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Staphylococcus aureus Pneumonia in Hamsters with Elastase-Induced Emphysema—the Virulence Enhancing Activity of Mucin (42698)

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Abstract. Staphylococcus aureus pneumonia was studied in hamsters with elastase-induced emphysema and in saline-treated controls. Emphysematous animals cleared endotracheally administered inocula of *S. aureus* in saline as rapidly as controls. After infection with *S. aureus* in 1% mucin, emphysematous animals had impaired clearance compared with controls; after infection with *S. aureus* in 5% mucin, emphysematous animals had decreased survival at 96 hr compared to controls (6/24 vs 15/24, $P < 0.01$ Fisher's exact test). Bronchoalveolar lavage of uninfected elastase-treated hamsters yielded twice as many cells per animal as uninfected controls ($P < 0.0001$, paired t test), and the cells contained a higher percentage of polymorphonuclear leukocytes (37.8% vs 3.8%, $P < 0.0001$). Lavage cells from both groups of animals were equally efficient per cell at killing opsonized *S. aureus* in an *in vitro* bactericidal assay. Hamsters with elastase-induced emphysema were resistant to infection with *S. aureus* alone despite marked structural abnormalities in the lung, possibly due in part to increased numbers of resident phagocytic cells. After infection with *S. aureus* in mucin as a virulence enhancing factor emphysematous animals had impaired clearance and decreased survival compared to controls.

Chronic obstructive pulmonary disease is associated with substantial morbidity and mortality in the elderly (1). Although the clinical impression of an increased incidence of infection in persons with emphysema is difficult to document (2–4), infections such as pneumonia and suppurative bronchitis can precipitate hospitalization and death in these patients (4). In practice, the effects of pure emphysema are often confounded by the effects of smoking, which alters inflammatory and immune effector cells and is associated with an increased incidence of upper respiratory infection (5, 6).

Current theories on the pathogenesis of emphysema suggest that noxious stimuli elicit an inflammatory response and recruit polymorphonuclear leukocytes into the lung. These cells in turn release elastase and other proteolytic enzymes which destroy connective tissue, leading to emphysema (1). In hamsters, peroral intratracheal administration of porcine elastase produces panacinar emphysema within 30 days. The lesions produced by human neutrophil elastase are not as severe (7). Attempts to increase the dose of neutrophil elastase to produce a more severe lesion result in the death of animals from pulmonary hemorrhage (8). The anatomic and functional derangements seen in these models have been described in detail (1, 7–9).

We utilized the porcine elastase-induced hamster model of emphysema to study the course of infection following peroral endotracheal challenge with *Staphylococcus aureus* (SA). Elastase-treated animals were compared to saline-treated controls with respect to *in vivo* clearance of SA, the histopathology of pneumonia, and the natural history of infection. Bronchoalveolar lavage (BAL) was performed to define the resident cell population in these animals before and after infection. The capacity of these cells to kill opsonized SA was assessed in an *in vitro* bactericidal assay.

Mucin was added as a virulence enhancing factor to create a more severe infection and thus accentuate the differences in *in vivo* bacterial clearance and survival between emphysematous animals and controls.

Methods. Adult male Golden Syrian hamsters (Engle Laboratory Animals, Inc., Farmersburg, IN) approximately 8 weeks of age and weighing 110 to 120 g were housed in individual cages and given food and water *ad*

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libitum. Animals received either porcine elastase (courtesy of Dr. Philip J. Stone, Boston University School of Medicine, Boston, MA) at a dose of 0.2 mg/0.5 ml of 0.15 *M* NaCl/100 g body weight, or 0.5 ml of 0.15 *M* NaCl/100 g body weight, by peroral endotracheal instillation as previously described (9). The method of extraction of porcine pancreatic elastase has been previously described (10, 11). The elastase was found to have less than 0.1% trypsin or chymotrypsin-like activity and active site titrations revealed the enzyme to be fully active. Elastase administration in the dose and manner described above consistently results in the formation of subpleural blebs visible to the naked eye; microscopically there is panacinar emphysema with increase in alveolar size that correlates with increase in the mean linear intercept and internal surface area. To allow full development of emphysema, all experiments were performed 30 days after elastase or saline administration. In selected experiments, normal untreated hamsters received lipopolysaccharide (4.0 μg/0.4 ml *Escherichia coli* serotype 026:B6; L-3755, Sigma Chemical Co., St. Louis, MO) (LPS); this was the minimal dose required to elicit the maximal yield of cells by bronchoalveolar lavage at 24 h.

Reynold, a clinical blood isolate of SA was provided by Dr. Walter Karakawa, Pennsylvania State University (College Park, PA) (12); rabbit antiserum was prepared as previously described (13). Organisms were grown in Columbia broth with 1% NaCl at 37°C for 6 hr, washed three times in phosphate-buffered saline (PBS), and resuspended in normal saline. The concentration of SA was adjusted by spectrophotometry to 10^8 cfu/0.2 ml and confirmed by plating serial dilutions. Porcine mucin (M-2378, Sigma) was prepared in normal saline (wt/vol) and the standard inoculum of 10^8 cfu in 0.4 ml was obtained by mixing equal volumes of SA with saline or mucin solutions as indicated. Porcine mucin is prepared from porcine stomach, has 75% mucin content, less than 0.1% *N*-acetylneuraminic acid before hydrolysis and 0.6% after hydrolysis, and the percentage mucin content is 1% lower in an alcohol-treated sample than an untreated sample. Periodic cultures of mucin solutions did not reveal bacterial or fungal contamination.

In vivo clearance and survival experiments were performed on sets of eight emphysematous (E) and eight control (C) animals for each inoculum. Animals were anesthetized by an intraperitoneal injection of methohexital sodium (Eli Lilly & Co., Indianapolis, IN), the inocula given by peroral endotracheal instillation (9) and the animals held vertically suspended for 5 min to allow uniform spread by gravity. Quantitative pour plate blood cultures were obtained by puncture of the retro-orbital plexus under CO_2 narcosis. Animals were sacrificed in a CO_2 chamber at 24, 48, 72, and 96 hr, the lungs dissected free and homogenized in an electric blender (Waring commercial blender, Dynamics Corp, New Hartford, CT) with distilled water to facilitate tissue lysis. Quantitative lung cultures were obtained by serial dilution and plating of the homogenate.

Bronchoalveolar lavage was performed by anesthetizing the hamsters in a CO_2 chamber and exsanguinating them by transection of the abdominal aorta. The trachea was exposed by a neck incision and cannulated and lavage was performed with 5-ml aliquots of PBS with heparin (10 U/ml) to a total volume of 30 ml. BAL recovery was aided by gentle massage and gravity; animals with grossly bloody lavage fluid were discarded. BAL fluids from five animals were pooled and centrifuged at 300*g* for 10 min at 4°C, the supernatant was decanted, and the cells were washed twice in cold Hanks' balanced salt solution (HBSS). After the final wash, the cell pellet was resuspended in 0.8 ml of HBSS containing 0.4% bovine serum albumin (A-7638, Sigma) (HBSS-BSA). Cells were counted in a hemocytometer and viability was determined by exclusion of 0.1% trypan blue dye. Cytocentrifuged cell preparations were stained with Wright–Giemsa stain (EM Science, Gibbstown, NJ) and a differential count was performed on 200 consecutive cells.

Bactericidal killing of *S. aureus* by BAL cells was performed in 12 × 75-mm polystyrene tubes (No. 2058, Falcon Plastics, Oxnard, CA) containing 0.7 ml of the cell suspension (7×10^6 cells/ml), 0.1 ml of a 1:10 dilution of heat-inactivated immune rabbit serum, 0.1 ml of fresh hamster serum as a source of complement, and 0.1 ml of bacteria. Organisms were grown in broth for 18 hr,

washed, resuspended in HBSS, and adjusted to give a bacteria to cell ratio of 2:1. Control tubes with no cells and no sera were also prepared. Prior to incubation, 0.1 ml of fluid was removed from each tube, added to 1 ml of 0.1% BSA in distilled water, vortexed thoroughly, held for 2 min to lyse white cells, and then serially diluted and plated to obtain a baseline bacterial count. The tubes were then gassed with 10% CO_2 and incubated at 37°C on a mechanical tumbler site. Samples (0.1 ml) were removed after 3 hr of incubation and processed as described.

Specimens for pathological examination were prepared by anesthetizing the animals with CO_2 narcosis and exsanguinating them by transection of the abdominal aorta. The trachea was cannulated and the lungs were insufflated with 5 ml of freshly prepared fixative (4% formalin and 1% glutaraldehyde in 0.1 *M* sodium cacodylate buffer). Heart and lungs were removed and fixed *en bloc* and mounted lung sections were stained with hematoxylin and eosin. Statistical analysis of data was performed on a microcomputer (Compaq Computer Corp., Houston, TX) using CRISP (Crunch Software Corp., San Francisco, CA) to perform paired *t* tests, Fisher's exact test, and analysis of variance (ANOVA) (14); $P < 0.05$ was considered significant.

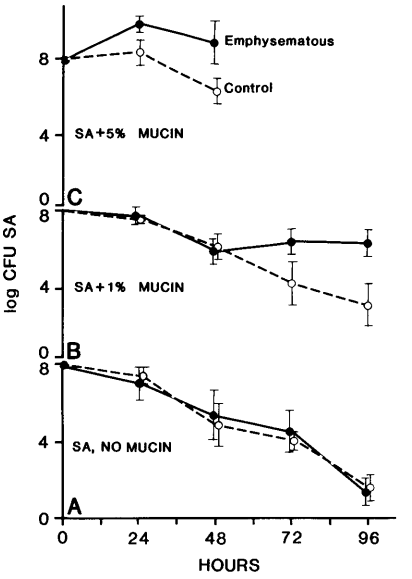

FIG. 1. Pulmonary clearance in emphysematous and control hamsters after inoculation with 10^8 cfu *S. aureus* (SA) in (A) saline, (B) 1% mucin, and (C) 5% mucin. With SA in 1% mucin, clearance was greater in control hamsters at 72 and 96 hr ($P = 0.03$, ANOVA). With SA in 5% mucin, clearance was significantly greater in control hamsters at 24 and 48 hr ($P < 0.1$, ANOVA); data not available beyond 48 hr because of spontaneous mortality. See text for details.

Results. In preliminary experiments normal hamsters were given 10^8 cfu of SA intratracheally. Among animals sacrificed immediately, the administered inocula were recovered quantitatively; however, the organisms were cleared rapidly, with 10^4–10^5 cfu recovered at 48 hr. The animals did not appear ill and were not bacteremic. SA in mucin at concentrations of 0.1 and 1% gave similar results. Following SA in 5% mucin, bacterial counts persisted for 48 hr and then fell. Animals given this inoculum appeared ill; some died spontaneously. Administration of 5% mucin alone caused the animals to appear ruffled and tachypneic for 24 hr; none died and the lungs of these animals were sterile.

In vivo clearance of S. aureus. Both E and C animals cleared inocula of 10^8 SA alone (Fig. 1). E animals receiving SA in 1% mucin showed normal clearance for 48 hr and then decreased clearance at 72 and 96 hr ($P = 0.03$, ANOVA) (Fig. 1). Emphysematous hamsters appeared listless and tachypneic with this infection while the controls appeared normal. Bacteremia was observed only in one E animal.

After challenge with SA in 5% mucin, bacterial counts increased acutely in E animals and were significantly greater than in C animals through 48 hr ($P < 0.01$, ANOVA) (Fig. 1). Spontaneous deaths in E hamsters beyond this time point prevented the collection of reliable clearance data. All animals appeared ill; bacteremia was found in one E animal at 24 hr and in one E and one C animal at 48 hr.

Natural history of SA with 5% mucin pneumonia. Hamsters were inoculated with SA in 5% mucin and survivors were counted daily. To avoid the potential for anesthesia contributing to deaths, these animals were not bled. All deaths among C animals occurred by Day 2, whereas E animals continued to die through Day 4 (Fig. 2). At that time survival was 63% (15/24) among C compared with 25% (6/24) among E (*P*

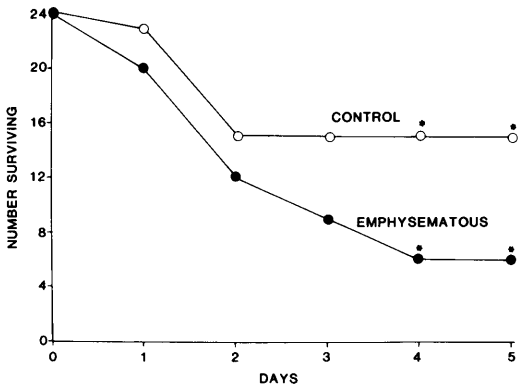

FIG. 2. Survival in emphysematous (E) and control (C) hamsters after inoculation with 10^8 cfu *S. aureus* in 5% mucin. Results were derived from three experiments each with eight E and eight C hamsters. There were no further deaths through Day 14. (*Survival greater in control hamsters, $P < 0.01$, Fisher's exact test.)

< 0.01, Fisher's exact test). There were no further deaths through Day 14 when the experiment was terminated.

Pathology of SA pneumonia. Multiple histological sections of both lungs were studied 24 hr after inoculation. The production of a uniform panacinar emphysema in elastase-treated animals was confirmed. Both E and C animals challenged with SA alone showed bronchopneumonia, without any apparent difference between the two groups; 5% mucin alone also resulted in patchy bronchopneumonia. SA with 5% mucin produced consolidation of the lung with an intense inflammatory infiltrate consisting predominantly of PMNs. E animals had greater necrosis and appreciably more early abscess formation than C animals.

Bronchoalveolar lavage. Thirty days after injection of saline or elastase, BAL of resting hamsters yielded 6.72 ± 0.11 $\log_{10}$ cells/animal from C and 7.00 ± 0.07 $\log_{10}$ cells/animal from E ($P < 0.0001$, paired t test) (Table I). Differential cell counts revealed 96.2% pulmonary alveolar macrophages (PAMs) and 3.8% polymorphonuclear leukocytes (PMNs) among BAL cells from C compared with 62.2% PAMs and 37.8% PMNs from E ($P < 0.0001$, paired t test). Twenty-four hours after challenge with SA in 5% mucin, C and E animals had similar numbers of BAL cells (7.12 vs 7.06 $\log_{10}$ cells/animal, respectively) with similar differentials (82% vs 85% PMNs). For comparison, the maximal response elicited from normal hamsters 24 hr after endotracheal LPS was 7.94 ± 0.12 $\log_{10}$ cells/animal; these cells were 91.3% PMNs. The PMN response could be elicited by SA alone in the absence of mucin (Table I).

In vitro bactericidal assay. Bactericidal assays were performed using BAL cells from unstimulated C and E animals; BAL cells elicited by LPS from normal hamsters served as positive controls. In three separate experiments, cells from C and E animals exhibited comparable bacterial killing at 3 hr ($86 \pm 4\%$ vs $81 \pm 8\%$, respectively). The LPS-elicited BAL cells gave $97 \pm 1\%$ kill. Control tubes without cells and either with or without antibody and complement had increasing bacterial counts.

Discussion. Bacterial pneumonia and influenza remain the leading infectious causes of death in the elderly (4). The measurable effects of aging on immunity (15, 16) seem less important in the development of pneumonia in the elderly than the high prevalence of underlying diseases, particularly chronic lung disease (4, 17–19). The clinical impression that patients with emphysema have an increased incidence of pneumonia is difficult to prove (2, 3). However, patients with emphysema who develop pneumonia clearly experience an increased morbidity and mortality (20, 21).

The elastase-induced emphysema model is useful for assessing the effects of emphysema without the confounding effects of smoking; the derangements in respiratory function produced (increases in compliance, functional residual capacity) are very similar to those seen in patients with emphysema (22). Infection in the hamster emphysema model has not been previously examined. In this study, both E and C animals quickly cleared an intratracheal challenge of 10^8 SA. Although previous studies in mice have shown brisk clearance of SA (23–25), the rapid elimination of SA by E animals was striking in light of their anatomical derangements.

BAL revealed that E hamsters in the resting state had twice as many cells per animal as C and a significantly higher percentage of PMNs (Table I). These findings, previously unreported, are consistent with the impression that there is persistent inflammation as-

TABLE I. BRONCHOALVEOLAR LAVAGE CELL YIELDS AND DIFFERENTIALS ELICITED FROM HAMSTERS AFTER DIFFERENT TREATMENTS AND STIMULI

Stimulus[a]	Prior treatment[b]	n	Log_{10} cells/ animal[c]	Differential[d]	
				% PAM	% PMN
None	Saline	9[e]	6.72 ± 0.11*	96.2	3.8
	Elastase	9[e]	7.00 ± 0.07*	62.2	37.8*
SA	Saline	9	7.01 ± 0.11	17.8	82.2
	Elastase	9	7.06 ± 0.07	18.1	81.9
SA + 5% mucin	Saline	4	7.06 ± 0.08	18.0	82.0
	Elastase	4	7.12 ± 0.09	15.0	85.0
LPS	None	9	7.94 ± 0.12	8.2	91.3
Mucin	None	9	6.87 ± 0.03	23.6	76.4

[a] Stimulus administered by peroral endotracheal instillation 24 hr before bronchoalveolar lavage. SA + 5% mucin, 10^8 cfu *S. aureus* in 5% mucin; SA, 10^8 cfu *S. aureus;* LPS, 4.0 μg *E. coli* lipopolysaccharide. See Methods for details.

[b] Prior treatment given by endotracheal instillation 30 days before stimulus. Elastase produced animals with emphysema; saline was given to controls. See Methods for details.

[c] Results expressed as means ± standard deviation.

[d] Differential counts of 200 cells on a Wright–Giemsa stain preparation.

[e] Nine experiments each, with a pool of five animals for each treatment.

* $P < 0.0001$, paired t test, comparing saline and elastase groups.

sociated with the evolution of the elastase-induced injury (22); similar cellular changes are noted on BAL in smokers with chronic bronchitis and emphysema (26). The BAL cells obtained from resting animals of both groups were equally efficient on a per cell basis in an *in vitro* bactericidal assay; the increased percentage of PMNs among the cells from E animals was not reflected in increased killing. Following bacterial challenge the total yields of BAL cells and the percentages of PMNs increased to comparable levels in both E and C animals (Table I). Thus, the BAL cells from E animals in the resting state were closer in number and differential to the BAL cells elicited during infection. The increased numbers of cells and the higher percentage of PMNs present in the alveolar spaces of E hamsters may therefore have conferred increased bactericidal capability *in vivo* that might partially offset the structural derangements present in these animals. This dual phagocytic system involving both PAMs and PMNs is considered important in pulmonary bacterial defense (27).

Mucin was added as a virulence enhancing factor to accentuate the differences between E and C animals. Bacterial pneumonia often begins by the microaspiration of colonizing pathogens along with oropharyngeal mucus secretions producing a bronchoembolus (4). Further, overproduction of bronchial mucus is part of the bronchoalveolar response to injury by inhaled irritants such as cigarette smoke and pollutants (28). The mechanical properties of porcine mucin may encourage bronchial plugging and atelectasis in a manner analogous to bronchial mucus. Chemically, however, porcine mucin does not compare with the complex protein and glycoprotein structure of bronchial mucus. Mucin has been previously used in animal models of pneumonia and other infections to enhance virulence (29–33). We confirmed that mucin is an effective virulence enhancing agent and used this effect to demonstrate a difference in susceptibility to infection between E and C animals; a difference that otherwise would not be apparent.

After inoculation of SA with 1% mucin, E hamsters demonstrated impaired late clearance of bacteria; given SA with 5% mucin, E animals had both acutely impaired clearance and increased mortality compared with C animals (Figs. 1 and 2). Histologically, E animals had lung necrosis and abscess formation, whereas C animals showed only consolidation. Thus, once infection was established, E animals were more severely affected. These results are congruent with the clinical evidence that emphysema does not necessarily make the host more susceptible

to infection, but that once infection is established, the presence of emphysema is associated with greater morbidity and mortality.

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