Subcellular Localization and Further Characterization of a New Elastase Inhibitor from Pneumococci

MICHAE VERED,1 SANFORD R. SIMON,2 AND AARON JANOFF1*

Departments of Pathology1 and Biochemistry,2 State University of New York, Stony Brook, New York 11794

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Streptococcus pneumoniae contains an inhibitor of human neutrophil elastase. The agent does not inhibit other proteases, including neutrophil cathepsin G and pancreatic elastase. It is active in the presence of insoluble elastin as well as synthetic elastase substrates. The inhibitor is present in the pneumococcal cell membrane. [125I]elastase binding studies and inhibition experiments with intact bacterial autolysates suggest that this agent has its elastase-binding site(s) exposed on the outside of the bacterial cell membrane. Native and randomized membrane vesicles also show equal inhibitory activity. Active inhibitor can be solubilized from pneumococcal membranes by treatment with a dipolar ionic detergent and can then be reconstituted, in active form, within artificial liposomes. Complex formation between the neutrophil elastase inhibitor and neutrophil elastase may involve noncovalent interactions. Although elastase containing a covalently bound substrate analog no longer binds the pneumococcal inhibitor, the present study shows that complex formation is nevertheless independent of neutrophil elastase catalytic activity. Specific inhibitor activity and inhibitor release during bile salt-stimulated autolysis are greater in a nonnecrotizing pneumococcal strain (type I) than when they are in a necrotizing strain (type III) or in Klebsiella pneumoniae. These results may help explain the frequent resolution of some pneumococcal pneumonias, despite the presence in the early pneumonic exudate of many neutrophils containing an elastolytic protease capable of injuring lung connective tissue.

We previously reported finding an inhibitor of human neutrophil elastase in extracts of Streptococcus pneumoniae and some other pyogenic bacteria (25). The pneumococcal agent acted non-competitively, behaved like a high-molecular-weight substance in gel-permeation chromatography, and inhibited neutrophil elastase, but not pancreatic elastase or trypsin. Because neutrophil elastase can digest elastin (11), types III and IV collagen (17), proteoglycans (12), and fibronectin (18), it has been implicated as a mediator of connective tissue injury in many inflammatory disorders (10). We therefore reasoned that the microbial elastase inhibitor might be one factor affecting the degree of tissue injury associated with pyogenic infections. Indeed, crude extracts of pneumococci can protect mice against acute lung injury induced by human neutrophil granule extracts (24).

To better understand the potential role of this inhibitor in protecting host tissue against neutrophil-mediated injury, we wished to determine the subcellular localization of the agent in pneumococci so as to evaluate its ability to interact with neutrophil protease in pneumonic exudates. We also wished to compare the specific activity of pneumococcal inhibitor and its release from necrotizing and nonnecrotizing pneumococcal strains. In the present report we address these two questions and provide further characterization of the inhibitor’s specificity, its mechanism of complex formation with leukocyte elastase, and its interaction with that enzyme in the presence of insoluble elastin.

MATERIALS AND METHODS

General procedures. Sources of bacteria, culture conditions, methods for harvesting and washing of bacterial cells, and enzyme inhibition assays have been described in detail in our previous report (25).

Localization of the pneumococcal inhibitor. (i) Subcellular fractionation of pneumococci. In some experiments, washed pneumococcal cells were incubated in protoplast buffer (0.05 M Tris [pH 7.5] containing 0.625 M sucrose and 0.01 M MgCl2) for up to 5 h at 37°C to activate cell wall autolysis (13). The lysed cell wall products and mesosomes were recovered as the supernatant fraction after centrifugation at 35,000 × g for 15 min. The centrifuged pellet, representing intact autolysates preserved in high-osmolarity sucrose, was then suspended in a low-ionic-strength solution (0.01 M Tris, pH 8.0) to induce osmotic shock and cell lysis. The resultant fraction contained membrane vesicles and cytoplasmic constituents.

Alternatively, pneumococci suspended in phosphate-buffered saline (pH 7.0) were subjected to mechanical disintegration by 15 1-min cycles of high-speed rotary mixing in the presence of 0.2-μm-diameter glass beads (VWR Scientific Inc., South Plainfield, N.J.) (6). After the homogenate was centrifuged at 35,000 × g for 10 min, the pellet, containing primarily ruptured cell walls and some unbroken cells, was suspended and sonicated (sonifier cell disruptor; Heat Systems-Ultrasound, Inc., Plainfield, N.Y.). The supernatant of the original homogenate, containing cytoplasmic constituents, mesosomes, and membrane fragments, was subjected to centrifugation (160,000 × g for 3 h) to separate membranes plus mesosomes (high-speed pellet) from soluble cytoplasmic components (high-speed supernatant). The membrane pellet was then suspended and subjected to sonication.

All fractions obtained with either of the above methods were finally normalized by dilution to constant volume (the starting autolysate volume or the volume of original homogenate). The fractions were then analyzed for inhibitory activity against neutrophil elastase and for protein content.

(ii) Analysis of subcellular fractions. The inhibitory activity of pneumococcal cell fractions against pure human neutrophil elastase (Elastin Products Co., St. Louis, Mo.) was determined in a spectrophotometric, kinetic assay with
succinyl-trialanyl-p-nitroanilide as the substrate (1). Protein concentration of the same cell fractions was measured by the method of Lowry et al. (16) with bovine serum albumin as the standard.

(iii) Preparation of pneumococcal autoplasts, "native" membrane vesicles, and "randomized" membrane vesicles. To study the availability of inhibitory sites for neutrophil elastase on intact pneumococcal autoplasts, the latter were prepared by incubation of washed bacteria in protoplast buffer (see above) (13), followed by centrifugation to collect the protoplasts freed of walls and capsules. A portion of these autoplasts was suspended in protoplast buffer and received no further treatment (intact autoplasts). A second portion was suspended and sonicated in protoplast buffer to form membrane vesicles containing integral proteins primarily in their native orientation (5). A third portion was suspended and sonicated in low-ionic-strength Tris buffer (0.01 M, pH 8.0) to produce membrane vesicles containing randomly oriented integral proteins (5). Both kinds of membrane vesicles were then collected by high-speed centrifugation (160,000 × g, 3 h) and suspended in their respective buffers (with and without 0.625 M sucrose). They were diluted to the same volume as the intact autoplasts, and all three preparations were then used immediately.

(iv) Studies on binding of labeled elastase by pneumococcal autoplasts. The availability of specific enzyme-binding (inhibitory) sites was also tested by measuring the uptake of radiolabeled neutrophil elastase by pneumococcal autoplasts. Uptake of radiolabeled pancreatic elastase served as a control for nonspecific binding, since the activity of pancreatic elastase is not affected by the pneumococcal inhibitor (25). For purpose of radiolabeling, neutrophil elastase and pancreatic elastase (Elastin Products Co., St. Louis, Mo.) were each reacted with Na125I (Research Products International Corp., Mt. Prospect, III.) that had first been treated with Na25I (Research Products International Corp., Mt. Prospect, Ill.) according to the manufacturer’s directions. The final specific activities were 3.1 and 1.3 μCi/nmol for the neutrophil and pancreatic enzymes, respectively. The activity of each enzyme versus succinyl-trialanyl-p-nitroanilide remained unaffected by the labeling procedure.

Various amounts of type I autoplasts were determined by protein assay were incubated for 30 min at 37°C with either 0.25 nmol of the labeled pancreatic enzyme or with 0.17 nmol of labeled neutrophil elastase. In some neutrophil elastase incubation tubes, a 20-fold excess of unlabeled neutrophil enzyme was added to test for displacement of the labeled elastase. As a control for nonspecific competition, other such tubes contained a 20-fold excess of unlabeled pancreatic elastase. After incubation, cells were sedimented at 35,000 × g for 15 min, and radioactivity was counted in both supernatants and pellets.

(v) Solubilization and reconstitution of the membrane inhibitor. Pneumococcal membrane pellets were prepared by high-speed centrifugation of the cytoplasmic fraction of mechanically disintegrated cells (see above). The membrane vesicles were suspended and incubated for 20 min at 37°C in 0.01 M Tris buffer (pH 8.0) containing 0.15 M NaCl and 0.01 M CHAPS (Sigma Chemical Co., St. Louis, Mo.), a dipolar ionic detergent which can solubilize a variety of membrane proteins (reviewed in reference 8). After this treatment, soluble membrane materials were separated from sedimentable membrane vesicles by high-speed centrifugation. Both fractions were then tested for inhibitory activity against neutrophil elastase to ascertain the degree of solubilization of the inhibitor after detergent treatment.

In other experiments, detergent-solubilized inhibitor was reconstituted in artificial liposomes composed of soybean phospholipids (Asolectin; Sigma) as previously described (19). After detergent removal by dialysis, the artificial liposomes were collected by high-speed centrifugation and tested for elastase inhibition. Activity was compared with activity remaining in the supernatant fraction after liposome sedimentation and with activity of liposomes composed of Asolectin alone.

Comparisons of specific inhibitor activity and inhibitor release in necrotizing and nonnecrotizing strains. Type I Streptococcus pneumoniae (which rarely causes lung necrosis), type III S. pneumoniae (which sometimes produces injury), and Klebsiella pneumoniae (a pyogenic, gram-negative rod which almost always causes destruction of lung tissue) have all been previously shown to contain inhibitors of neutrophil elastase (25). These organisms were compared quantitatively for their specific inhibitory activity (units of inhibitor activity per milligram of bacterial protein) and for release of elastase inhibitor under standardized conditions.

(i) Specific inhibitory activity. Washed bacteria were suspended in 0.01 M Tris (pH 8.0) containing 0.15 M NaCl and repeatedly sonicated. The sonicated suspensions were then mixed with CHAPS to a final detergent concentration of 0.01 M. Samples of these extracts were directly analyzed for protein by the Lowry et al. method (16), whereas other samples were tested for inhibition of neutrophil elastase in the presence of detergent. Expressed inhibitory activity was finally expressed per milligram of bacterial protein.

(ii) Release of inhibitory activity during bile salt-mediated autolysis. Washed bacteria were suspended in phosphate-buffered saline; at 0 min, sodium deoxycholate was added to a final concentration of 0.01% (wt/vol). This bile salt activates the murein hydrolase [N-acetyl muramic acid-L-alanyl amidase] of the pneumococcus, leading to cleavage of peptidoglycan cross-links and eventual cell lysis (23). At various times after the addition of the bile agent, duplicate samples were removed and centrifuged at 17,000 × g for 4 min, and the resultant supernatants were assayed for inhibitory activity against neutrophil elastase in the presence of 0.01 M CHAPS. The inhibitory activity released was expressed as specific activity, based on protein values determined for the starting suspensions as described above. The entire experiment was carried out twice.

Further characterization of inhibitor specificity. In a previous study (25), we showed that concentrations of pneumococcal extract protein that completely inhibited neutrophil elastase were totally ineffective against pancreatic trypsin and pancreatic elastase. Experiments were now undertaken to test the inhibitor against two additional proteases. Bovine pancreatic alpha-chymotrypsin was obtained from Worthington Diagnostics (Freehold, N.J.). Neutrophil chymotrypsin (cathepsin G) was a generous gift from James C. Travis (University of Georgia, Athens). (Cathepsin G is another serine protease present in human neutrophils and is also contained in the primary or azurophil granules of these cells, as is the elastase.) Inhibition of both enzymes by pneumococcal extracts was assayed with benzoyl-tyrosine-ethyl ester as the substrate (Sigma) by the methods of Hummel (9). Enzymes were preincubated alone or with increasing amounts of pneumococcal membrane fraction (protein measurement) for 30 min at 37°C before adding the substrate. Preincubations and assays were carried out without the addition of CHAPS.

Mechanism of complex formation between pneumococcal
inhibitor and neutrophil elastase. Our earlier study (25) showed that neutrophil elastase inactivated by treatment with an elastase specific, active site-directed irreversibly binding peptide chloromethyl ketone no longer formed complexes with pneumococcal inhibitor, suggesting that an unblocked substrate-binding pocket or an active catalytic site in the enzyme (or both) was required for complex formation to take place. Experiments were now undertaken to distinguish between these two possibilities and also to determine whether covalent or noncovalent associations were involved in complex formation.

Pure neutrophil elastase was reacted with diisopropylphosphofluoridate (DiPF) (New England Nuclear Corp., Boston, Mass.) to block the active site serine hydroxyl in the protease while leaving the substrate-binding pocket free. After the reaction, excess DiPF was removed by dialysis, and inactivation of the enzyme was confirmed by assaying activity versus succinyl-(alanil)-p-nitroanilide. The DiPF was labeled with \(^{3}H\) (specific activity, 27.5 nCi/nmol) to facilitate subsequent resolution of free versus complexed [\(^{3}H\)DiP]-elastase by gel permeation chromatography. Thus, after incubating the labeled enzyme with pneumococcal extracts, column chromatography was carried out through Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N.J.); the radioactivity of column fractions was monitored to determine whether the elastase appeared in elution volumes corresponding to the higher-molecular-weight enzyme-inhibitor complexes or the lower-molecular-weight free enzyme.

In other experiments, \(^{125}I\)-labeled neutrophil elastase prepared as described previously (25) was mixed with pneumococcal inhibitor and allowed to form complexes. The latter were verified by gel permeation chromatography as described previously (25) and were then subjected to polyanacrylamide gel electrophoresis (PAGE) in the presence of 0.1% (wt/vol) sodium dodecyl sulfate (SDS), a mild denaturant which can break noncovalent associations between proteins. Gradient (5 to 20%) PAGE was carried out in slab gels by the method of Laemmli (14). The electrophoreograms were subsequently developed by autoradiography to locate the \(^{125}I\)-labeled enzyme.

Activity of pneumococcal elastase inhibitor in the presence of insoluble elastin substrate. All previous experiments designed to test pneumococcal extracts for inhibitory activity against neutrophil elastase were based on assays with a synthetic substrate (25) (see above). Experiments were now undertaken to explore the effect of insoluble elastin as substrate on the ability of the pneumococcal agent to inhibit elastase.

Reaction mixtures containing enzyme alone or enzyme plus increasing concentrations of inhibitor-enriched pneumococcal cell fractions were preincubated in 0.01 M Tris buffer (pH 8.0) containing 0.15 M NaCl at 37°C for 30 min and then added to vessels containing 2 mg of insoluble elastin from bovine ligamentum nuchae (Elastin Products Co., St. Louis, Mo.) suspended in the same buffer. This substrate was in the form of finely divided particles (less than 400 mesh). Incubation was continued at 37°C for 2 h in a gyratory shaking water bath at 70 cycles per min. Reactions were terminated by the addition of trichloroacetic acid to a final concentration of 5% (wt/vol). After an additional 30 min at 0°C, the samples were centrifuged at 1,000 \(\times \) g for 15 min, and the supernatant fractions were analyzed for trichloroacetic acid-soluble peptides by the Lowry et al. assay (16).

As a positive control for elastase inhibition in the presence of insoluble elastin, various concentrations of purified human alpha-1-antitrypsin (Sigma) were employed in place of the pneumococcal fraction.

RESULTS

Localization of the pneumococcal inhibitor. Experiments were carried out with types I and III S. pneumoniae to compare elastase inhibitory activity in different pneumococcal subcellular fractions. Figures 1 through 3 show that the major portion of the inhibitory activity is located in the membrane fraction of these bacteria, suggesting that it may be an integral protein of the pneumococcal cell surface. In this location, the inhibitor presumably could interact with neutrophil enzymes discharged into phagocytic vacuoles or secreted extracellularly, provided that inhibitor active sites are exposed on the outside of the cell membrane and provided that cell wall components (e.g., capsular polysaccharides) do not interfere. (In this connection, it is worth noting that type III pneumococci, which not infrequently produce lung necrosis, have thicker capsules than do type I pneumococci, which rarely cause lung damage.)

Figure 1 specifically shows that the cytoplasm and membrane fraction obtained by lysis of pneumococcal autolysates in low-ionic-strength medium is more active against neutrophil elastase than are cell wall components and mesosomes, released during wall autolysis in protoplasmic buffer (see above). In Fig. 1, the inhibitory activity has been normalized to a constant fraction of the starting autolysate volume to illustrate how the total activity is distributed among the different fractions obtained by the autolysis preparation procedure.

Figure 2, based on an independent cell fractionation technique, reveals greater inhibitory activity in the fraction containing cytoplasm plus membranes and mesosomes (low-speed supernatant after mechanical disintegration; see
tions that were used to sediment these membranes originally.

When the solubilized inhibitor is mixed with soybean phospholipids (Asolectin) as described previously (19), and the detergent is removed by dialysis, inhibitory activity becomes associated with the newly formed artificial phospholipid vesicles (liposomes) and disappears from the solution phase. Empty Asolectin liposomes (formed in the absence of detergent-solubilized pneumococcal membrane proteins) showed no elastase-inhibitory activity (Fig. 5).

Exposure of inhibitor active site(s) on the surface of the cell membrane. (i) [125I]elastase binding experiments. "Naked" protoplasts of type I pneumococci, prepared as described above (induction of autolysis in hypertonic sucrose to yield autoplasts), bind radiolabeled neutrophil elastase, but do not bind radiolabeled pancreatic elastase (Fig. 6). Binding of the labeled neutrophil enzyme can be inhibited by excess unla-
beled neutrophil elastase, whereas excess unlabeled pancreatic elastase has no effect on uptake of the labeled neutrophil enzyme (Fig. 6).

Neutrophil elastase is very hydrophobic (more so than pancreatic elastase) and, for this reason, may enter phospholipid blayers more readily than the latter molecule. Therefore, the foregoing binding experiments must be interpreted with caution. However, the results of these binding experiments may also be due to complexing of neutrophil elastase to its specific inhibitor in the pneumococcal cell membrane. According to this second interpretation, it might be predicted that intact pneumococcal protoplasts (autoplasts) should be capable of inhibiting neutrophil elastase activity. Moreover, if the inhibitor is exposed on the outside of the membrane, sonicated membrane vesicles formed in the presence of hypertonic sucrose to maintain

![Graph showing inhibitory activity against neutrophil elastase in subcellular fractions of pneumococci prepared by homogenization](image)

**FIG. 2.** Inhibitory activity against neutrophil elastase in subcellular fractions of pneumococci prepared by homogenization (see the text). The ordinate and abscissa are as in Fig. 1. All test fractions were diluted to the original homogenate volume. Symbols: △, cell walls; □, cytoplasm plus mesosomes plus membranes; ■, membranes plus mesosomes alone; ●, cytosol alone. This experiment was carried out three times.

above) compared with the cell wall fraction (low-speed pellet). Also shown in Fig. 2 are the results obtained when membranes and mesosomes were separated from soluble cytoplasmic constituents by high-speed centrifugation and then separately tested. Much greater activity is again displayed by the fraction containing cell membranes. In Fig. 2, as in Fig. 1, the inhibitory activity has been normalized to a constant fraction of the starting volume of homogenized cell suspension to demonstrate how the total activity is distributed among the different fractions obtained by the mechanical disruption procedure.

Figure 3 is a histogram showing the specific activity of the pneumococcal inhibitor in various cell fractions, expressed on the basis of protein content of the fraction. The results support the conclusion that the highest concentrations of elastase inhibitor are in the membrane protein fraction of the pneumococci. The bulk of the pneumococcal proteins (51%) are found in the cytosol, accounting for the low specific inhibitory activity found in any fraction that includes these proteins. The membrane fraction contains only 11% of the total protein, but contains 79% of the total inhibitory activity. The weaker inhibitory activity present in some other cell fractions (Fig. 1 through 3) may be due to the fact that the inhibitor is not exclusively present in membranes or else to partial contamination of these fractions with membrane materials. Further work will be required to distinguish between these two possibilities.

**Solubilization and reconstitution of the membrane inhibitor.** Disruption of pneumococcal membranes with the zwitterionic detergent CHAPS (see above) leads to solubilization of elastase-inhibitory activity (Fig. 4). This activity can then be recovered in the high-speed supernatant after centrifugation of detergent-treated membranes under the same condi-
native orientation of their membrane proteins should inhibit elastase as well as or better than vesicles formed by sonication of membranes in low-ionic-strength buffer to randomize the orientation of these proteins. All of the foregoing predictions were next subjected to experimental test.

(ii) Elastase inhibition by intact pneumococcal autolysates and native membrane vesicles compared with randomized membrane vesicles. When intact pneumococcal autolysates or pneumococcal membrane vesicles were solubilized in protoplast buffer to preserve native orientation of membrane proteins, they were incubated with neutrophil elastase, the resultant enzyme inhibition was indistinguishable from that given by the usual preparation of membrane vesicles sonicated in low-ionic-strength buffer (randomized orientation of membrane proteins) (Fig. 7). These data support the conclusion derived from binding experiments (see above) that the pneumococcal elastase inhibitor has active site(s) exposed on the surface of the bacterial cell membrane. Penetration of the hydrophobic enzyme into membrane phospholipids would not (by itself) inhibit the activity of neutrophil elastase, since only liposomes containing pneumococcal inhibitor block this activity, whereas control liposomes do not (Fig. 5).

Further characterization of pneumococcal elastase inhibitor. (i) Nature of the complexes formed with neutrophil elastase. When neutrophil elastase was treated with [1-\(^{3}H\)]DiPF to block the active site serine-195 hydroxy of the protease and the labeled, inactivated enzyme was then incubated with pneumococcal extracts containing elastase-inhibitory activity, complexes of enzyme and inhibitor could be demonstrated by gel permeation chromatography through Sephadex G-100 (Fig. 8).

Furthermore, complexes of \(^{125}\)I-labeled enzyme and inhibitor (see above) were readily dissociated during PAGE in the presence of the detergent SDS (Fig. 9).

These results suggest, first, that an active catalytic site in elastase is not required for complex formation with pneumococcal inhibitor to occur, even though an unblocked substrate-binding pocket in the enzyme is a prerequisite for such complex formation (25). Second, the complexes not only form independently of enzyme acylation via covalent linkage of the active site serine-195, but also appear to involve no other covalent bonds between the enzyme and the inhibitor.

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FIG. 4. Solubilization of pneumococcal elastase inhibitor from the cell membrane fraction by treatment with a dipolar ionic detergent (see the text). The ordinate and abscissa are as in Fig. 1. Symbols: ■, control cell membrane pellet suspended in 0.01 M Tris buffer (pH 8) containing 0.15 M NaCl and incubated at 37°C for 20 min and then sedimented at 160,000 x g for 3 h and finally suspended and sonicated in the same buffer; □, treated cell membrane pellet, processed exactly as above, except that the incubation buffer contained 0.01 M CHAPS; ▲, supernatant of the detergent-treated membrane pellet, after sedimentation of these membranes under the same conditions as above. All of the foregoing fractions were diluted to equal volumes and assayed for elastase inhibition in the presence of 0.01 M CHAPS with a synthetic substrate (see the text). Note the transfer of inhibitory activity to the soluble phase (supernatant) after exposure of membranes to the detergent. This experiment was done twice, with essentially identical results.

FIG. 5. Reconstitution of solubilized pneumococcal inhibitor into artificial liposomes. The ordinate and abscissa are as in Fig. 1. Symbols: ■, solubilized proteins in the supernatant of the detergent-treated pneumococcal membranes (as in Fig. 4) incorporated into Asoslectin liposomes (detergent removed by dialysis); □, soluble phase of the foregoing suspension, after sedimentation of the Asoslectin liposomes; ▲, Asoslectin liposomes prepared in the absence of pneumococcal membrane proteins. The experiment was done on two separate occasions with the same results.

FIG. 6. Binding of \(^{125}\)I-labeled enzymes by pneumococcal autolysates (type I). The ordinate shows the bound fraction of added radioactivity. The abscissa shows the amount of autolysate protein. Symbols: ●, binding of \(^{125}\)I-labeled neutrophil elastase; ○, binding of \(^{125}\)I-labeled pancreatic elastase; ▲, competition for \(^{125}\)I-labeled neutrophil elastase binding by a 20-fold excess of unlabeled neutrophil enzyme; ♦, competition for \(^{125}\)I-neutrophil elastase binding by 20-fold excess of unlabeled pancreatic enzyme. Incubation was for 30 min at 37°C in all cases. The amounts of labeled enzymes added per reaction were 0.17 nmol (0.53 μCi of \(^{125}\)I) of neutrophil elastase and 0.25 nmol (0.33 μCi of \(^{125}\)I) of pancreatic elastase. A second binding experiment was also done, with comparable results.
Again, these results must be interpreted with caution because of the hydrophobic character of leukocyte elastase. If \(^{[\text{H}]\text{DiP}}\) elastase dissolves readily in membrane vesicles and the latter are excluded from the G-100 Sephadex gel, the partition of labeled enzyme shown in Fig. 8 would be expected to occur. Similarly, the dissociation of labeled enzyme during electrophoresis in the presence of SDS (Fig. 9) could simply be due to SDS solubilization of membrane vesicles containing entrapped leukocyte elastase.

(ii) Additional tests of inhibitor specificity. Concentrations of pneumococcal membrane protein that inhibit neutrophil elastase do not affect the activity of bovine alpha-chymotrypsin or, more important, of neutrophil cathepsin G, a chymotrypsin-like enzyme found together with elastase in the primary granules of these cells (Fig. 10). Lack of inhibitory activity of the pneumococcal agent was previously reported for bovine trypsin and porcine pancreatic elastase (25).

(iii) Inhibitor activity in the presence of insoluble elastin. The pneumococcal agent can also inhibit neutrophil elastase when insoluble elastin is used as the substrate rather than the low-molecular-weight synthetic tripeptide, succinyltrialanyl-p-nitroanilide (Fig. 11). Thus the noncovalent complexes of enzyme and bacterial inhibitor, formed during preincubation of neutrophil elastase with pneumococcal extracts (see above), remain relatively stable (at least for 2 h) after addition to elastin. Insoluble elastin has very high affinity for elastases and has been reported to slowly dissociate pancreatic elastase from its complex with alpha-1-antitrypsin (21). Yet, under the conditions of our experiment, both alpha-1-antitrypsin (which forms very stable complexes with neutrophil elastase) and the pneumococcal inhibitor remained effective in the presence of elastin particles (Fig. 11).

Comparisons of inhibitor amount and release from necro-

Rather, the enzyme-inhibitor complexes are probably stabilized by noncovalent associations, which can be disrupted when the proteins are denatured by chaotropic agents like SDS (Fig. 9).

FIG. 7. Inhibition of neutrophil elastase by pneumococcal autolasts and membrane vesicles. The ordinate and abscissa are as in Fig. 1. Symbols: \(\bigtriangleup\), intact pneumococcal autolasts in hypertonic sucrose; \(\bigtriangleup\), autolast membrane vesicles prepared in hypertonic sucrose to preserve the native orientation of membrane proteins; \(\square\), autolast membrane vesicles prepared in hypotonic medium to randomize the orientation of membrane proteins. Both vesicle preparations were diluted to the same volume as the starting autolast suspension. A second experiment gave similar results.
tizing and nonnecrotizing organisms. The type I pneumococcus, a strain which rarely produces permanent lung injury during the course of infection, contains more neutrophil elastase inhibitor than does the type III pneumococcus, which can produce permanent lung injury in humans (Fig. 12). Also, when stimulated to undergo autolysis by incubation in 0.01% deoxycholic acid, type I pneumococci release more inhibitor than type III pneumococci (as expressed per milligram of bacterial protein). K. pneumoniae, a gram-negative rod which almost always gives rise to a necrotizing pneumonia, contains and releases even less inhibitor than do the two pneumococcal strains. The data presented in Fig. 12 suggest that type III pneumococci and K. pneumoniae both release their total extractable inhibitor during incubation with deoxycholic acid, but that the much lower starting content of inhibitor in these organisms results in the differences shown, relative to type I pneumococci.

Although this is an unlikely explanation for the foregoing results, it should be pointed out that the extraction of inhibitor from all three microorganisms was accomplished by sonication and addition of CHAPS to solubilize membrane proteins (see above). In addition, supernatants of bacterial suspensions incubated with deoxycholate were also treated with detergent before assay for elastase inhibition. It is thus possible that differences in inhibitory activity among the tested strains relate to variations in the effect of the detergent upon the interaction of their inhibitors with elastase, rather than to true differences in the amount of inhibitor present or released.

DISCUSSION

An inhibitor of human neutrophil elastase was found earlier in extracts of S. pneumoniae and other pyogenic microorganisms (25). It was partially characterized and shown to act non-competitively and to be ineffective against trypsin and pancreatic elastase. The agent appeared capable of protecting mice against acute lung injury (hemorrhage, edema) induced by intratracheal instillation of crude extracts of human neutrophil granules (24).

In the present study, this pneumococcal substance has been more extensively characterized. Its inhibitory specificity has been shown to exclude chymotrypsin and neutrophil cathepsin G. It has been found capable of inhibiting elastase in the presence of insoluble elastin. The agent has been localized to the pneumococcal membrane by a combination of previously established cell fractionation techniques for pneumococci, binding studies with radio-labeled-enzyme, inhibition experiments with autolysates and membrane vesicle preparations, and demonstration of solubilization with a membranolytic detergent. The mechanism of complex formation between neutrophil elastase and pneumococcal inhibitor has been clarified. It involves noncovalent associations and is independent of peptide bond cleavage or acyl-enzyme formation. Earlier studies (25) showed that an unoccupied binding pocket in elastase was essential for its interaction with the pneumococcal inhibitor. Finally, preliminary results suggest that different strains of pneumococci and other pyogenic bacteria may vary in their content of this elastase inhibitor and in their capacity to
release the agent extracellularly. This variability may help to explain the different propensities of these microorganisms to produce permanent lung injury as a sequela of pneumonia. Although variation in content and release of neutrophil elastase inhibitor from different strains of pneumococci and other organisms could represent one factor influencing the degree of lung injury associated with pyogenic infection, other factors surely also play a role in the outcome. These may include elastase inhibitors of host origin, either produced locally (4) or present in plasma exudate (15). It may be of interest, in this connection, that some pneumococci can decrease the activity of the major plasmatic elastase inhibitor, alpha-1-antitrypsin (alpha-1-proteinase inhibitor), by desialation of this glycoprotein (22). Since neutrophils can also inactivate alpha-1-proteinase inhibitor, in this case by oxidation of its active site methionine residue (3), different intensities of the neutrophil respiratory burst brought on by interaction of these cells with different bacterial organisms in the lung could constitute another factor in the complex interplay between host and invader leading to resolution versus damage. In addition, different microorganisms may induce different levels of elastase secretion from phagocytosing neutrophils; the more heavily encapsulated organisms (e.g., type III pneumococci and Klebsiella) possibly are more stimulatory as a result of being more difficult to engulf (7). Simultaneously, the thicker capsules surrounding these organisms could limit interaction between the inhibitor located within their membranes and the released protease.

Finally, some bacteria produce and secrete proteases of their own, and these enzymes are also potential participants in the process. Clearly, much additional work will be required to sort out the relative importance of each of these factors.

Our previous data on the pneumococcal inhibitor’s molecular weight were difficult to interpret. On the one hand, estimation of its mass by SDS-PAGE of fractions enriched in elastase-inhibitory activity provided a value of 140 kilodaltons. However, when similar samples were subjected to gel permeation chromatography (in the absence of deter-
gent), inhibitory activity was regularly recovered in the excluded volume of large-pore matrices such as Sephacryl S-300 or Sepharose 2B (25). We now believe that the abberant elution behavior of the agent during gel permeation chromatography was due to the presence of the inhibitor within large aggregates of membrane phospholipid, whereas electrophoresis in the presence of a detergent (SDS) dissociated these aggregates and facilitated more accurate determination of the size of the inhibitor.

The presence of a molecule in the pneumococcal cell membrane containing an exposed binding site for human neutrophil elastase raises the question of the possibility that pneumococci have developed this agent to protect themselves against the bactericidal effects of neutrophil elastase (2). However, an opposite possibility also exists, namely, that neutrophil elastase has been modified over the course of the evolution of the host so as to avidly bind to a major protein in the pneumococcal membrane, perhaps to facilitate the cytotoxic effect of elastase upon these bacteria. This view presupposes that the antibacterial mechanism of neutrophil elastase is independent of its enzymatic activity. This speculation has precedent in the case of another cationic protease of the neutrophil, cathepsin G (20). The place of the pneumococcal elastase inhibitor in the biology of host-parasite interaction deserves further study.

In summary, the elastase of human neutrophils (a potent hydrolase capable of producing severe tissue damage at neutral pH [10]) can be specifically inhibited by a factor present in the cell membrane of pneumococci. A pneumococcal strain (type III) that is associated with higher risk of lung necrosis during infection contains less of the inhibitor per unit cell protein than does a strain (type I) that is rarely implicated in tissue damage. Further study is needed to clarify the significance of these findings.

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LITERATURE CITED