Abstract. Inflammatory cells are capable of degrading extracellular matrix macromolecules in vivo in the presence of proteinase inhibitors. We and others have hypothesized that such proteolysis is permitted in large part by mechanisms operative in the immediate pericellular environment, especially at zones of contact between inflammatory cells and insoluble matrix components. To further test this hypothesis in vitro, we have used a model system in which viable polymorphonuclear neutrophils (PMN) are allowed to contact a surface coated with proteinase-sensitive substrate, and in which PMN interaction with the surface can be modulated. We have evaluated proteolysis of the surface-bound protein in the presence and absence of proteinase inhibitors. Our results were: (a) In the presence (but not in the absence) of proteinase inhibitors, proteolysis was confined to sharply marginated zones subjacent to the cells; (b) opsonization of the surface enhanced spreading of the PMN, (c) opsonization diminished the effectiveness of alpha-1-proteinase inhibitor (alpha-1-PI) and alpha-2-macroglobulin as inhibitors of proteolysis of surface-bound protein; (d) anti-oxidants did not alter the effectiveness of alpha-1-PI in inhibiting proteolysis of opsonized substrate by PMN; and (e) PMN could restrict entry of alpha-1-PI into zones of contact with opsonized surfaces. We conclude that: (a) In the presence of proteinase inhibitors, PMN can express sharply margmated and exclusively pericellular proteolytic activity; (b) locally high proteinase concentrations and/or exclusion of proteinase inhibitors from pericellular microenvironments may be important mechanisms for pericellular matrix degradation by PMN; and (c) these observations may have general relevance to extracellular matrix remodeling by a variety of inflammatory and other cell types.

Proteinases derived from inflammatory cells have been considered to play major roles in extracellular matrix turnover both in health and disease. Since the plasma and interstitial space are replete with inhibitors of a spectrum of proteinases (48), hypotheses regarding the mechanisms for proteolysis of extracellular matrix must allow for the occurrence of proteolysis in the presence of proteinase inhibitors (II).

Considerable recent attention has focused upon polymorphonuclear neutrophils (PMN), and especially upon the elastase of PMN (human leukocyte elastase, or HLE; EC 3.4.21.11) in considerations of matrix turnover at inflammatory foci (1, 23, 45–47, 53). HLE confers upon PMN the potential for powerful proinflammatory and matrix degradative capacity through its ability to degrade a spectrum of matrix components in addition to elastin (5, 6, 21, 22, 28, 33, 34, 38, 42, 43, 54). Evidence for HLE activity in the extracellular space has been provided by the demonstration of a fibrinopeptide A-containing peptide (fibrinopeptide A-alpha 1-21) in plasma (54). The sites of this activity are unknown; the mechanisms permitting such extracellular HLE activity in the presence of proteinase inhibitors are of considerable interest.

It has been suggested that PMN might degrade extracellular matrix in the presence of inhibitors: (a) by inactivating the HLE-inhibitory capacity of alpha-1-proteinase inhibitor (alpha-1-PI) by means of the myeloperoxidase/H2O2/halide system (12, 14); (b) by oxidative inactivation of antileukoproteinase (13, 26, 27, 31, 39); and/or (c) by oxidant-independent mechanisms at sites of cell-substrate contact (7, 10, 11, 46, 50). The first two mechanisms could be operative at sites where alpha-1-PI and/or anti-leukoproteinase were the major or only effective inhibitors, while the latter may be a more general mechanism.

We have hypothesized that inflammatory cell-derived proteolysis of extracellular matrix in the presence of proteinase inhibitors is limited to the immediate pericellular zone (10, II). To evaluate this hypothesis in vitro and to further explore local mechanisms for proteolysis in the presence of inhibi-
MATERIALS AND METHODS

MATERIALS

N-hydroxy succinimidobiotin, avidin, dichlorotriazinylaminofluorescein dihydrochloride, BSA, catalase, superoxide dismutase, and dimethyl sulfoxide were obtained from Sigma Chemical Co. (St. Louis, MO) Methoxysuccinyl ala-ala-pro-val chloromethyl ketone was purchased from Enzyme Systems Products, (Dublin, CA).

HUMAN PROTEINS

Purified human plasma fibronectin (FN) was generously provided by Dr. J. A. McDonald, Washington University Medical Center (St. Louis, MO). Purified alpha-1-PI was obtained from Dr. J. A. Pierce, Washington University Medical Center. Purified human plasma alpha-2-macroglobulin (32) was provided by Dr. J. S. Huong, St. Louis University (St. Louis, MO). Human serum albumin (HSA) was obtained from the American Red Cross. Human PMN were obtained from peripheral blood by ficoll-Hypaque centrifugation and dextran sedimentation (4). Differential counting was performed on cytocentrifuge preparations (Cytospin 2, Shandon Southern Instruments, Sewickley, PA), and revealed >95% PMN, with the remainder of the cells being eosinophils. These preparations did not contain monocytes, lymphocytes, or platelets.

PROTEINASE ASSAY

FN proteolysis by PMN was quantified in a solid phase assay as previously described (10). Briefly, 96-well microtiter plates (Nunc Immunoplate II) were coated with FN (5 µg/well). The FN was labeled with [125I]NaI (100 mCi/ml; Amersham Corp. Arlington Heights, IL) by the Enzymobead method (Bio-Rad, Richmond, CA). The [125I]FN, after mixing with unlabeled FN to yield a specific activity of ~16,000 CPM/µg, was added to microtiter wells which were then dried at room temperature and exhaustively washed to remove unbound FN. Proteolytic activity was quantified by measuring release of [125I]-labeled fibrinogen degradation products into the wells (Model 1272 Clino gamma, LKB-Wallace) after 3 h, 37°C, 5% CO2 in air. This solid phase assay system has been shown to be sensitive to nanogram quantities of proteinases, to be specific for proteinase activity, and to accurately quantify FN proteolytic activity of viable PMN (10). PMN-derived proteolytic activity in this model system is nearly completely attributable to HLE, as demonstrated by >90% inhibition of PMN-derived proteolysis by the elastase-specific inhibitor methoxy-ala-ala-pro-val chloromethyl ketone (10). Additionally, in preliminary experiments, SDS-polyacrylamide gels followed by autoradiography revealed that 125I released during incubation with PMN consisted of FN peptides characteristic of those resulting from catalytic activity of HLE, but no intact FN. Assays were incubated in RPMI-1640 salts (The Tissue Culture Support Center, Washington University Medical Center) unless otherwise specified, at 37°C in 5% CO2/air. Previous studies have documented that PMN introduced into this assay system rapidly settle (<5 min) onto the FN-coated surface and adhere (10).

Opsonization of FN

To modulate PMN interaction with the FN-coated surfaces, the microtiter wells were incubated with rabbit anti-FN IgG or (as a control) anti-FN Fab fragments for 90 min at 37°C, then the wells were washed exhaustively to remove unbound antibody. All anti-FN antibodies were generously provided by Dr. J. A. McDonald (Washington University Medical Center). Preliminary experiments with a direct enzyme-linked immunosorbent assay technique (horseradish peroxidase-coupled goat anti-rabbit IgG; Cappel, Malvern, PA) established that 5 µg IgG or Fab' yielded maximal binding of immunoglobulins to the surface; this amount was used in the described experiments. Viable PMN (20,000/well) or purified HLE (20 µg/well) were introduced into coated wells in the presence or absence of proteinase inhibitors.

IMMUNOLOCALIZATION OF FN PROTEOLYSIS

To evaluate the hypothesis that FN proteolysis in the presence of inhibitors occurred subjacent to the cells, FN was coated onto glass microscope slides (Lab-Tek chamber slides; Miles Laboratories, Naperville, IL). FN, 15 µg in 200 µl, was added to the chambers and dried at room temperature, then the slides were exhaustively washed to remove unbound FN. To modulate PMN attachment and spreading, the FN-coated surface could then be overlaid with rabbit anti-FN IgG or Fab' as described above (10 µg/chamber). Proteinase inhibitors (either alpha-1-PI or alpha-2-macroglobulin) were added simultaneously with the PMN where specified.

After incubation with PMN (50,000 PMN/chamber, 45 min, 37°C, 5% CO2/air), the cells were fixed and permeabilized with absolute ethanol. Residual surface-bound FN was then localized by indirect immunofluorescence using murine monoclonal anti-FN Fab' fragments followed by species-specific goat anti-mouse F(ab')2 fragments conjugated to fluorescein (Cappel Laboratories, Malvern, PA). After removal of the chambers from the slides, coverslips were applied over a mounting medium of 25% glycerol in PBS containing 250 µg/ml p-phenylene diamine to reduce photobleaching. The slides were examined by phase contrast and epifluorescence microscopy (Leitz Dialux 20, Ernst Leitz Westar GmbH).

PMN SPREADING

PMN were introduced into Miles Lab-Tek chamber slides (Miles Laboratories) which were first coated with FN (15 µg/well). Some of the FN-coated chambers were overlaid with either: (a) rabbit polyclonal or murine monoclonal anti-FN IgG; (b) anti-FN Fab' fragments; (c) murine monoclonal anti-FN IgM; or (d) anti-FN IgM followed by fresh human serum (1:250 dilution) as a source of complement. All of the chambers were exhaustively washed to remove nonadherent proteins.

PMN were introduced into the chambers and observed by phase contrast optics (Nikon Diaphot-TMD) via a video camera (Series 67, Dage-MTI, Michigan City, IN) interfaced with microcomputer-based image analysis software (SMI Microcomp) and a digitizing pad (Hipad, Houston Inst., Austin, TX). After incubation for 2 min, PMN were fixed with 3% glutaraldehyde (30 min, 4°C), then the area of spreading of the cells was quantified for ~100 cells in each chamber. In additional experiments, PMN were fixed with glutaraldehyde after spreading and examined using Hoffman modulation contrast optics (Leitz).

EXCLUSION OF ALPHA-1-PI FROM ZONES OF CONTACT BETWEEN NEUTROPHILS AND SURFACES

To evaluate the possibility that PMN might be able to exclude proteinase inhibitors from diffusing freely into their zones of contact with surfaces, alpha-1-PI penetration beneath adherent PMN was examined. Miles Lab-Tek chamber slides were coated with a mixture of FN (8 µg) and avidin (50 µg). Where desired, this could be overlaid with rabbit anti-FN IgG or Fab' fragments as described above. The chambers were exhaustively washed, then PMN were added and allowed to adhere and spread for 5 min. Biotinylated, fluoresceinated alpha-1-PI was then added for an additional 10 min. The slides were washed, fixed with absolute ethanol, then examined by phase contrast and epifluorescence microscopy. This experimental design allowed binding of the biotinylated inhibitor to the avidin-coated surface wherever it had access, and allowed detection of the bound inhibitor by its fluorescence. To exclude the possibility that failure of binding of biotinylated inhibitor to the surface might be due to avidin proteolysis by the PMN, control cells were fixed and permeabilized with absolute ethanol before addition of the biotinylated, fluoresceinated alpha-1-PI. This allowed penetration of the biotinylated inhibitor to zones subjacent to the fixed cells, where it could then bind to avidin present on the surface.
Figure 1. Inhibition of proteolysis of surface-bound FN by alpha-1-PI. (a) Inhibition of activity of 20 ng of purified HLE by varying amounts of inhibitor. Note that inhibition is nearly complete when 62.5 ng of inhibitor (~1.41 molar ratio of inhibitor to enzyme) was added simultaneously with enzyme, and that higher amounts of inhibitor completely inhibited enzymatic activity. Bars represent means, ±SD (n = 4). (b) Inhibition of proteolytic activity of viable PMN. 20,000 PMN were added simultaneously with varying amounts of inhibitor. PMN were unstimulated except for effects of contact with the surface. Note that marked excess of inhibitor yields incomplete inhibition of proteolytic activity of the PMN on the FN (control, open bars) surface. Inhibition of proteolysis of the FN-Fab' surface (hatched bars) was similar to control. For all inhibitor concentrations, inhibition of proteolysis of the FN-IgG surface (solid bars) was markedly reduced. Error bars = SD; n = 3.

Results

Proteolysis in the Absence of Proteinase Inhibitors
Proteolytic activity of unstimulated PMN obtained on a given day and assayed using the same lot of FN-coated plates showed a mean coefficient of variance of 11% in five separate experiments. In these experiments, the FN proteolysis by 20,000 unstimulated PMN (n = 15) was 1,981 ± SEM 344 CPM. For comparison, 20 ng of HLE released 1,867 ± 99 CPM 125I in parallel assays (n = 3).

In the absence of proteinase inhibitors, proteolysis by PMN when the FN was opsonized with anti-FN IgG (FN-IgG) was not significantly changed; the mean proteolysis was 103 ± SEM 7% of paired unstimulated control values (n = 15). Similarly, proteolysis by PMN of FN coated with anti-FN Fab' fragments (FN-Fab') was 102 ± 3% of control (n = 15). For comparison, proteolysis of non-opsonized FN by PMN stimulated with phorbol myristate acetate (PMA, 10 ng/ml) was 295 ± 29% of control (n = 15, P < 0.001 by t-test). Thus, in contrast to the response to PMA, contact of PMN with an opsonized surface did not enhance proteolytic activity in this model in the absence of proteinase inhibitors.

Effect of Alpha-1-PI Upon FN Proteolysis
Proteolytic activity of 20 ng of leukocyte elastase was inhibited 92% by 62.5 ng of alpha-1-PI (~1.4 molar excess of inhibitor added simultaneously with the enzyme), and essentially no proteolytic activity was observed with higher amounts of inhibitor (Fig. 1 a). In marked contrast, alpha-1-PI was less effective in inhibiting FN proteolysis by viable PMN. Results of one of five experiments performed are shown in Fig. 1 b; all gave similar results. As shown by the open bars, complete inhibition of proteolysis was not achieved by amounts up to 12.5 μg of inhibitor (62.5 μg/ml). The FN-coated surface was also overlaid with anti-FN Fab' fragments or with anti-FN IgG in an attempt to modify the interaction of PMN with the surface. Fig. 1 b additionally shows inhibition, by amounts of alpha-1-PI up to 200 μg assay (1.0 mg/ml), of PMN-mediated proteolysis of the FN on these surfaces. Proteolytic inhibition of PMN on FN (control) and FN-Fab' surfaces was similar. Inhibition of PMN proteolysis on FN-IgG, however, was markedly less complete for all alpha-1-PI concentrations tested. Further studies (not shown) demonstrated that alpha-1-PI inhibition of PMN proteolytic activity toward FN was not significantly changed in comparison with control when the FN was overlaid with murine monoclonal anti-FN IgM or with anti-FN IgM plus dilute human serum (as a source of complement). Since PMN on FN-IgG did not express more proteolytic activity in the absence of inhibitors, these data indicated that Fc receptor-mediated interaction of PMN with the opsonized surface reduced the capability of alpha-1-PI to inhibit the PMN-derived elastase.

Effect of Anti-Oxidants Upon Inhibition of Neutrophil Proteolysis of Opsonized FN
Preliminary experiments demonstrated that a mixture of anti-oxidants (catalase, 1,000 U/ml; superoxide dismutase, 50 μg/ml; and DMSO, 1% vol/vol) maximally impaired the ability of PMA-stimulated PMN to oxidatively inactivate alpha-1-PI. This anti-oxidant mixture had no effect upon FN proteolysis by either viable PMN or purified HLE, and also had no effect upon inhibition by alpha-1-PI of the activity of purified HLE against FN (not shown).

The effects of this anti-oxidant mixture upon inhibition of PMN proteolysis of FN can be seen in Fig. 2. Anti-oxidants did not enhance inhibition of PMN proteolysis of FN, FN-Fab', or FN-IgG. This finding indicates either that oxidative inactivation of the alpha-1-PI did not contribute to the incomplete inhibition of PMN proteolysis on the FN-IgG surfaces, or that oxidative inactivation of the inhibitor was occurring at sites which were not accessible to the anti-oxidants (49).
Figure 2. Effect of anti-oxidants upon inhibition of PMN proteolysis by 3 μg of alpha-1-PI. Inhibition of proteolytic activity was measured in the absence (open bars) or presence (hatched bars) of a mixture of anti-oxidants (catalase, 1,000 U/ml; superoxide dismutase, 50 μg/ml; and DMSO, 1% vol/vol). Error bars = SD; n = 3.

**Neutrophil Spreading**

We have previously shown that PMN introduced into our microtiter well assay system settle to the bottom surface of the well, adhere, and spread on the surface within 5 min (10). PMN spreading, as assessed by Hoffman modulation contrast microscopy, was strikingly enhanced when the cells were in contact with FN-IgG as compared with either FN or FN-Fab' (Fig. 3). The cells on FN or FN-Fab' displayed a rounded morphology, and were loosely adherent to the surface. This is in agreement with studies by reflection contrast microscopy (29) which showed very small contact patches of unstimulated PMN with substratum. In marked contrast, the cells on FN-IgG were highly spread and flattened.

Quantification of spreading of PMN by phase contrast microscopy is shown in Fig. 4. The areas of spreading of the PMN were 122 ± 43 (mean ± SD; n = 97) and 263 ± 86 (n = 106) for FN-Fab' and FN-IgG, respectively. These differences were highly significant (P < 0.001 by t-test), and clearly demonstrate that adherence to the FN-IgG surface, in contrast to FN-Fab', markedly enhanced the observed cell spreading. The mean area of spreading of PMN on FN-IgM, and FN-IgM + complement, was not greater than that of cells on FN-Fab' (not shown). Enhanced spreading was observed only when the FN was overlaid with anti-FN antibodies which contained Fc fragments. These data indicate that contact of PMN with the FN-IgG surface markedly increased cell spreading, mediated by Fc receptor binding.

**Immunolocalization of Proteolysis**

Preliminary experiments (not shown) demonstrated that in the absence of inhibitors, PMA-stimulated PMN gradually degraded FN in expanding zones around the cells over time until no immunoreactive FN remained after 1-2 h. The effect of proteinase inhibitors (not shown) was to protect substrate in the areas remote from the cell surface; however, proteolysis subjacent to the cells was still observed. Fig. 5 shows the contrast between proteolysis of FN-Fab' and of FN-IgG in the presence of alpha-1-PI (250 μg/ml) in this experiment, but the concentration of inhibitor was not critical to the results obtained. A reasonably uniform coating of the surface with immunoreactive FN was observed in the absence of added cells (Fig. 5a). Minimal to absent nonspecific fluorescence was observed when nonimmune Fab' fragments were substituted for anti-FN Fab' fragments in the immunostaining (not shown). When unstimulated PMN were added, rare localized areas of FN proteolysis on the FN-Fab' surface (Fig. 5b) were associated with rounded adherent cells by

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*Figure 3. Modulation contrast microscopy of PMN allowed to adhere and spread on various surfaces. See text for details. (A) Surface coated with FN only. (B) Surface coated with FN + anti-FN Fab' fragments. (C) FN-IgG surface. Note that PMN on FN-IgG are more tightly adherent and spread than cells on either the control or FN-Fab' surface. Bar = 10 μm.*
or irregularly shaped and varied in size down to <1.0 \mu m.

Zones of proteolysis were rounded usually sharply delimited zones of total clearing of immuno-
tin saturation of the cells and the substrate. It was of interest that, in the ab-

PMN were allowed to adhere and spread for 20 min at 37°C in the presence of alpha-1-PI (50 \mu g/ml), then fixed with glutaraldehyde; the area of spreading was quantified by a microcomputer-based image analysis system. PMN in contact with FN-IgG spread avidly on the surface (P < 0.001 in comparison with FN-Fab' by t-test).

In the absence of cells (Fig. 7 a), a uniform lawn of bound fluorescent probe was observable. When avidin was absent from the surface (Fig. 7 b), minimal nonspecific binding of the fluorescent probe was detected. When PMN were adherent to FN-Fab', an uninterrupted lawn of fluorescence was again observed, despite the presence of attached cells (Fig. 7, c and d). In contrast, when PMN were allowed to attach to FN-IgG, zones of restricted access of the probe were identified which corresponded to the area immediately subjacent to each cell, as identified by phase-contrast microscopy (Fig. 7, e and f).

To exclude the possibility that the reduced fluorescence subjacent to the cells resulted from proteolysis of avidin in those zones, control preparations were fixed and permeabilized with ethanol before addition of the probe (Fig. 7, g and h). An uninterrupted lawn of bound fluorescent probe was seen, indicating that avidin was still present beneath the cells. The reduced or absent subcellular binding of the biotinylated probe seen in Fig. 7 e was thus due to restricted access of inhibitor to the area beneath the cells, rather than to avidin proteolysis.

**Discussion**

The experiments described extend our prior observations of proteolytic activity of PMN while in contact with a susceptible substrate (10). In earlier work, we found that alpha-1-PI and alpha-2-macroglobulin incompletely inhibited proteolysis of surface-bound FN in a similar model when the cells were allowed to contact the substrate. We hypothesized that the inhibitors were partially excluded from the interface between PMN and the substrate, allowing relatively unrestrained proteolysis to occur in that microenvironment (10). In the present work, we have confirmed that while proteinase inhibitors (alpha-1-PI and alpha-2-macroglobulin) prevent PMN-derived proteolysis in zones distant from the cell surface, they are ineffective in protecting substrate immediately subjacent to the cells.

We chose to use a stimulus to PMN activation (contact with an opsonized surface) which is relevant to acute inflammation in a variety of disease states. Our work demonstrated that substrate opsonization, mediated by PMN Fc receptor binding, markedly enhanced spreading of the cells on the surface and reduced penetration of alpha-1-PI (and presumably also alpha-2-macroglobulin, which has approximately a 13-fold larger molecular radius) into zones of contact between the cells and the substrate. It was of interest that, in the absence of inhibitors, proteolytic activity of PMN spread on an opsonized surface was not different from that of cells on control surfaces. In contrast, when proteinase inhibitors (either alpha-1-PI or alpha-2-macroglobulin) were present, substrate opsonization markedly diminished the effectiveness of the inhibitors. This indicates that, in this model, the predominant effect of opsonization of the surface was to promote extensive and close interaction of the cells with the surface,
Figure 5. Immunolocalization of PMN proteolysis in the presence of alpha-1-PI (250 μg/ml). PMN (50,000/chamber) were introduced into chambers of Miles glass chamber slides (Miles Laboratories) coated with human plasma FN. The slides, with adherent PMN, were fixed with ethanol and stained for FN by indirect immunofluorescence following a 45-min incubation. See text for details. (A) Lawn of fibronectin observed when PMN were omitted. (B) Rare zone of absent FN, associated with rounded cell by phase contrast (not shown), observed when unstimulated PMN were present during the incubation on FN-Fab'. (C and D) Epifluorescence and phase contrast, respectively, of otherwise unstimulated PMN on FN-IgG, showing strikingly localized zones of FN proteolysis corresponding only to zones of PMN migratory paths. Note that FN is protected by the inhibitor in the zones between cells, and that proteolytic zones are very sharply margined. Quite elongated PMN profiles were often revealed by phase contrast microscopy. (E and F) Epifluorescence and phase contrast microscopy, respectively, of PMN on FN-IgG, showing details of proteolysis by two PMN. Again note strikingly localized and sharply marginated zones of proteolysis, ranging in size down to ≤1 μm. Bars, 10 μm.

such that local proteolysis could not be prevented by inhibitor. The poorer inhibition by alpha-2-macroglobulin in comparison with alpha-1-PI, and the broader zones of PMN proteolysis in the presence of alpha-2-macroglobulin in the present work, would be consistent with poorer penetration of the larger inhibitor into areas beneath the cells.

We (10, 11) and others (40, 44, 46, 50) have earlier suggested the possibility that proteolysis by PMN, especially in the presence of proteinase inhibitors, takes place in the immediate pericellular space. The studies reported here confirm that hypothesis, and indicate that proteinase inhibitors are effective only at sites remote from appropriately stimulated cells.

Oxidative and Nonoxidative Mechanisms of Proteolysis in the Presence of Inhibitors

As indicated by the present work, partitioning of proteinase inhibitors may provide a partial explanation for extracellular proteolysis in the presence of inhibitors. In agreement with
Figure 6. Immunolocalization of FN proteolysis in the presence of alpha-2-macroglobulin (50 \(\mu\)g/ml). Methods were as described for Fig. 5. (a) Epifluorescence; (b) phase contrast. Note that alpha-2-macroglobulin prevented proteolysis in the zones between cells, but that sharply marginated zones of absent FN are seen and correspond to the location of PMN. Bar, 10 \(\mu\)m.

our data, monocyte-derived macrophages adherent to an opsonized surface have been shown to exclude molecules as small as Fab fragments \((M_r = 50,000)\) from penetrating into zones of contact between the cells and the surface \((55)\). These cells have been thought to have the capacity to create a "closed compartment," in which the efficacy of secretory products is maximized, at sites of contact with the surface \((24)\). Chapman and Stone \((15)\) found that the elastolytic activity of PMN, in contrast to that of macrophages, was readily inhibited by proteinase inhibitors. However, in those studies the PMN were unstimulated and presumably poorly adherent to the elastin particles used in the assay.

Weiss \((50)\) has classified PMN proteolysis in the presence of proteinase inhibitors as being permitted by either oxidative or nonoxidative mechanisms. In addition to partitioning of proteinase inhibitors, other nonoxidative mechanisms may have contributed to the proteolytic activity in our studies. Based upon reasonable assumptions, HLE can be estimated to be present in millimolar concentrations within azurophil granules of PMN \((7, 8)\). Thus, at sites of azurophil granule release from the cells, very high concentrations of enzyme will be present, at least transiently, in the pericellular space. The rate of diffusion of enzyme away from local sites may vary depending upon the closeness of contact of the cell with target macromolecules; however, even brief exposure of extracellular proteins to such high concentrations of HLE may result in significant proteolysis. In support of the possibility of high local enzyme concentrations, Henson \((25)\) has reported that azurophil granule extrusion from PMN occurred predominantly at sites where PMN membrane was adherent to immune complexes, and occurred minimally along the free, non-adherent membrane. The possibility thus exists that locally very high HLE concentrations might occur at sites of contact of PMN membrane with immune complexes. Finally, it is possible that a portion of the HLE might remain on the PMN surface membrane when azurophil granules are released, as has been reported for alkaline phosphatase \((25)\).

In endotoxin-stimulated murine macrophages studied by Chapman et al., membrane-bound plasminogen activator was resistant (while soluble plasminogen activator was sensitive) to inhibition by fluid-phase inhibitors \((16)\). Membrane binding of HLE might occur due to the cationic and relatively insoluble nature of the enzyme, or by means of binding to PMN surface receptors for HLE which have been described \((19)\).

Oxidative mechanisms which might permit proteolysis by PMN in the presence of either alpha-1-PI \((12, 14, 18, 40, 53)\) or anti-leukoproteinase \((13, 31)\) have received considerable attention. Oxidants released by PMN during respiratory burst activity have been shown to functionally inactivate these inhibitors, resulting in loss of their ability to inhibit released HLE. The predominant oxidizing species released by PMN appear to be the myeloperoxidase/H2O2/halide system \((18, 36, 37)\), or hypochlorous acid/chloroamine products of myeloperoxidase activity \((51, 52)\). In vivo, the extent and importance of oxidative inactivation of proteinase inhibitors may depend upon: (a) the intensity of PMN respiratory burst activity, which varies with different stimuli, (b) the local presence of anti-oxidants or competitive antagonists of inhibitor inactivation \((2, 9, 50)\); and (c) the local availability of alternative proteinase inhibitors such as alpha-2-macroglobulin.

Anti-oxidants have improved the effectiveness of inhibition by alpha-1-PI in some previous in vitro models \((40, 50, 53)\), but not in the present and some other previous studies \((10, 44, 46)\). These variable results may relate to failure of anti-oxidants to penetrate to sites of some oxidant activity in the pericellular microenvironment \((49)\), or to technical details of the in vitro assay systems. However, the proteolytic activity of PMN from chronic granulomatous disease patients \((50)\) and the local ineffectiveness of alpha-2-macroglobulin in the present and previous \((10)\) work demonstrate that oxidative inhibitor inactivation is not necessary for local proteolytic activity by PMN when in contact with an insoluble substrate. Based upon the available evidence, it seems most reasonable to conclude that both oxidative and nonoxidative mechanisms may permit local proteolytic activity when PMN are in close contact with insoluble substrates, but that oxidative inhibitor inactivation may be necessary for proteolytic activity more remote from the cell surface.

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Figure 7. Exclusion of alpha-1-PI from penetrating into zones subjacent to PMN. See text for details of methods. (A) Lawn of biotinylated, fluoresceinated alpha-1-PI bound to surface coated with FN + avidin in the absence of PMN. (B) Minimal to absent fluorescence when avidin was not present on the surface. (C and D) Epifluorescence and phase contrast, respectively, of PMN adherent to avidin + FN-Fab'; note relatively uninterrupted fluorescence, with considerable nuclear autofluorescence. (E and F) Epifluorescence and phase contrast, respectively, of PMN adherent to avidin + FN-IgG. Zones of reduced fluorescence correspond exactly to margins of spread PMN, indicating areas of reduced binding of fluoresceinated biotin-alpha-1-PI. (G and H), epifluorescence and phase contrast, respectively, of PMN adherent to avidin + FN-IgG, but fixed and permeabilized before addition of the biotinylated, fluoresceinated inhibitor. Uninterrupted fluorescence indicates that zones of reduced fluorescence in E could not be attributed to avidin proteolysis by PMN, but rather were due to restricted access of the probe to zones subjacent to the cells. Bar, 10 μm.
Sharply Delimited and Continual Proteolytic Activity of PMN

One of the most intriguing observations in the current experiments was the patchy and sharply demarcated proteolysis which occurred as PMN traversed the surface-bound substrate. These zones varied in size down to 1-2 μm, and larger zones may have represented confluence of a number of smaller zones of proteolysis. Zones of partial or poorly demarcated proteolysis were uncommonly observed (Fig. 5). The mechanisms giving rise to this pattern are unclear, but the zones of proteolysis may: (a) occur at sites in close proximity to release of an azurophil granule or granules; and/or (b) occur at sites of close contact between the migrating PMN and the surface. With regard to the former possibility, it is noteworthy that the size of the zones of proteolysis would be in agreement with the size of PMN azurophilic granules (~0.3–0.5 μm). The latter possibility is of interest because the closeness of contact between PMN and an opsonized surface is quite varied over the area of spreading (29, 30). Similar studies of fibroblasts by reflection contrast microscopy have revealed that the estimated distance between the fibroblast cell membrane and a glass substrate varies from <50 to >600 angstroms. Chen et al. have identified very localized zones of FN proteolysis subjacent to transformed fibroblasts; these occur at sites of expression of p60A α inhibi- 
gen and at sites of rosette contacts with the surface (17). In this study, we were not able to assess the possibility that localized proteolysis occurred at sites of specialized contacts between PMN and the subjacent surface.

Another interesting observation was the controlled and continual release of proteolytic enzyme by PMN during the 45-min migration on the opsonized surface. Although time-lapse photography was not possible with our experimental design, it was apparent that proteolytic activity was continu- ally expressed by the PMN during migration, since localized areas of proteolysis were observed at all points along their paths.

Although we studied proteolysis of FN, we have previ- ously shown (10) that most or all of the FN degradation in this model system is attributable to HLE in models utilizing other substrates (44, 46, 53) HLE has also been found to provide nearly all of PMN-derived proteolytic activity. Our results thus may be relevant to the turnover of a number of extracellular matrix components, since HLE has a broad substrate specificity which includes not only FN and elastin, but also types III and IV collagen (33, 34, 41, 42) and proteoglycans (28).

The extensive pericellular proteolysis in our experiments which occurred in the presence of protease inhibitors may be highly relevant to in vivo proteolysis and suggest: (a) that the role of protease inhibitor(s) may be to spatially confine proteolytic activity; and (b) that examination of “protease-inhibitor balance” at a tissue or organ level may incorrectly suggest that protease inhibitors will be effective in prevent- ing extracellular proteolysis. Indeed, recent work by Weitz et al. (54) has indicated that HLE activity exists in the extracellular space and that the products of such activity can be detected. Hopefully, additional studies using intact cells in vitro, in model systems such as the one used in the present work, will lead to further insights into mechanisms of extracellular matrix turnover in health and disease.

This work was supported by grants from the National Institutes of Health (NIH) (HL 30341 and HL 29594) and the Council for Tobacco Research, Inc.

Received for publication 20 July 1987, and in revised form 2 November 1987.

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