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In vivo suppression of immune complex-induced alveolitis by secretory leukoproteinase inhibitor and tissue inhibitor of metalloproteinases 2

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ABSTRACT **The pulmonary tree is exposed to neutrophil-derived serine proteinases and matrix metalloproteinases in inflammatory lung diseases, but the degree to which these enzymes participate in tissue injury remains undefined, as does the therapeutic utility of antiproteinase-based interventions. To address these issues, an *in vivo* rat model was examined in which the intrapulmonary deposition of immune complexes initiates a neutrophil-mediated acute alveolitis. *In vitro* studies demonstrated that rat neutrophils can release neutrophil elastase and cathepsin G as well as a neutrophil progelatinase, which was subsequently activated by either chlorinated oxidants or serine proteinases. Based on structural homologies that exist between rat and human neutrophil proteinases, rat neutrophil elastase and cathepsin G activities could be specifically regulated *in vitro* by recombinant human secretory leukoproteinase inhibitor, and rat neutrophil gelatinase activity proved sensitive to inhibition by recombinant human tissue inhibitor of metalloproteinases 2. When either of the recombinant antiproteinases were instilled intratracheally, *in vivo* lung damage as assessed by increased permeability or hemorrhage was significantly reduced. Furthermore, the coadministration of the serine and matrix metalloproteinase inhibitors almost completely prevented pulmonary damage while effecting only a modest decrease in neutrophil influx. These data support a critical role for neutrophil-derived proteinases in acute lung damage *in vivo* and identify recombinant human secretory leukoproteinase and recombinant human tissue inhibitor of metalloproteinases 2 as potentially efficacious interventions in inflammatory disease states.**

Neutrophils play a critical role in the development of inflammatory lung diseases by infiltrating the pulmonary tree and releasing tissue-destructive mediators (1–3). Although the primary species used by neutrophils to damage host tissues *in vivo* remain undefined, the lung is exposed to an increased burden of leukocyte-derived proteolytic enzymes in disease states ranging from respiratory distress syndromes to cystic fibrosis (1–3). *In vitro* studies have demonstrated that neutrophil serine and metalloproteinases can degrade the extracellular matrix, hydrolyze serum proteins associated with the clotting and complement cascades, and mediate cytotoxic effects (1, 2). However, despite their destructive potential *in vitro*, the degree to which neutrophil proteinases participate in pulmonary damage *in vivo* remains the subject of conjecture.

In a rat model of acute alveolitis, the intrapulmonary deposition of immune complexes initiates a proinflammatory cascade wherein infiltrating neutrophils compromise endothelial and epithelial permeability barriers while simultaneously disrupting structural barriers established by the extracellular matrix (4, 5). Because rat and human neutrophil proteinases display considerable homology (6), we reasoned that the rat model might afford the opportunity to assess the role of leukocyte-derived proteolytic enzymes in lung damage *in vivo* and evaluate the therapeutic efficacy of antiproteinases relevant to human intervention. Herein, we demonstrate that the serine proteinase inhibitor, secretory leukoproteinase inhibitor (SLPI; refs. 7 and 8), and the matrix metalloproteinase inhibitor, tissue inhibitor of metalloproteinases 2 (TIMP-2; refs. 9 and 10), are able to specifically regulate homologous rat proteinases *in vitro* and can, when administered *in vivo*, potently suppress immune complex-induced alveolitis.

METHODS

Neutrophil Preparation. Neutrophils were isolated from glycogen-induced peritoneal exudates in Long–Evans male rats (Charles River Breeding Laboratories; 300–350 g) 4 hr after the i.p. injection of sterile 1% glycogen (4, 5). Harvested neutrophils were suspended in Hanks' balanced salt solution (pH 7.4; GIBCO).

Reaction Conditions. Neutrophils were stimulated either with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml), surface-bound immune complexes [prepared with bovine serum albumin (BSA; Boehringer Mannheim), and rabbit anti-BSA polyclonal antisera (Cappel) as described (11)] or with N-formylmethionylleucylphenylalanine (fMet-Leu-Phe; 1 μM) in the absence or presence of cytochalasin B (10 μg/ml), catalase (20 μg/ml), azide (1 mM), L-methionine (5 mM), recombinant (human) [r(h)] SLPI (Synergen, Boulder, CO) or r(h)TIMP-2 (Amgen). Hypochlorous acid (HOCl) and *N*-chloramine generation was quantiated as described (12).

Proteinase Assays. Neutrophils (1×10^6 cells per ml) were stimulated for 90 min at 37°C as described above and the cell-free releasates were assayed for rat neutrophil elastase (RNE) and cathepsin G activity with methoxysuccinyl-alanylalanylprolylvalyl-*p*-nitroanilide (1.0 mM; Calbiochem) and succinyl-alanylalanylprolylphenyl-*p*-nitroanilide (1.0 mM; Calbiochem), respectively, as described (13). Results are expressed as nmol of substrate cleaved per hr at 25°C.

For matrix metalloproteinase assays, neutrophils (20×10^6 cells per ml) were stimulated as described above and the cell-free releasates were treated with α_1-proteinase inhibitor (25 μg/ml; Calbiochem) and phenylmethylsulfonyl fluoride (1 mM) to inhibit serine proteinase activity (13). Latent matrix metalloproteinases were activated with 4-aminophenylmercuric acetate (APMA; 0.5 mM) for 0.5–3 hr at 37°C (14). Type

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Abbreviations: APMA, 4-aminophenylmercuric acetate; BSA, bovine serum albumin; PMA, phorbol 12-myristate 13-acetate; r(h), recombinant (human); RNE, rat neutrophil elastase; SLPI, secretory leukoproteinase inhibitor; TIMP-2, tissue inhibitor of metalloproteinases 2.

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I collagenolytic and gelatinolytic activities were determined by incubating releasates with rat tail type I collagen (1 μM) or gelatin (1 μM) for 18 hr at 25°C and 3 hr at 37°C, respectively. Substrate hydrolysis was determined by SDS/PAGE as described (14). Zymography was performed under nonreducing conditions in SDS/8.5% polyacrylamide gels impregnated with either β-casein (1 mg/ml; Sigma) or gelatin (1 mg/ml; Sigma) (14).

Acute Alveolitis Model. Lung injury was induced in rats by the i.v. injection of 10 mg of BSA and the intratracheal instillation of 2.5 mg of rabbit anti-BSA polyclonal antisera (4, 5). Tissue injury was assessed 4 hr after injection as changes in vascular permeability or hemorrhage (4, 5). Permeability values and hemorrhage were defined as the ratio of ^{125}I-labeled BSA or ^{51}Cr-labeled erythrocytes in lungs, respectively, to the amount present in 1.0 ml of blood (4, 5). Where indicated, proteinase inhibitors were coinstilled intratracheally with antisera. Lungs were extracted for myeloperoxidase as an indicator of neutrophil content as described (5).

Statistical Analysis. All values are expressed as mean ± SD unless indicated otherwise. Data were analyzed by analysis of variance and a comparison of means was determined by the least significant difference test. Statistical significance was defined at $P < 0.05$.

RESULTS

Characterization of Serine and Metalloproteinase Release from Stimulated Neutrophils. Releasates recovered from neutrophils incubated with surface-bound immune complexes or PMA contained increased quantities of RNE and cathepsin G activities (Fig. 1). Under identical conditions, 1×10^6 rat neutrophils generated 28.4 ± 9.4 nmol of HOCl ($n = 9$) and 1.4 ± 0.2 nmol of *N*-chloramines ($n = 4$). In comparison, PMA-stimulated cells generated 33.6 ± 10.0 nmol of HOCl and 2.1 ± 0.8 nmol of *N*-chloramines, respectively ($n = 5$). As previously observed with human neutrophils (13), when chlorinated oxidants were scavenged by triggering rat neutrophils in the presence of methionine (5 mM), the RNE and cathepsin G activities in the releasates increased 2.5 ± 0.9-fold ($n = 4$) and 3.6 ± 0.9-fold ($n = 5$), respectively.

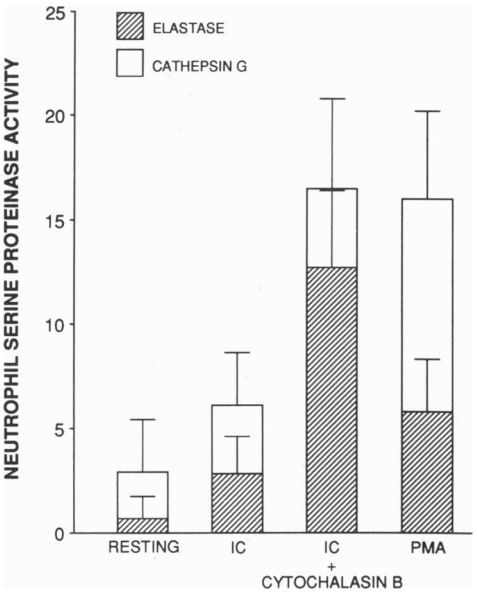

FIG. 1. Serine proteinase release from rat neutrophils. Approximately 1×10^6 neutrophils were incubated alone, with surface-bound immune complexes (IC) in the absence or presence of cytochalasin B (10 μg/ml), or with PMA (50 ng/ml) for 90 min at 37°C. Cell-free releasates were recovered and assayed for RNE or cathepsin G amidolytic activity. Activity is expressed as nmol of substrate cleaved per hour. Results are expressed as the mean ± SD of nine experiments with the exception of those performed in the presence of cytochalasin B ($n = 3$).

Human neutrophils degrade collagenous substrates by releasing and activating the metalloproteinase zymogens, neutrophil procollagenase and neutrophil progelatinase (1, 14). To determine the ability of rat neutrophils to release collagenase, supernatants were analyzed initially by β-casein zymography (14). A single faint band of proteolytic activity was detected at ≈100 kDa (Fig. 2*A*, lane 1), but the organomercurial activator, APMA, failed to induce a shift in the molecular mass of the proteinase (lane 2) and the caseinolytic activity was not inhibited by EDTA (lane 3). Furthermore, when cell-free releasates were incubated with native type I collagen in the absence or presence of APMA, no collagenolytic activity could be detected (Fig. 2*B*, lane 2). Additional studies revealed that the caseinolytic activity could be attributed to a serine proteinase–proteinase inhibitor complex (unpublished observation) while attempts to detect collagenase activity in peripheral blood rat neutrophils were similarly unsuccessful.

Despite the absence of collagenase activity in neutrophil releasates, gelatin zymography revealed two major bands of proteolytic activity at ≈97 and ≈230 kDa (Fig. 2*A*, lane 4). When cell-free supernatants were incubated with APMA prior to zymography (Fig. 2*A*, lane 5), these bands decreased in intensity and a major new band at ≈75 kDa appeared in a manner consistent with the autoproteolytic conversion of rat progelatinase to its major active form (15, 16). After APMA activation, gelatinolytic activity, as assessed by zymography or proteolytic assay, was inhibited completely by EDTA (Fig. 2 *A*, lane 6, and *B*, lane 4).

Oxidative and Proteolytic Pathways of Neutrophil Progelatinase Activation. In human neutrophils, progelatinase can be activated by either HOCl- or serine proteinase-dependent pathways (17–19). As shown in Fig. 3, rat neutrophils released and activated the zymogen as demonstrated by the appearance of gelatinolytic activity. If, however, HOCl generation was inhibited with catalase or azide or chlorinated

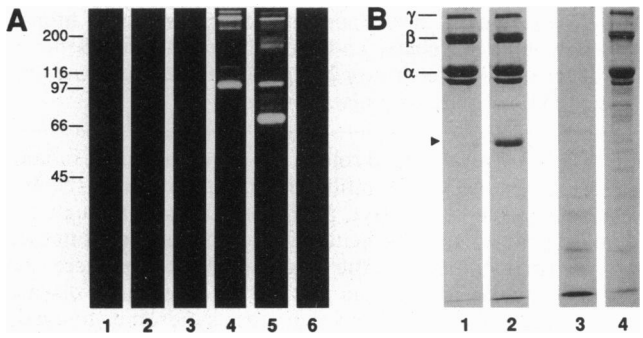

FIG. 2. Metalloproteinase profile of rat neutrophils. (*A*) Zymography of neutrophil releasates. Cell-free supernatants from PMA-stimulated neutrophils were analyzed on β-casein (lanes 1–3)- or gelatin (lanes 4–6)-impregnated gels. Supernatants were treated with serine proteinase inhibitors and assayed directly (lanes 1 and 4), after a 30-min preincubation with APMA (lanes 2 and 5), or after a preincubation with APMA followed by 10 mM EDTA (lanes 3 and 6). (*B*) Collagenolytic and gelatinolytic activity of rat neutrophil releasates. Cell-free supernatants from PMA-stimulated cells were treated with serine proteinase inhibitors and APMA and incubated with type I collagen or gelatin, and proteolysis was assessed by SDS/PAGE. Native collagen alone (lane 1), collagen incubated with APMA-treated releasate (lane 2), gelatin incubated with APMA-treated releasate (lane 3), or gelatin incubated with APMA-treated releasate in the presence of 10 mM EDTA (lane 4). The γ, β, and α bands depict collagen or gelatin trimers, dimers, and monomers, respectively, while the band identified by the arrowhead is human α_1-proteinase inhibitor.

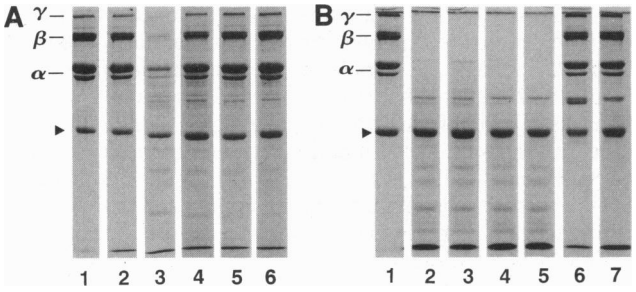

FIG. 3. Oxidative and proteolytic activation of progelatinase. (*A*) Cell-free supernatants from PMA-stimulated neutrophils were treated with serine proteinase inhibitors, incubated with type I gelatin, and analyzed by SDS/PAGE. Native gelatin (lane 1) was incubated with supernatant from resting cells (lane 2), with supernatant from cells stimulated with PMA (lane 3), or with supernatant from cells stimulated in the presence of catalase (10 μg/ml; lane 4), 1 mM azide (lane 5), or 5 mM methionine (lane 6). (*B*) Gelatin was incubated alone (lane 1) or with neutrophil releasates recovered from cells stimulated with fMet-Leu-Phe and cytochalasin B in the absence or presence of catalase, azide, or methionine (lanes 2–5, respectively). In lanes 6 and 7, neutrophils were stimulated in the presence of methionine and human α_1-proteinase inhibitor (25 μg/ml) or methionine and r(h)SLPI (50 μg/ml). The band identified by the arrowhead is α_1-proteinase inhibitor.

oxidants were scavenged by methionine, then gelatinase activity was inhibited. Catalase, azide, or methionine did not affect gelatinase release or the activity of the fully processed enzyme (data not shown). Rat neutrophils triggered with immune complexes similarly activated progelatinase (20.6 ± 8.2 μg of gelatin degraded per 3 hr; $n = 3$) via an HOCl-dependent process that was inhibited completely by either azide or methionine (i.e., <1 μg of gelatin degraded per 3 hr).

In contrast to the results obtained with PMA or immune complex stimulation, rat neutrophils were able to activate progelatinase in the presence of antioxidants when serine proteinase release was enhanced by pretreating the cells with cytochalasin B (Fig. 3). Under these conditions, however, the addition of the serine antiproteinase, α_1-proteinase inhibitor, completely prevented progelatinase activation. Thus, rat neutrophils can activate progelatinase via HOCl- or serine proteinase-dependent pathways.

Regulation of Neutrophil Proteinases by r(h)SLPI and r(h)TIMP-2 *in Vitro*. SLPI is a nonglycosylated ≈12-kDa serine proteinase inhibitor believed to be one of the major defenses against proteolytic damage mediated by either neutrophil elastase or cathepsin G in medium and large airways (7, 20). When 1×10^6 rat neutrophils were stimulated with fMet-Leu-Phe (1 μM) in the presence of cytochalasin B and the cell-free releasates were incubated with r(h)SLPI (50 μg/ml), RNE and cathepsin G activities were inhibited by 90.1 ± 4.2% and 93.2 ± 1.2%, respectively ($n = 4$). Furthermore, despite the fact that r(h)SLPI has been reported to be sensitive to oxidative inactivation (21), both RNE and cathepsin G were effectively inhibited when 1×10^6 neutrophils were stimulated with PMA or immune complexes in the presence of r(h)SLPI at 50 μg/ml (i.e., RNE and cathepsin G activities were inhibited 100 ± 0% and 100 ± 0%, respectively, when cells were triggered with PMA and were inhibited 100 ± 0% and 93 ± 12%, respectively, with immune complexes as the stimulus; $n = 4$). r(h)SLPI did not affect HOCl generation, the oxidative activation of progelatinase, or gelatinase activity in the cell-free releasates (data not shown), but it effectively inhibited the serine proteinase-dependent activation of progelatinase (Fig. 3*B*, lane 7).

Like SLPI, TIMP-2 is also a small (≈22 kDa) nonglycosylated protein, but its regulatory activities are restricted to members of the matrix metalloproteinase family (9, 10, 22). Thus, while r(h)TIMP-2 affected neither RNE or cathepsin G activities in the cell-free releasates nor HOCl generation (data not shown), it completely inhibited neutrophil gelatinase activity in cell-free releasates (Fig. 4). Similarly, when neutrophils were triggered in the presence of r(h)TIMP-2 (2 μg/ml), gelatinase activated by the oxidative- or serine proteinase-dependent pathways was completely inhibited.

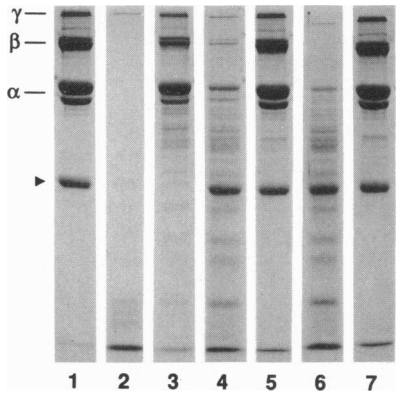

FIG. 4. Regulation of neutrophil gelatinase by r(h)TIMP-2. Type I gelatin was incubated alone (lane 1), with APMA-treated supernatant recovered from PMA-stimulated cells (lane 2), or with APMA-treated supernatants incubated with 1 μg of r(h)TIMP-2 (lane 3). Gelatinase activated by neutrophils stimulated with PMA (lane 4) or fMet-Leu-Phe and cytochalasin B (lane 6) was completely inhibited when cells were triggered in the presence of r(h)TIMP-2 at 2 μg/ml (lanes 5 and 7, respectively). The band identified by the arrowhead is α_1-proteinase inhibitor.

Protective Effects Exerted by r(h)SLPI and r(h)TIMP-2 in Immune Complex-Induced Lung Damage *in Vivo*. Given the effectiveness with which r(h)SLPI and r(h)TIMP-2 inhibited their cognate targets *in vitro*, the ability of the antiproteinases to ameliorate neutrophil-mediated damage *in vivo* was assessed. After the intravenous injection of BSA and the intratracheal instillation of anti-BSA polyclonal antisera, pulmonary damage could be detected after 4 hr, as assessed by the ≈3-fold and ≈5-fold increases in the permeability and hemorrhagic indices, respectively (Fig. 5 *A* and *B*). In marked contrast, when 1 mg of r(h)SLPI was coadministered with the anti-BSA polyclonal antiserum, the permeability index was reduced by ≈54% and hemorrhage decreased by ≈71%. Increasing the dose of r(h)SLPI to 2 mg did not significantly improve its protective effect (data not shown). As described (23), intratracheal injections of control proteins (e.g., superoxide dismutase or inactivated catalase) in doses ranging from 1 to 5 mg had no effect on pulmonary permeability or hemorrhage in immune complex-treated animals.§ When r(h)TIMP-2 (1 mg) was substituted for r(h)SLPI, the permeability and hemorrhagic indices were likewise reduced by 48% and 61%, respectively (Fig. 5). Significantly, the tandem administration of r(h)SLPI and r(h)TIMP-2 exerted the greatest tissue-salvaging effect, as reflected by a 69% decrease in the permeability index and an 83% decrease in pulmonary hemorrhage.

In agreement with quantitative assessments of pulmonary damage, histologic analyses revealed significant decreases in fibrin deposition and hemorrhage in the antiproteinase-treated animals, but neutrophil influx into the intraalveolar space appeared blunted (data not shown). To quantitatively assess the size of the neutrophil infiltrate, lung tissues from antiproteinase-treated and untreated rats were extracted and assayed for myeloperoxidase content. Interestingly, the ad-

§Although both recombinant antiproteinases can be inactivated by reduction and alkylation, reduced r(h)SLPI increased elastase and cathepsin G activities whereas reduced r(h)TIMP-2 formed an insoluble precipitate (unpublished observation).

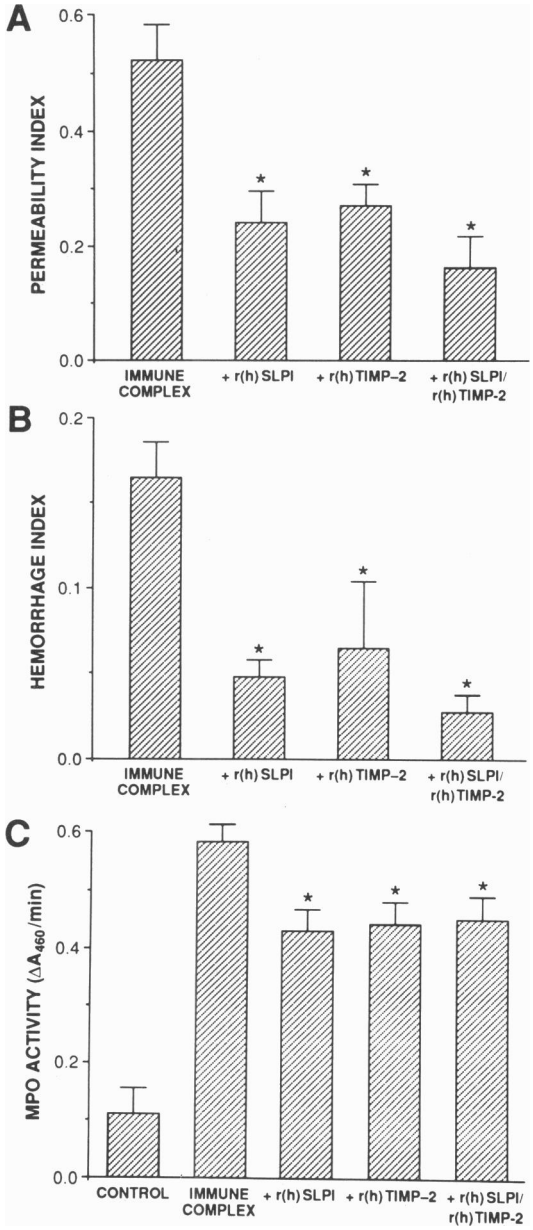

FIG. 5. Inhibition of pulmonary damage by r(h)SLPI and r(h)-TIMP-2. Immune complex-induced damage was monitored by changes in permeability (*A*) or hemorrhage (*B*) in the rat lung. The control values for permeability and hemorrhage (0.16 ± 0.02 and 0.04 ± 0.01, respectively) were subtracted from each group. In *C* the effect of r(h)SLPI and r(h)TIMP-2 on neutrophil influx was assessed by myeloperoxidase activity. Myeloperoxidase (MPO) activity is expressed as the change in A_{460} resulting from the H_2O_2-dependent oxidation of *o*-dianisidine. Results are expressed as the mean ± SD of four or more determinations. A statistically significant difference of $P < 0.05$ vs. control is indicated by an asterisk.

ministration of either r(h)SLPI or r(h)TIMP-2 reduced lung myeloperoxidase content by ≈30% relative to animals receiving anti-BSA polyclonal antisera alone (Fig. 5*C*). When the inhibitors were coadministered, no further reduction in myeloperoxidase content was detected. Thus, r(h)SLPI and r(h)TIMP-2 significantly inhibited immune complex-induced alveolitis while only modestly decreasing neutrophil influx.

DISCUSSION

SLPI and TIMP-2 exhibit narrow inhibitory specificities that include those proteinases most frequently implicated in proinflammatory damage (1, 7, 22). In addition, both antiproteinases display high inhibitory affinity constants for their cognate proteinases, allowing pseudoirreversible conditions to be achieved at micromolar concentrations *in vitro* and *in vivo* (20, 24–27). Given the ability of the human antiproteinases to inhibit rat neutrophil proteinases without affecting other parameters of cell function,[¶] the therapeutic potential of SLPI and TIMP-2 was examined in an *in vivo* model of acute alveolitis. Significantly, when administered alone, or in combination, the recombinant antiproteinases effectively inhibited neutrophil-dependent pulmonary damage. These results extend earlier reports that lung damage in the rat model can also be attenuated by antioxidants (23). Given the facts that (*i*) chlorinated oxidants can simultaneously depress α_1-proteinase inhibitor function while activating latent matrix metalloproteinases (1) and (*ii*) oxidative metabolism is unaffected by either SLPI or TIMP-2,[‖] we postulate that reactive oxidants indirectly mediate damage *in vivo* by regulating proteolytic activity. Although endogenous SLPI and TIMP presumably exert some protective effect in pulmonary tissues, the concentration of these antiproteinases *in vivo* is low and they are susceptible to oxidative or proteolytic attack (21, 30, 31).

The protective effects exerted by r(h)SLPI are consistent with the ability of neutrophil serine proteinases to mediate damage *in vivo* (1, 2), but little is known with regard to the proinflammatory potential of matrix metalloproteinases. However, *in vitro* studies have demonstrated that neutrophil gelatinase can degrade native type IV and V collagen, type I collagen telopeptides, and insoluble elastin (22, 32). Although it is tempting to speculate that the regulation of neutrophil proteinases directly affected the ability of the cells to traverse connective tissue barriers, neither *in vitro* nor *in vivo* studies have identified a direct role for serine or matrix metalloproteinases in diapedesis (33, 34). Because neutrophil elastase can elicit a chemotactic response by inducing interleukin 8 synthesis (3) and neutrophil gelatinase can generate potent chemotaxins by hydrolyzing either extracellular matrix macromolecules (35) or α_1-proteinase inhibitor (14, 36), the reduced neutrophil influx may have occurred as an indirect consequence of the inhibition of the cognate serine and matrix metalloproteinases.

In preliminary attempts to monitor the proteolytic milieu *in vivo*, we have detected large increases in the concentrations of RNE, cathepsin G, and the 96-kDa gelatinase in bronchoalveolar lavage fluids recovered from immune complex-treated animals (unpublished observation). However, enzymic activities recovered in the fluid phase could only be detected with small molecular mass synthetic substrates, indicating that the neutrophil proteinases were irreversibly entrapped by plasma α-macroglobulins that diffused into the inflammatory site (37, 38). α-Macroglobulin-associated proteinase activities in lavage fluids from antiproteinase-treated animals were not further decreased (unpublished observation), but reversible inhibitors (like SLPI and TIMP-2) readily transfer bound proteinases to α-macroglobulins (39). *In vivo*, tissue-destructive proteinases are more realistically expected to be associated with the extracellular matrix where plasma-borne antiproteinases display only a limited ability to regulate proteolysis (8, 40, 41). Interestingly, *in vitro* studies have already demonstrated that SLPI can effectively inhibit neutrophil elastase prebound to elastin (24, 40, 42).

In summary, by utilizing endogenous rather than synthetic proteinase inhibitors (43), advantage was taken of those

[¶]SLPI is unable to regulate a third serine proteinase found in human neutrophils (i.e., proteinase 3; refs. 8 and 28), but a rat homologue was not detected (unpublished observation).

[‖]r(h)SLPI has been reported to increase glutathione levels in sheep epithelial lining fluid (29), but similar effects were not observed in rats (unpublished observation).

evolutionary pressures that dictated the bioengineering of highly specific and nontoxic antiproteinases capable of binding their cognate proteinases at rates designed to protect host tissues. The protective effects exerted by SLPI and TIMP-2 should have important implications for our understanding of the pathophysiology of acute lung injury and for the further development of therapeutic interventions designed to suppress inflammatory disease processes *in vivo*.

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