured using mouse-specific immunoenasys (R&D Systems, Minneapolis, Minn.). The sensitivities were 39.06 pg/ml for MCP-1, 7.81 pg/ml for MIP-2, and 19.5 pg/ml for TNF-α.

(iv) MPO. MPO levels in lung homogenates were measured by a colorimetric method with guaiacol as the substrate (Sigma). Briefly, 16.6 μl of peritoneal fluid was added to 1 ml of reaction buffer (0.4% H2O2–0.429 M guaiacol in 50 mM potassium phosphate buffer [pH 7]). The change in optical density at 470 nm was measured over 1 min on a spectrophotometer, and the slope was calculated to generate units per milliliter per minute.

Histopathological analysis. Each tissue slide was evaluated by two investigators, who were unaware of the treatment groups. The slides were graded according to the following criteria: no definite damage meant no histological changes or minor changes, including unequal distension of alveolar units, mild thickening of the alveolar septa, and perivascular and peribronchiolar edema; and definite damage meant lung consolidation, thickened alveolar septae, and the presence of intra-alveolar inflammatory infiltrates.

Statistical analysis. Comparisons between continuous variables were performed with Fisher’s exact test. Comparisons between nominal variables (histopathological data) were performed with the chi-square test.

RESULTS

All of the animals survived for the duration of the experiments. Both strains of mice cleared E. coli and S. aureus from the lungs over 12 h but failed to clear S. pneumoniae (Fig. 1). Following aerosolization of E. coli, bacterial recovery from the lungs of lpr mice was virtually identical to that from C57BL/6 mice at 6 and 12 h (Fig. 1A). Following aerosolization of S. aureus and S. pneumoniae, there were significantly fewer bacteria in the lungs of lpr mice at 6 h, but there were no differ-
ences in bacterial recovery at 12 h (Fig. 1B and C). Thus, Fas deficiency did not lead to higher bacterial loads in the lungs at any of the times studied.

The effect of Fas deficiency on polymorphonuclear leukocyte (PMN) recruitment and migration depended on the bacterial species. Neutrophil migration into the airspaces was assessed by BALF total PMN counts, whereas total pulmonary PMN counts were determined by measuring MPO activity in lung homogenates. Following E. coli aerosolization, the lpr mice had significantly more PMNs in BALF at 6 and 12 h (Fig. 2A) as well as higher levels of MPO activity in lung homogenates (Fig. 3A) than the C57BL/6 mice. In contrast, at 6 and 12 h after S. pneumoniae aerosolization, the lpr mice had fewer PMNs in BALF than the C57BL/6 mice and similar levels of lung homogenate MPO activity (Fig. 2C and 3C). These findings suggest that Fas deficiency reduced PMN migration into the airspaces in response to S. pneumoniae without affecting PMN entrapment in the lung vasculature. There were no significant differences in either BALF total PMN counts or levels of MPO activity at 6 or 12 h after S. aureus exposure (Fig. 2B and 3B).

The concentrations of the cytokines TNF-α, MIP-2 (a murine analog of melanocyte growth stimulating activity [GRO]), and MCP-1 were measured in lung homogenates using mouse-specific immunoassays (Table 1). The concentrations of TNF-α were similar in C57BL/6 and lpr mice at each of the times tested, regardless of the type of bacteria aerosolized. At 6 h following E. coli infection, the concentration of MIP-2 was significantly higher in the lpr animals than in the C57BL/6 animals (P = 0.03). There were no significant differences between the animal groups in MIP-2 concentrations at the other times. There was a trend toward lower concentrations of MCP-1 in the lpr animals than in the C57BL/6 animals. This trend reached statistical significance at 12 h following S. pneumoniae infection (P = 0.03).

Changes in alveolar permeability were assessed by measuring the BALF concentrations of HSA. HSA was administered intraperitoneally 1 h prior to euthanasia. The HSA concentra-

FIG. 1. : Bacterial clearance from the lungs of C57BL/6 (C57) and lpr mice, expressed as the percentage of bacterial recovery immediately after aerosol exposure. Bacterial deposition at time zero (T = 0) was 1.44 × 10^6 CFU/lung for E. coli, 1.81 × 10^8 CFU/lung for S. aureus, and 4.55 × 10^4 CFU/lung for S. pneumoniae. There were six or more animals in each group at each observation time. Data are means and standard errors and are expressed as bacterial recovery (CFU) per gram of lung. Asterisks indicate a P value of <0.05.
tions were higher in the BALF of the wild-type mice than in that of the Fas-deficient mice (Fig. 4). This trend reached statistical significance 6 and 12 h following *E. coli* exposure and at 6 h following *S. aureus* aerosolization.

Lung tissue preparations were evaluated in a masked fashion by two investigators. Each tissue slide was classified as showing either no definite damage or definite damage. Immediately following *E. coli* aerosolization, no animal showed evidence of tissue damage (Fig. 5A and B). Six hours later, five of six animals in the wild-type group showed definite tissue damage, versus one of six animals in the *lpr* group (Fig. 5C to F) (chi-square value, 0.02). There were no differences in tissue changes at any of the other times, regardless of the bacterial species.

**DISCUSSION**

The main goal of this study was to determine whether the Fas/FasL system plays a role in pulmonary host defenses against specific, clinically relevant gram-positive and gram-negative bacteria. C57BL/6 mice or Fas-deficient, *lpr* mice were exposed to aerosols of *E. coli*, *S. aureus*, or *S. pneumoniae*. For each bacterial species studied, bacterial clearance was the same or faster in the Fas-deficient, *lpr* animals. However, for each bacterial species studied, evidence of tissue damage was less severe in the Fas-deficient, *lpr* mice, measured as albumin permeability. Histological evidence of inflammation and epithelial damage was significantly milder in the *lpr* mice. The results show that the Fas/FasL system is not involved in bacterial clearance at the bacterial inocula used. However, the Fas system appears to have an important role in the tissue response to bacterial infection, and this is particularly true for *E. coli*.

Several lines of evidence suggest that the Fas/FasL system may be involved in host defenses. In addition to triggering apoptosis and independently of its proapoptotic function, Fas activation can also lead to NF-κB translocation and cytokine release (22). Rabbit alveolar macrophages and human bronchiolar epithelial cells produce interleukin 8 (IL-8) following...
Fas ligation (7; 18). Mice treated with intrapulmonary monoclonal antibody Jo2, a Fas agonist, develop neutrophilic infiltration and expression of inflammatory cytokines. In contrast, mice lacking Fas are insensitive to the Jo2 antibody and do not develop these changes (20). In rabbits, endobronchial instillation of human recombinant FasL results in the development of inflammatory infiltrates and increased expression of IL-8 by alveolar macrophages (18). Moreover, Fas-deficient

![Graphs showing MPO activities for C. coli, S. aureus, and S. pneumoniae](image)

**FIG. 3.** MPO activities (units per milliliter per minute) in lung homogenates from C57BL/6 (C57) and lpr mice at 0, 6, and 12 h following aerosolization of *E. coli*, *S. aureus*, or *S. pneumoniae*. Data are means and standard errors.

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<th>Cytokine</th>
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<td>TNF-α</td>
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<td>MCP-1</td>
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* The P value was <0.05 compared to the data for the C57BL/6 animals.
mice treated with intranasal LPS have lower numbers of BALF PMNs and lower BALF concentrations of MIP-2 than wild-type mice (Matute-Bello et al., abstract). These studies have raised the possibility that impairment of the Fas/FasL system could result in deficiency of the neutrophilic response and increased susceptibility to bacterial infections.

The present study shows that Fas deficiency does not impair the clearance of aerosolized *E. coli*, *S. aureus*, and *S. pneumoniae* from the lungs. At all times following aerosolization of bacteria, the pulmonary bacterial recovery in the Fas-deficient, *lpr* mice was either similar to or lower than that in the C57BL/6 mice, suggesting that membrane Fas does not contribute to bacterial clearance at the bacterial doses tested. In fact, in response to *E. coli*, the Fas-deficient mice recruited more PMNs into the airspaces than the wild-type mice. This response was associated with significantly higher concentrations in the lungs of the CXC chemokine MIP-2, a murine GRO analog which is a major neutrophil chemoattractant in mice. Interestingly, the neutrophilic response to the gram-positive cocci was different from the response to *E. coli*. Following gram-positive bacterial (*S. pneumoniae*) exposure, the Fas-deficient animals showed a trend toward slower PMN recruitment into the airspaces. However, the MPO activity measurements showed no significant differences between the strains of mice. These results suggest that both strains of mice recruited similar numbers of PMNs to the pulmonary vasculature and/or interstitium but that PMN migration into the airspaces was less efficient in the absence of Fas. The differences in BALF PMN numbers were not associated with significant differences in MIP-2 concentrations in BALF.

The different results from this study and previous studies show that the inflammatory responses elicited by live bacteria are more complex than the responses elicited by single agents, such as sFasL or LPS. Prior studies tested direct Fas activation using FasL, activating antibodies, or bacterial LPS. However, live bacteria may trigger inflammatory responses by activating
different and redundant pathways. For example, *E. coli* infection can result in PMN recruitment through activation of CD14-dependent pathways, with the generation of endogenous chemotactants, such as IL-8 (4, 28). Bacterial peptides also interact directly with the formyl-methionyl-leucyl-phenylalanine receptor on PMNs (15). In addition to changing PMN function, Fas deficiency could enhance PMN survival, as the Fas/FasL system is an important determinant of PMN apoptosis (13).

Despite the fact that the Fas-deficient mice recruited more PMNs in response to *E. coli*, they also showed less evidence of tissue injury, as assessed by BALF HSA determinations and histopathological analyses. The development of tissue injury was not due to direct effects of bacteria, because bacterial counts were similar in both strains of mice. In addition, tissue injury was not due to neutrophils, which were actually more numerous in the *lpr* mice. Therefore, tissue injury is associated with activation of the Fas system by a mechanism that remains
to be determined. These findings extend those of previous studies suggesting a role for Fas-mediated apoptosis in the pathogenesis of lung injury and alveolar wall damage secondary to apoptosis of endothelial and epithelial cells (5, 8, 19, 20).

The cellular events that mediate Fas-dependent alveolar epithelial injury in vivo need to be clarified. The alveolar epithelial cell line MLE-15 expresses Fas on the membrane surface and becomes apoptotic in response to the Fas agonist Jo2 (3, 5). The expression of Fas in alveolar epithelial cell lines seems to be controlled by transcription factor Sp3 (21) and modulated by the renin-angiotensin system (29). In mice, the administration of Jo2 leads to apoptosis of alveolar epithelial cells and pulmonary fibrosis (11). In humans, elevated concentrations of sFasL have been found in lung fluids from patients with ARDS, and BALF from patients with ARDS induces Fas-mediated apoptosis of primary human distal lung epithelial cells (19). Interestingly, the proapoptotic effect of the Fas/FasL system on the alveolar epithelium seems to be limited to sFasL, as cell-bound FasL fails to induce apoptosis of alveolar type II cells, either in cultures or in vivo (14). This finding is important because monocyte-derived macrophages rapidly release preformed sFasL upon activation, raising the possibility that in the lungs, activated macrophages could release sFasL (10a). Thus, the data suggest that Fas is involved in epithelial injury and raises the possibility that inhibiting the Fas system could be effective in limiting some types of lung injury.

A recent study by Grassme et al. suggested that Fas-mediated apoptosis of epithelial cells may have a role in preventing systemic dissemination of bacteria from the lungs (6). In that study, wild-type mice treated with intranasal P. aeruginosa developed apoptosis of airway epithelial cells. In contrast, Fas-deficient mice treated with the same organism did not show apoptosis of airway epithelial cells and had a higher incidence of sepsis and increased mortality during the week following bacterial exposure. The authors showed that the increased incidence of sepsis was not due to deficiencies of the immune system in the lpr mice, because chimeras with the lpr background and wild-type immune cells were not protected. The authors concluded that P. aeruginosa resulted in epithelial damage by apoptosis and postulated that the mechanism for the increased incidence of sepsis could be either that apoptosis of epithelial cells favors control of the infection or that triggering of inflammatory pathways by Fas activation is important for control of the infection. The results of the present study, although obtained using different protocols, suggest that the first mechanism is the most likely (apoptosis of epithelial cells favors control of the infection).

In conclusion, the data show that at the bacterial doses tested, the Fas/FasL system is not necessary for the clearance of E. coli, S. aureus, and S. pneumoniae from the lungs and that Fas contributes to pathological changes in lung tissue.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grants HL30542 and HL 62995 from the National Institutes of Health, by the American Heart Association of Washington (C.W.F.), by the Francis Families Foundation (C.W.F.), and by the Medical Research Service of the U.S. Department of Veterans Affairs.

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