The Effect of Lysozyme on Elastase-Mediated Injury

JEROME O. CANTOR,[1]*† BRONISLAVA SHTEYNGART,* JOSEPH M. CERRETA,‡ AND GERARD M. TURINO*

*St. Luke's-Roosevelt Hospital Center, New York, New York; †Maimonides Medical Center, Brooklyn, New York; and ‡St. John's University, Jamaica, New York

Previous studies by this laboratory demonstrated that lysozyme is increased in human pulmonary emphysema, and that it preferentially binds to elastic fibers, which undergo degradation in this disease. In the current investigation, the relationship between lysozyme and elastic fiber injury was further examined, both *in vitro* and *in vivo*. The effect of exogenously administered egg-white lysozyme on pancreatic elastase-induced injury was determined using a biosynthetically radiolabeled extracellular matrix preparation mainly composed of elastic fibers. Although matrix treated with lysozyme showed attachment of the protein to elastic fibers, there was no significant increase in elastolysis compared with untreated controls following exposure to either 1 μg/ml or 100 ng/ml of pancreatic elastase. However, lysozyme did impair the ability of hyaluronan (HA) to prevent elastase injury to elastic fibers. Matrix samples sequentially treated with lysozyme and HA, then incubated with 1 μg/ml or 100 ng/ml of pancreatic elastase, showed significantly increased elastolysis compared with those treated with HA alone. Since HA is closely associated with elastic fibers *in vivo*, the ability of lysozyme to enhance elastolysis was further tested in an animal model of emphysema induced by intratracheal administration of porcine pancreatic elastase. Animals exposed to aerosolized lysozyme prior to elastase administration showed significantly increased airspace enlargement. The mean linear intercept of the lysozyme-treated animals was 123 μm compared with 75 μm for controls receiving aerosolized water ($P < 0.0001$). These findings suggest that lysozyme may not be an innocuous component of the inflammatory response associated with pulmonary emphysema, but may actually play a role in the pathogenesis of the disease.

[Exp Biol Med Vol. 227(2):108–113, 2002]

Key words: lysozyme; elastic fibers; elastase; emphysema

This work was supported by the Ned Doyle Foundation, by the Charles A. Mastronardi Fund, by the Franklyn Bracken Fund, by the James P. Mara Center for Lung Disease at the St. Luke's-Roosevelt Hospital Center, and by the Maimonides Research and Development Foundation.

[1] To whom requests for reprints should be addressed at Maimonides Medical Center, 4802 Tenth Avenue, Brooklyn, NY 11219. E-mail: JOCANTOR@pol.net

Received May 4, 2001.
Accepted October 8, 2001.

1535-3702/02/2272-0108$15.00

As previously shown by this laboratory, lysozyme was significantly increased in human lung tissues with pulmonary emphysema, and the protein was specifically associated with elastic fibers, which undergo breakdown in this disease (1). Furthermore, hyaluronidase treatment of normal lung tissue enhanced the binding of lysozyme to elastic fibers, suggesting that degradation of extracellular matrix components, as occurs in pulmonary emphysema, may expose binding sites for lysozyme on these fibers (1). Since lysozyme is a strongly cationic protein that binds to polysaccharides (2–4), this process could affect the structural and biochemical relationships between elastic fibers and other extracellular matrix components, thereby interfering with the repair process in pulmonary emphysema. In particular, impairment of the normal association between elastic fibers and hyaluronan (HA) could make such fibers more susceptible to degradation by elastases. HA has been shown by this laboratory to bind to elastic fibers and protect them from elastase-induced injury (5–8).

The current study further examines the potential role of lysozyme in the pathogenesis of pulmonary emphysema. Using a radiolabeled matrix preparation (5), lysozyme was tested for its effects on both elastase-induced injury to elastic fibers and the prevention of elastolysis by HA. Concomitant *in vivo* experiments studied the ability of an aerosolized preparation of lysozyme to alter airspace enlargement induced by intratracheal instillation of elastase. The results suggest that deposition of lysozyme in the lung extracellular matrix may enhance the progression of pulmonary emphysema.

Materials and Methods

Preparation of a Radiolabeled Cell-Free Tissue Culture Matrix. Rat pleural mesothelial cells, obtained from the American Type Culture Collection (Rockville, MD), were cultured in 75-cm^2 plastic flasks using Nutrient Mixture Ham's F-12 medium supplemented with 15% fetal bovine serum, 1% glutamine, 20 units/ml streptomycin, and 20 units/ml penicillin G. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells

and extracellular matrix were radiolabeled for 6 weeks with ^{14}C-lysine (6.25 μCi per flask). At the end of the labeling period, the cultures were washed with phosphate-buffered saline (PBS) and the cells were lysed with 0.5% sodium deoxycholate and EGTA. Following removal of the cellular material, the matrix was rinsed with PBS and was allowed to air dry. The plastic surface containing the radiolabeled matrix was then cut into 2 × 2-cm squares.

The Effect of Lysozyme on Elastolysis. Matrix squares were overlaid with 0.5 ml of 1 mg/ml hen egg-white lysozyme (Sigma Chemical, St. Louis, MO) in PBS for 30 min at room temperature. Controls were treated with PBS alone. Following removal of the liquid, the matrices were dried and incubated for 3 hr at 37°C with 0.5 ml of either 1.0 μg/ml or 0.1 μg/ml porcine pancreatic elastase (Elastin Products Co., Owensville, MO) in 0.1 M Tris buffer, pH 8.0. The liquid was then removed, combined with a single 0.5-ml PBS wash of the matrix, and measured for radioactivity in a liquid scintillation spectrometer. Results were expressed as net cpm (per matrix square) after subtracting background radioactivity released from samples treated with PBS and Tris buffer alone.

The ability of lysozyme itself to degrade elastic fibers was determined by incubating the radiolabeled matrix with either 1 mg/ml or 100 μg/ml of egg-white lysozyme in 0.5 ml of PBS at 37°C for 3 hr, and then measuring the release of radioactivity. The results of samples treated with PBS alone were used to calculate net cpm.

The Effect of Lysozyme on HA-Mediated Reduction of Elastolysis. Matrix squares were treated with egg-white lysozyme as described above, washed with PBS, and incubated with 1 mg/ml streptococcal HA (Carbo-Mer, Westborough, MA) in 0.5 ml of PBS for 30 min at room temperature. As in previous studies (6–8), a low-molecular weight (50–100 kDa) preparation of HA was used.

After removal of the liquid, the matrices were dried and incubated for 3 hr at 37°C with 0.5 ml of 1.0 μg/ml or 100 ng/ml of porcine pancreatic elastase (Elastin Products) in 0.1 M Tris buffer, pH 8.0. Controls were treated with PBS instead of lysozyme prior to incubation with HA and elastase. Release of radioactivity (net cpm) was determined as previously described.

The order of treatment with lysozyme and HA was then reversed. Matrix squares were first incubated with 1 mg/ml HA in 0.5 ml of PBS for 30 min at room temperature, then treated with lysozyme, exposed to 1.0 μg/ml of pancreatic elastase, and measured for release of radioactivity.

Determination of Lysozyme Binding to the Matrix. Matrix samples, prepared from cells grown on glass coverslips, were incubated with either 1 mg/ml egg-white lysozyme in PBS or with buffer alone (controls) for 30 min at room temperature. After washing the coverslips with PBS, the presence of lysozyme was determined immunohistochemically. The samples were fixed in acetone, treated with rabbit serum for 30 min, washed with PBS, and then incubated with rabbit anti-lysozyme (hen egg-white) antiserum (Chemicon International, Temecula, CA) for 1 hr and again washed with PBS. Following treatment with goat serum for 30 min, a secondary, fluorescein-labeled goat anti-rabbit IgG antibody was applied for 1 hr. The matrix samples were then washed with PBS, mounted on glass slides, and examined with a fluorescence microscope.

Identification of Elastic Fibers in the Matrix. Matrix elastic fibers were identified by immunofluorescence using a primary goat anti-rat lung α-elastin antibody (Elastin Products) and a secondary, fluorescein-labeled rabbit anti-goat IgG antibody (Zymed Laboratories, San Francisco, CA). Matrix samples, prepared from cells grown on glass slide coverslips, were fixed in acetone, treated with goat serum for 30 min, and washed with PBS. The samples were then incubated with goat anti-rat lung elastin antiserum for 1 hr and again washed with PBS. After treatment with rabbit serum for 30 min, a secondary, fluorescein-labeled rabbit anti-goat IgG antibody (Zymed Laboratories) was applied for 1 hr. The matrix samples were then washed with PBS, mounted on glass slides, and examined with a fluorescence microscope.

The Verhoeff-Van Gieson stain was also used to determine the presence of matrix elastic fibers on the coverslips and to assess loss of these fibers following treatment with HA and elastase or elastase alone.

Exposure of Elastase-Treated Animals to Aerosolized Lysozyme. Syrian hamsters, weighing approximately 100 g, were placed inside a dual-port Plexiglas chamber and were exposed to aerosolized egg-white lysozyme (20 mg in 20 ml of water) for 50 min via a Whisper-Jet nebulizer (Marquest Medical Products, Englewood, CO) attached to a compressed air source. Controls were exposed to 20 ml of water alone for 50 min. Thirty minutes following aerosolization, the animals were anesthetized with ketamine and were instilled intratracheally with 40 units of porcine pancreatic elastase (Elastin Products) dissolved in 0.2 ml of normal saline solution. The elastase was delivered into the trachea via a 26-gauge needle mounted on a 1-ml syringe.

The animals were sacrificed 1 week following aerosol treatment, and their lungs were fixed *in situ* by inserting a catheter into the trachea and instilling 10% neutral-buffered formalin at a pressure of 20 cm H_2O. After 2 hr, both the lungs and the heart were removed from the chest as a single block and were additionally fixed in 10% formalin for several days. The lungs were then dissected free of extraparenchymal structures, sectioned randomly, and processed for histology. Slide sections stained with hematoxylin and eosin were coded, and mean linear intercept measurements were made by an experienced morphologist (J.M.C.), according to published procedures (9).

Identification of Aerosolized Lysozyme in the Lung. Syrian hamsters were exposed to either chicken egg-white lysozyme or water (controls) as previously described. The animals were sacrificed either 30 min or 24 hr after exposure, and their lungs were fixed *in situ* and were

processed for histology. Slide sections were immunostained for lysozyme and were examined with a fluorescence microscope. Additional sections were stained with hematoxylin and eosin to determine possible inflammatory changes in the lungs.

Statistical Analysis, The two-sample t test was used to determine statistically significant differences ($P < 0.05$) between two treatment groups. Analysis of three or more treatment groups was performed with the Newman-Keuls multiple comparisons test.

Results

***In Vitro* Studies.** The rat pleural mesothelial cells used in these studies were previously shown to abundantly synthesize elastin and form a matrix largely composed of elastic fibers (5, 10). Immunofluorescence studies, using anti-elastin antibodies, demonstrate the variability and complexity of this fiber network (Fig. 1).

Matrix treated with lysozyme and subjected to immunofluorescence studies with anti-lysozyme antibodies revealed a similar pattern of fluorescence, demonstrating that lysozyme binds to these fibers (Fig. 2). No significant immunofluorescence was seen in matrix samples that were not pretreated with lysozyme.

To determine if such binding protects elastic fibers from injury, radiolabeled matrix samples were treated with lysozyme, and were then exposed to 1 μg/ml or 100 ng/ml of pancreatic elastase. Release of radioactivity was not significantly affected by lysozyme treatment at either elastase concentration, indicating that the protein does not reduce the susceptibility of elastic fibers to degradation (Fig. 3). Indeed, exposure to lysozyme resulted in a small increase in elastolysis. However, treatment of the matrix with lysozyme alone produced no significant release of radioactivity from radiolabeled matrix (less than 50 cpm with 1.0 mg/ml lysozyme; $n = 4$), demonstrating that the protein itself does not degrade elastic fibers.

Since HA was previously shown to bind to this matrix and reduce elastase injury (5), lysozyme was tested for its capacity to alter this protective effect. As in previous studies, treatment of radiolabeled matrix with HA significantly decreased elastase-induced release of radioactivity compared with untreated controls (Fig. 3). A 31% reduction was seen with 1 μg/ml of pancreatic elastase (2325 vs 3353 cpm; $P < 0.05$) and a 60% decrease was seen with 100 ng/ml of the enzyme (596 vs 1479 cpm; $P < 0.05$). The diminished release of radioactivity correlated with increased numbers of intact elastic fibers in the HA-treated matrix samples compared with untreated controls (Fig. 4).

Radiolabeled matrix exposed to lysozyme prior to treatment with HA did not show a similar decrease in elastolysis and, in fact, produced a significant increase in release of

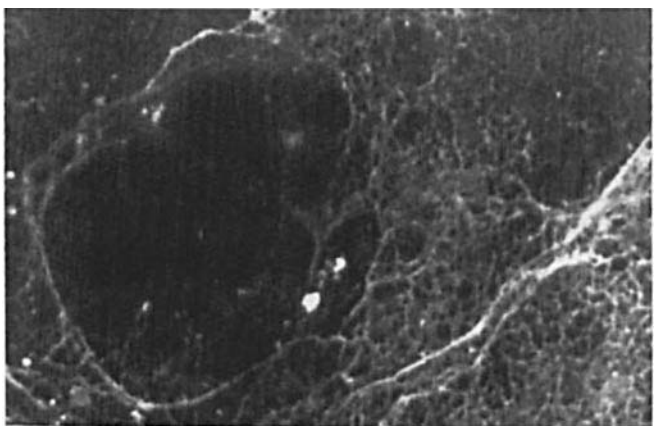

Figure 1. Immunofluorescence studies with anti-elastin antibodies revealed a complex pattern of matrix elastic fibers. Original magnification: ×200.

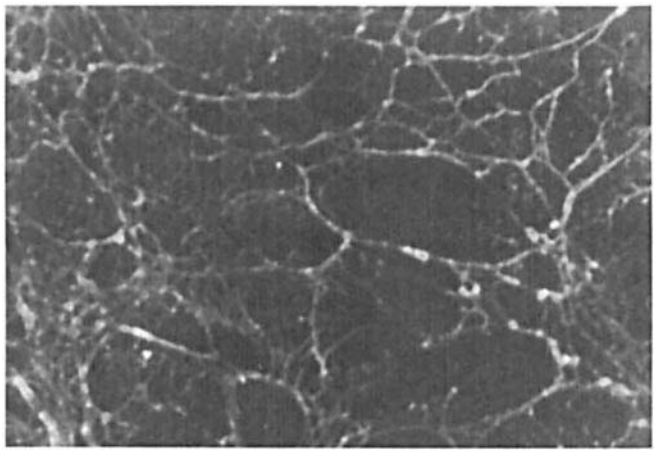

Figure 2. Immunofluorescence studies with anti-lysozyme antibodies, performed after treatment of the matrix with lysozyme, revealed a pattern of fluorescence similar to that seen with anti-elastin antibodies (Fig. 1), demonstrating that lysozyme binds to elastic fibers. Original magnification: ×400.

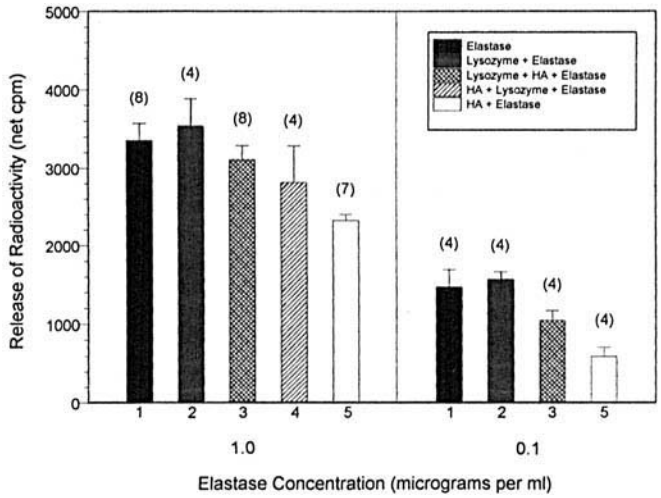

Figure 3. Treatment of radiolabeled matrix with lysozyme, HA, or a combination of both agents produced different effects on elastolysis, as measured by release of radioactivity. Although lysozyme did not significantly affect elastic fiber breakdown by pancreatic elastase (bar 1 vs 2), it did interfere with the protective effect of HA (bars 3 and 4 vs 5). However, a significant increase in elastolysis ($P < 0.05$) was observed only when lysozyme was applied prior to HA (bar 3 vs 5). Figures in parentheses refer to number of samples tested. T-bars indicate SEM.

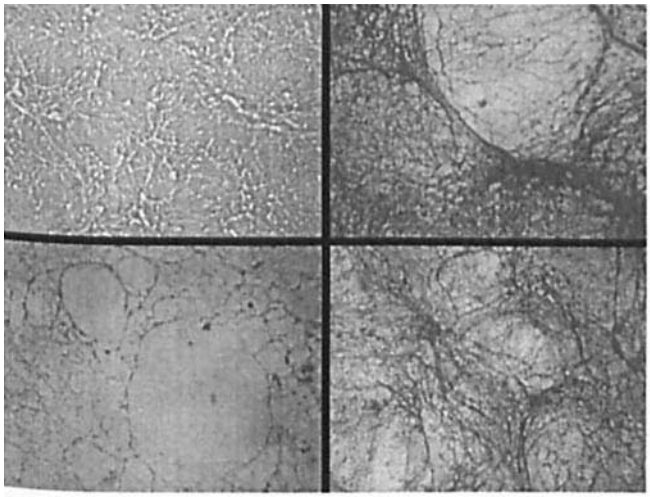

Figure 4. (Upper left) Photomicrograph of unstained, cell-free matrix preparation showing variable pattern of matrix deposition. (Upper right) Matrix elastic fibers stained with Verhoeff-Van Gieson (VVG) stain. (Lower left) Matrix treated with elastase showing marked loss of elastic fibers (VVG stain). (Lower right) Matrix pretreated with HA prior to elastase exposure showing protective effect of HA (VVG stain). Original magnification: ×200.

radioactivity compared with samples treated with HA alone (Fig. 3). The loss of protection was noted with both 1 μg/ml elastase (3106 vs 2325 cpm; $P < 0.05$) and 100 ng/ml of the enzyme (1055 vs 596 cpm; $P < 0.05$). Application of lysozyme following treatment of the matrix with HA was less effective in counteracting the protective effect of HA. The increase in elastolysis compared with samples treated with HA alone was not statistically significant (2816 vs 2325 cpm; Fig. 3).

***In Vivo* Studies.** Hamsters exposed to aerosolized lysozyme for 50 min showed no inflammatory lung changes at 24 hr. Immunofluorescence studies using anti-lysozyme antibodies demonstrated that the exogenous lysozyme was present in the lung 30 min after completion of the aerosol exposure and could still be detected at 24 hr (Fig. 5). The fluorescence was seen diffusely throughout the lung and did not appear to be specifically associated with a particular tissue component. However, an increase in fluorescence was noted within alveolar macrophages at 24 hr. Control animals receiving aerosolized water alone did not show significant lung fluorescence.

Exposure to aerosolized lysozyme greatly enhanced airspace enlargement induced by intratracheal instillation of pancreatic elastase (Fig. 6). The mean linear intercept of animals treated with lysozyme prior to elastase administration was 64% larger than that of controls receiving aerosolized water (123 [$n = 6$] vs 75 μm [$n = 5$]; $P < 0.0001$).

Discussion

In an earlier study from this laboratory, tissue sections from normal, fibrotic, and emphysematous human lungs were evaluated for differences in lysozyme content (1). An increase in extracellular lysozyme was specifically observed in lung tissues with pulmonary emphysema, and the protein was preferentially associated with elastic fibers (1). Similar findings have been reported in sun-damaged skin where changes in elastic fibers are also observed (11–13).

The most likely sources of the increased lung lysozyme content in emphysema are inflammatory cells, which are recruited to the lung in this disease. Endogenous lung cells also secrete lysozyme (e.g., alveolar epithelium), but in our earlier investigation of human emphysematous lungs, intracellular immunostaining for lysozyme was mainly associated with alveolar macrophages (1, 14).

The attachment of lysozyme to elastic fibers may be related to its strong cationic charge. The protein binds to anionic molecules in the extracellular matrix (2–4), including polysaccharides that are associated with elastic fibers such as HA (15, 16). *In vitro* studies have shown that lysozyme limits the self-aggregation of HA (17).

Functionally, the lysozyme proteins from different species appear to be similar, but antibody cross-reactivity may vary (18). In a previous investigation, we found cross-reactivity between egg-white and human lysozyme using

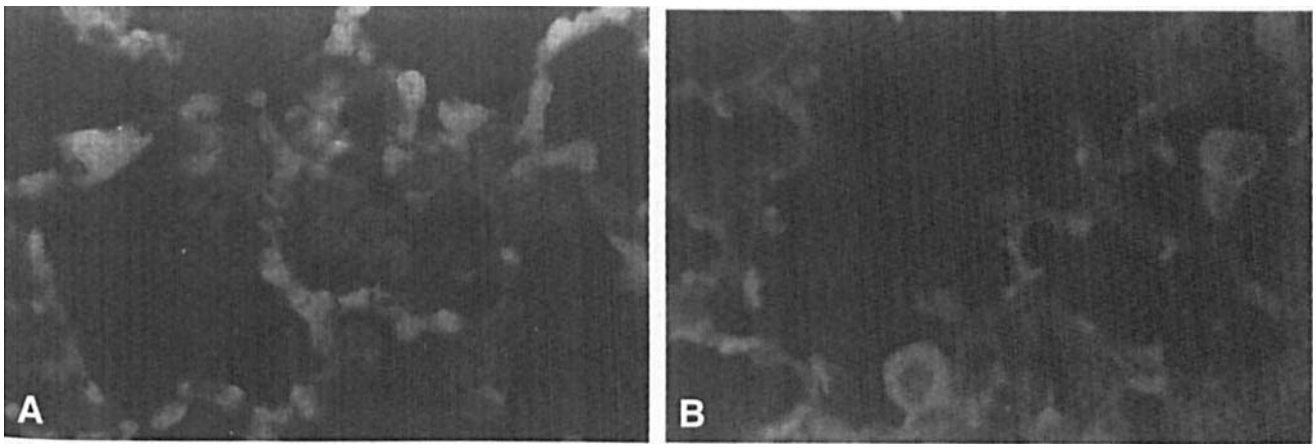

Figure 5. (A) Immunofluorescence studies using anti-lysozyme antibodies demonstrated that inhaled lysozyme was present in the pulmonary interstitium 30 min after completion of the aerosol exposure. (B) The fluorescence was still visible at 24 hr, especially within alveolar macrophages. Original magnification: ×400.

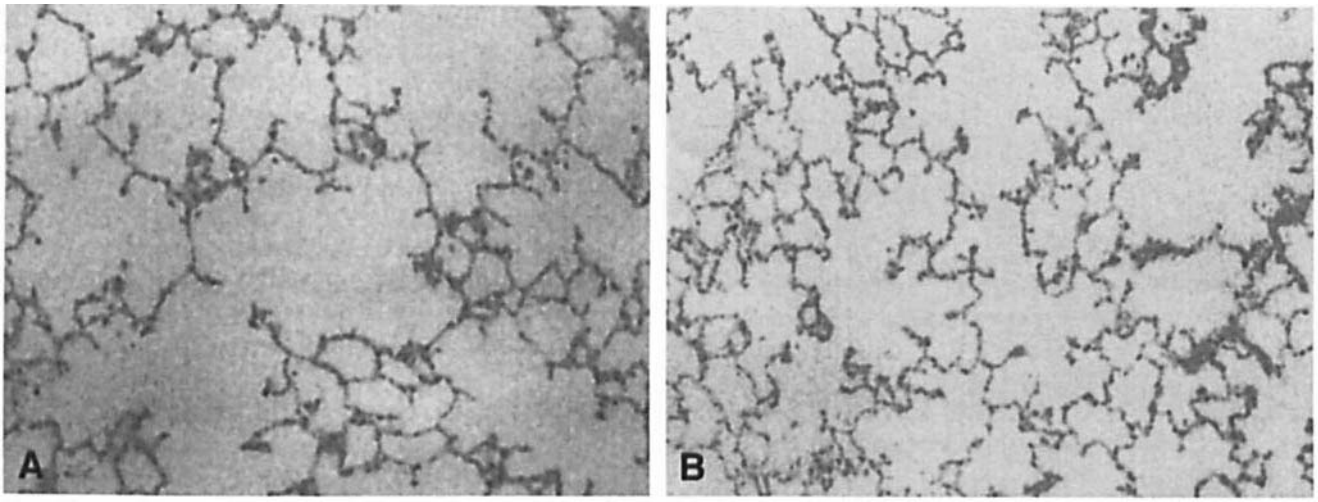

Figure 6. (A) The lungs of animals exposed to aerosolized lysozyme prior to elastase instillation showed markedly dilated airspaces. (B) In contrast, animals receiving aerosolized water prior to elastase instillation had significantly less airspace enlargement. Original magnification: ×100.

polyclonal anti-human lysozyme antibodies (1). However, others have reported little or no cross-reactivity with certain antibody preparations, and cross-reactivity may be dependent on the specific characteristics of the anti-lysozyme antibodies (18). Since cross-species functionality is preserved, we believe it is valid to use egg-white lysozyme. Studies using intratracheally administered egg-white lysozyme to treat chronic bronchitis suggest that the protein does retain activity in the respiratory tract (19). Furthermore, the binding of lysozyme to elastic fibers may be more related to its cationic or enzymatic nature than its antigenicity.

The current studies using the elastase model of emphysema support the concept that attachment of lysozyme to elastic fibers may increase their susceptibility to injury. In this experimental model, damage to elastic fibers results in a loss of lung recoil, leading to airspace dilatation and rupture of alveolar walls. Exposure of animals to aerosolized lysozyme greatly increased airspace enlargement in experimental emphysema induced by intratracheal instillation of elastase. This effect was not due to direct degradation of elastic fibers by lysozyme because the protein itself did not have any elastolytic activity *in vitro*. Furthermore, pretreatment of the elastic fiber matrix with the protein did not significantly increase elastase injury. Such treatment did, however, reduce the protective effect of exogenously administered HA, suggesting that lysozyme may increase the susceptibility of elastic fibers to injury by altering their relationship with surrounding matrix constituents.

Immunofluorescence studies using anti-lysozyme antibodies indicated that lysozyme quickly enters the lung interstitium following aerosolization. This finding is consistent with other studies that have shown that molecules much larger than lysozyme (which has a molecular weight of approximately 14,500 Daltons) can rapidly cross the alveolar epithelial barrier (20, 21). Consequently, lysozyme would already be present when elastase was intratracheally instilled and it could readily bind to the matrix as lung injury progressed. Since the amount of HA in the lung rapidly increases following elastase instillation (22), it is possible that the primary effect of this protein is to interfere with the attachment of newly synthesized HA to elastic fibers, thereby increasing the accessibility of the fibers to elastase. This process might be similar to what was observed in the radiolabeled matrix studies, where lysozyme was more effective against HA that had not become bound to elastic fibers.

In contrast to the findings of the current investigation, previous work by another laboratory has shown that lysozyme prevents elastase-induced degradation of elastin, the main protein component of elastic fibers (23). In that study, it was proposed that lysozyme prevents elastolysis by competing with elastase for anionic binding sites on elastin. Since intact elastic fibers were not used as a substrate, it is difficult to compare the results of that investigation with the present findings. Lysozyme may interact differently with elastin than with elastic fibers due to the absence of surrounding matrix constituents. This concept is supported by previous studies from this laboratory demonstrating that hyaluronidase treatment of lung tissues increases the attachment of lysozyme to elastic fibers (1).

Aside from affecting the matrix surrounding elastic fibers, lysozyme may possibly increase lung injury by stimulating the inflammatory response to elastase. Although aerosolized lysozyme alone did not cause lung inflammation, it might act in concert with elastase to increase the influx of neutrophils and monocytes into the pulmonary interstitium. Specifically, the ability of lysozyme to bind to polyanionic compounds might inactivate elastase inhibitors such as heparin and heparin sulfate (24, 25). The resulting increase in elastic fiber breakdown would then attract more inflammatory cells into the lung because elastin fragments are known to be chemotactic (26–28). Such a self-propagating mechanism of injury could greatly amplify airspace enlargement.

Further understanding of the mechanism of action of

lysozyme will be important in determining its effect on potential treatments for pulmonary emphysema. Although most therapeutic approaches have focused on the imbalance between elastases and their inhibitors (29–32), this mechanism may only represent one component in the spectrum of emphysematous lung injury. By increasing the susceptibility of elastic fibers to degradation or by interfering with the resynthesis of these fibers, lysozyme could alter the balance between elastic fiber injury and repair, thus limiting the effectiveness of elastase inhibitors and other potential therapeutic agents.

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