Cell Surface–bound Elastase and Cathepsin G on Human Neutrophils: A Novel, Non-Oxidative Mechanism by Which Neutrophils Focus and Preserve Catalytic Activity of Serine Proteinases

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Abstract. Serine proteinases of human polymorphonuclear neutrophils play an important role in neutrophil-mediated proteolytic events; however, the non-oxidative mechanisms by which the cells can degrade extracellular matrix in the presence of proteinase inhibitors have not been elucidated. Herein, we provide the first report that human neutrophils express persistently active cell surface–bound human leukocyte elastase and cathepsin G on their cell surface. Unstimulated neutrophils have minimal cell surface expression of these enzymes; however, phorbol ester induces a 30-fold increase. While exposure of neutrophils to chemoattractants (fMLP and C5a) stimulates modest (two- to threefold) increases in cell surface expression of serine proteinases, priming with concentrations of lipopolysaccharide as low as 100 fg/ml leads to striking (up to 10-fold) increase in chemoattractant-induced cell surface expression, even in the presence of serum proteins. LPS-primed and fMLP-stimulated neutrophils have ~100 ng of cell surface human leukocyte elastase activity per 10^6 cells. Cell surface–bound human leukocyte elastase is catalytically active, yet is remarkably resistant to inhibition by naturally occurring proteinase inhibitors. These data indicate that binding of serine proteinases to the cell surface focuses and preserves their catalytic activity, even in the presence of proteinase inhibitors. Upregulated expression of persistently active cell surface–bound serine proteinases on activated neutrophils provides a novel mechanism to facilitate their egress from the vasculature, penetration of tissue barriers, and recruitment into sites of inflammation. Dysregulation of the cell surface expression of these enzymes has the potential to cause tissue destruction during inflammation.

CONTROLLED proteolytic activity of human polymorphonuclear neutrophils (PMN)[1] is involved in critically important processes, such as egress of the cells from the vasculature and their penetration of tissue barriers (55). However, tissue destruction and a variety of disabling diseases result when proteolysis of extracellular matrix components by PMN is excessive, inappropriate, or prolonged (2, 10, 37). Human leukocyte elastase and cathepsin G are two serine proteinases contained within the azurophil granules of PMN that degrade a variety of extracellular matrix macromolecules and play important roles in PMN-mediated proteolytic events.

In contrast to the wealth of available information about the structure and biochemistry of proteinases and their inhibitors, including the catalytic behavior of these enzymes when they are free in solution (15, 16, 35, 58, 59, 61–63), we have remarkably little information about the mechanisms by which PMN use and control their proteolytic enzymes to degrade extracellular matrix proteins in vivo. It is clear that degradation of extracellular matrix proteins by PMN in vivo must occur in an extracellular milieu that is replete with high concentrations of numerous proteinase inhibitors (63). We (9, 11) and others (54) have found that proteinase inhibitors confine proteolytic activity of PMN to the immediate pericellular microenvironment in vitro, but that they cannot inhibit degradation of proteins that are in direct contact with the cells.

PMN may locally degrade matrix macromolecules in the

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1. *Abbreviations used in this paper*: ALP, antileukoprotease; α_1-PI, α_1-proteinase inhibitor; CG/CMK, Z-gly-leu-phe chloromethylketone; FITC, fluorescein isothiocyanate; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; HBSS, Hanks' Balanced Salt Solution; HLE/CMK, methoxysuccinyl-ala-ala-pro-val chloromethylketone; LDH, lactate dehydrogenase; LPS, bacterial lipopolysaccharide; PBS, phosphate buffered saline; PMA, phorbol myristate acetate; PMN polymorphonuclear neutrophils; SLIPI, secretory leucoprotease inhibitor; TLCK, N-α-p-tosyl-L-lysine-chloromethyl ketone.

presence of inhibitors, in part, through oxidative inactivation of proteinase inhibitors in the pericellular environment. Both reactive oxygen species and metalloproteinases have been shown to inactivate α_1-proteinase inhibitor (13, 14, 22, 65) and other physiologic proteinase inhibitors (22, 23, 39, 53). PMN release oxidants locally at the site of their contact with a surface (64), and activated PMN secrete metalloproteinases (4, 24, 45). Thus, proteolysis in the microenvironment surrounding a cell may be facilitated by local inactivation of proteinase inhibitors.

Pericellular proteolysis by PMN may also proceed through modification(s) of the proteinases. We have considered the possibility that the catalytic activities of human leukocyte elastase and cathepsin G are focused and protected when these enzymes are bound to the cell surface of PMN (10).

Herein, we report that human leukocyte elastase and cathepsin G are expressed on the cell surface of PMN. These cell surface–bound enzymes are strikingly upregulated in activated PMN. Cell surface–bound human leukocyte elastase is catalytically active, and in marked contrast to enzyme that is freely released into the extracellular space, membrane-bound enzyme is remarkably resistant to inhibition by naturally occurring proteinase inhibitors. These observations indicate that PMN have the capacity to sharply focus the catalytic activity of human leukocyte elastase and cathepsin G, and provide a novel, non-oxidative mechanism by which the activity of these enzymes can be preserved in the presence of inhibitors.

Materials and Methods

Reagents

Aurobeads (30 nm diam) were purchased from Amersham Corp. (Arlington Heights, IL). Hypaque was purchased from Winthrop-Breon Laboratories, Sterling Drug Inc. (New York, NY). Rabbit anti–sheep $F(ab)_2$ and rabbit anti–sheep $F(ab)_2$ conjugated to FITC were obtained from Cappel (Malvern, PA). IgG fractions of monospecific sheep anti–human leukocyte elastase and anti-cathepsin G were provided by Dr. D. Burnett (University of Birmingham, Birmingham, UK). T-boc-ala-thiobenzyl ester, methyl-succinyl-phe-thiobenzyl ester, and Z-ala-ala-pro-aala-ONP were purchased from Enzyme Systems Products (Livermore, CA). Human serum albumin was obtained from the American Red Cross (Washington, DC). Permount was purchased from Fisher Scientific (Pittsburgh, PA). The limulus amebocyte lysate assay was purchased from Biowhittaker Inc. (Walkersville, MD). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Serine Proteinases and Proteinase Inhibitors

Human leukocyte elastase and cathepsin G were purified from purulent sputum, as described previously (42). The human leukocyte elastase preparation was 98% active when titrated against the active site titrant Z-ala-ala-pro-aala-ONP (51).

α_1-proteinase inhibitor was obtained from Bayer Corporation (New Haven, CT). Eglin C was obtained from Dr. H. P. Schnebli (Ciba-Geigy Limited, Basel, Switzerland). Antileukoproteinase (ALP) was a gift from Dr. J. Kramps (University of Leiden, Leiden, The Netherlands). Secretory leukocyte proteinase inhibitor (SLPI) was provided by Dr. J. Hoidal (University of Utah Health Sciences Center, Salt Lake City, UT). Methoxysuccinyl-ala-ala-pro-val chloromethylketone (HLE/CMK) and Z-gly-leu-phe chloromethyl ketone (CG/CMK) were purchased from Enzyme Systems Products (Livermore, CA).

The activity of the α_1-proteinase inhibitor was measured using active site–titrated human leukocyte elastase (3), and was shown to be 44% active. All concentrations of HLE and α_1-proteinase inhibitor indicated refer to the amount of active protein present.

PMN Isolation

Unless otherwise specified, human PMN were obtained from peripheral blood by ficoll-Hypaque and dextran sedimentation (5). Extreme care was taken to minimize endotoxin contamination in all reagents and buffers. For example, gloves were worn throughout the procedure, all glassware was baked at 180°C for 18 h, and cells were collected into sterile, endotoxin-free polypropylene tubes. All buffers were assayed for endotoxin using the amebocyte lysate method (36); they contained endotoxin concentrations that were below the lower limit of detection of the assay (3 pg/ml).

Differential counting was performed on cytocentrifuge preparations (Cytospin 2, Shandon Southern Instruments, Sewickley, PA), and revealed that greater than 95% of the cells were PMN. The remainder of the cells were eosinophils.

Where specified, PMN were also isolated by the plasma-Percoll gradient technique, as described elsewhere (32). These preparations were greater than 95% pure. The remainder of the nucleated cells were eosinophils.

PMN Priming and Stimulation

PMN were resuspended at 2×10^6/ml in HBSS, and then incubated for 5 to 60 min at 37°C, with and without the following stimuli: (*a*) phorbol myristate acetate (PMA, 50 ng/ml); (*b*) *N*-formyl-methionyl-leucyl-phenylalanine (fMLP, 10^{-8} M); (*c*) complement component 5a (C5a, 10^{-8} M); and (*d*) bacterial lipopolysaccharide from *Escherichia coli* 0111:B4 (LPS, 1 fg/ml to 100 ng/ml). Preliminary experiments established that the optimal incubation time for PMA and chemoattractants was 30 min, and this incubation time was used in all subsequent experiments. In additional preliminary experiments, we incubated PMN for 30 min at 37°C with and without fMLP at 10^{-10} to 10^{-6} M to establish the optimal concentration of fMLP for use in subsequent experiments reported here.

To assess the effect of priming PMN with LPS for subsequent stimulation with fMLP, cells were prewarmed to 37°C, and then incubated at 37°C for 5 min with varying concentrations of LPS. The cells were incubated for a further 30 min at 37°C with fMLP (10^{-8} M). To determine whether the stimuli adversely affected cell viability, cell-free supernatant fluids were harvested, extracts of unstimulated PMN were prepared in 0.05 M phosphate buffer containing 0.04% (vol/vol) Triton and 1 M NaCl, and samples were analyzed for lactate dehydrogenase (LDH) activity using a commercially available kit (Sigma). The results for cell-free supernatant fluids were expressed as a percentage of the amount of LDH activity contained within extracts of PMN.

Effect of Serum Proteins on Agonist-induced Upregulation of Cell Surface–bound Serine Proteinases

PMN were resuspended at 4×10^6 cells/ml in HBSS or HBSS supplemented with 20% autologous serum, as a source of naturally occurring proteinase inhibitors. Cells were primed for 5 min at 37°C with 10 pg/ml LPS then stimulated for 30 min at 37°C with 10^{-8} M fMLP. Control cells were incubated at 37°C in the absence of agonists.

Binding of Exogenous Serine Proteinases to PMN

PMN were resuspended in HBSS at 10^7 cells/ml, then incubated for 30 min at 4°C with and without exogenous human leukocyte elastase and cathepsin G (1 μg/10^6 cells). Cells were then washed with HBSS to remove unbound proteinases.

Immunofluorescence and Image Analysis

PMN were fixed for 3 min at 4°C in PBS containing 3% (wt/vol) paraformaldehyde and 0.25% (vol/vol) glutaraldehyde (pH 7.4), and then washed twice in HBSS. Cells (10^6) were incubated at 4°C in HBSS containing 1% (vol/vol) human serum albumin (pH 7.4) to reduce non-specific binding of antibodies. To stain for serine proteinases, the PMN were incubated for 45 min at 4°C with sheep anti–human leukocyte elastase $F(ab)_2$, sheep anti–cathepsin G $F(ab)_2$ or non-specific sheep $F(ab)_2$, and then washed twice to remove excess antibody. Cells were incubated for 45 min at 4°C with FITC-conjugated rabbit anti–sheep $F(ab)_2$, and then washed twice in HBSS. Cytocentrifuge preparations were mounted in 25% (vol/vol) glycerol in PBS containing 250 μg/ml *p*-phenylenediamine to reduce photobleaching (47), and coverslips were applied.

The immunostained cells were examined with phase–contrast and inci-

dent–light fluorescence microscopy (Leitz Dialux 20 with L2 filter set and Leitz NPL Fluotar 40×, N.A. 1.32 objective; Leica Westlar GMBH). The cells were imaged with a chilled charge-coupled device camera (Photometrics, Tucson, AZ) that was interfaced with a PC-compatible computer and Metamorph hardware/software for image processing and analysis (Universal Imaging, Inc., West Chester, PA). Images containing 15 to 25 cells per microscopic field were captured, and background was subtracted from each image before analysis. Preliminary results indicated that background subtraction yielded illumination intensities in the background with a coefficient of variation of ~0.4% over the microscopic field. For analysis, a region of interest was created that corresponded to each cell in the field. The integrated fluorescence intensity in the region of interest was calculated. For each cell analyzed, the integrated fluorescence intensity of an identical region of interest, located over an adjacent cell-free area of the image, was calculated and subtracted from the cell's fluorescence value. The fluorescence intensity of 100–150 cells in each group was quantified. To correct for non-specific staining, the mean fluorescence for the group of cells that were incubated with the non-specific sheep $F(ab)_2$ was subtracted from each of the values for cells that were incubated with the specific antibodies.

Immunogold Microscopy

To confirm that the serine proteinase antigens observed by immunofluorescence were located on the cell surface of PMN, cells were labeled with the primary $F(ab)_2$ antibodies, as described above, then incubated for 45 min at 4°C with rabbit anti-sheep $F(ab)_2$ that had been conjugated to colloidal gold particles of 30 nm diameter, as recommended by the manufacturer. The PMN were then washed twice in 0.25 M Tris containing 1% (vol/vol) bovine serum albumin and 0.05% (vol/vol) polyethylene glycol (pH 8.0). Cytocentrifuge preparations were mounted in Permount, coverslips were applied, and the slides were examined with polarization reflection microscopy (polarized incident light, when reflected from gold particles, passes through the cross polarizer in the Leitz RK reflection contrast 100×, N.A. 1.32 objective).

Enzyme Histochemistry

PMN were suspended at 2×10^6/ml in HBSS and stimulated for 20 min with fMLP (10^{-8} M), and then fixed for 30 min with 2% glutaraldehyde buffered with 0.1 M cacodylate-HCl (pH 7.4). The cells were washed twice in HBSS and then incubated for 30 min at 37°C in 3 ml of 0.1 M Tris-HCl, pH 7.8, containing 2 mg sodium aurothiosulfate (gold sulfate) and 1 mg of either t-boc-ala-thiobenzyl ester or met-succ-phe-thiobenzyl ester, which are substrates that react with human leukocyte elastase and cathepsin G, respectively (19, 20, 56). As a control, cells were incubated in buffer containing gold sulfate without substrate or with CBZ-arg-thiobenzyl ester, which is not hydrolyzed by any known PMN proteases. The PMN were washed twice in HBSS and postosmicated for 60 min with 2% aqueous osmium, followed by routine dehydration and embedding in low viscosity resin. Sections were examined and photographed with a Phillips 410 electron microscope at 80 kV.

Degradation of Fibronectin by Cell Surface–bound Enzyme

PMN were incubated for 5 min at 37°C with 10 pg/ml LPS, and then stimulated for 30 min with fMLP (10^{-8} M). After stimulation, cells were fixed for 3 min at 4°C with PBS containing 3% (wt/vol) paraformaldehyde and 1% (vol/vol) glutaraldehyde, washed twice in PBS, and resuspended at 4×10^7/ml in PBS. Fixed PMN (2×10^6) or purified human leukocyte elastase (50 ng) were added to fibronectin (20 μg) in the presence or absence of HLE/CMK or CG/CMK (60 μM), then incubated for 3 h at 37°C. After centrifugation at 300 *g* for 5 min, the cell-free supernatant fluids were harvested, reduced with 10% (vol/vol) β-2 mercaptoethanol, and subjected to SDS–polyacrylamide electrophoresis (38).

To exclude the possibility that cleavage of fibronectin by fixed PMN was due to release of human leukocyte elastase from intracellular granules, cells were incubated under identical conditions (4×10^7/ml in PBS for 3 h at 37°C). 200 μl aliquots of cell-free supernatant fluids were assayed in triplicate for human leukocyte elastase activity using 100 μM methoxysuccinyl-ala-ala-pro-val paranitroanilide (MSAPN) as a substrate. Absorbances were read at 410 nM with a V_{max} Kinetic Microplate Reader (Molecular Devices Corp., Palo Alto, CA). The assay was sensitive to release of 0.5 ng of human leukocyte elastase activity per 10^6 PMN.

Quantification of Cell Surface–bound Human Leukocyte Elastase

To obtain an estimate of the amount of human leukocyte elastase activity that is bound to the cell surface of PMN, cells were primed with LPS, stimulated with fMLP, and then fixed for 3 min at 4°C with PBS containing 3% (wt/vol) paraformaldehyde and 0.25% (vol/vol) glutaraldehyde, as described above. Varying numbers of cells (5×10^3 to 5×10^4) or varying amounts of purified human leukocyte elastase (1 to 5 ng) were incubated for 3 h at 37°C with fibronectin (10 μg). Cell-free supernatant fluids were harvested, then reduced with 10% (vol/vol) β-2 mercaptoethanol, and subjected to SDS–polyacrylamide electrophoresis to assess fibronectin degradation.

To confirm that fixation of PMN does not affect the catalytic activity of cell surface–bound serine proteinases, PMN were fixed for 3 min with 3% (wt/vol) paraformaldehyde and 0.25% (vol/vol) glutaraldehyde (to prevent leakage of endogenous proteinases), then incubated with exogenous human leukocyte elastase as described above. PMN were washed in HBSS to remove unbound proteinase, then aliquots of cell suspension were either fixed for a second time (as above) or held in PBS alone. Both groups of cells were washed, resuspended at 4×10^6/ml in HBSS, and then assayed in triplicate (100 μl/assay) for human leukocyte elastase activity using MSAPN.

Effect of Proteinase Inhibitors on the Activity of Cell Surface Serine Proteinases

PMN were resuspended at 4×10^6 cells/ml in HBSS and stimulated for 30 min at 37°C with PMA (50 ng/ml) or fMLP (10^{-8} M), after which, cells and cell-free supernatant fluids were harvested. The cells were fixed with paraformaldehye and glutaraldehyde as described previously, washed twice in PBS, then resuspended in PBS at 5×10^6 cells/ml. For assay, 100 μl of fixed cell suspension, cell-free supernatant fluid, or purified human leukocyte elastase (100 ng/assay) were dispensed into triplicate wells of a microtiter plate. 100 μl of the following inhibitors (or 100 μl of PBS, as a control) were then dispensed into the wells: (*a*) α_1-proteinase inhibitor (α_1-PI, 25 μg/ml); (*b*) eglin C (5 μg/ml); (*c*) antileukoprotease (ALP, 7.5 μg/ml); (*d*) secretory leukocyte proteinase inhibitor (SLPI, 6 μg/ml); (*e*) HLE/CMK (60 μM); (*f*) CG/CMK (60 μM); (*g*) N-α-p-tosyl-L-lysine-chloromethyl ketone (TLCK, 60 μM); and (*h*) PMSF (1 mM). After preincubation at room temperature for 10 min, MSAPN (100 μM) was added to each well, and absorbances were read at 410 nM with a V_{max} Kinetic Microplate Reader (Molecular Devices Corp., Palo Alto, CA).

To test the effects of fixation of PMN on the effectiveness of inhibitors against cell surface–bound serine proteinases, the cells were fixed with paraformaldehyde and glutaraldehyde (to prevent leakage of endogenous serine proteinases), washed, resuspended in HBSS at 2×10^7 cells/ml, and then incubated for 30 min at 4°C with exogenous human leukocyte elastase (1 μg/10^6 cells). Cells were then washed to remove unbound proteinase, and resuspended at 5×10^6/ml in HBSS, without a further fixation step. Cells (100 μl) were assayed in triplicate for cell surface–bound human leukocyte elastase activity using MSAPN (100 μM) as a substrate and purified HLE as the standard, as described above. Human leukocyte elastase (1.6 pmol), or 5×10^5 cells having cell surface–bound elastase with equivalent activity, were pre-incubated in triplicate (10 min, 25°C, total volume 150 μl) with HLE/CMK (60 μM), with varying concentrations of α_1-proteinase inhibitor, or with HBSS as a control. Residual catalytic activity of human leukocyte elastase was then quantified using MSAPN (100 μM).

Statistics

Data are expressed as mean ±SEM or mean ±SD. The results for paired and unpaired data were compared using the Mann-Whitney rank sum test for non-parametric data; *P* values less than 0.05 were considered significant.

Results

Elastase and Cathepsin G Are Expressed on the Surface of Human PMN

PMN were exposed to fMLP for 30 min, fixed, stained

with FITC for cell surface human leukocyte elastase and cathepsin G, and then examined by epifluorescence microscopy. Focal fluorescence was detected on the surface of cells incubated with anti–human leukocyte elastase and anti-cathepsin G $F(ab)_2$, but not on cells incubated with non-specific $F(ab)_2$ indicating that fMLP-stimulated cells express both human leukocyte elastase and cathepsin G on their cell surface.

Effect of PMA and Chemoattractants on Cell Surface Expression of Human Leukocyte Elastase and Cathepsin G by PMN

To examine regulation of cell surface expression of elastase and cathepsin G, cells were incubated for varying times with and without phorbol ester, fMLP, and C5a. Cells were then fixed, labeled with FITC for cell surface–bound elastase and cathepsin G, and then analyzed by quantitative fluorescence imaging. Unstimulated cells expressed minimal quantities of cell surface human leukocyte elastase and cathepsin G (Fig. 1). Exposure to PMA increased cell surface expression of human leukocyte elastase and cathepsin G by PMN within 15 min (Fig. 1 *A*). A maximum effect (30-fold increase) was observed after exposure of PMN to PMA for 30 min. In contrast, exposure of PMN to optimal concentrations of fMLP and C5a for 30 to 60 min stimulated statistically significant but modest (approximately twofold) increases in cell surface expression of human leukocyte elastase and cathepsin G (Figs. 1, *B* and *C*).

To determine the optimal concentration of fMLP for inducing increased expression of cell surface serine proteinases, cells were exposed to varying concentrations of fMLP for 30 min. FMLP stimulated a dose-dependent increase in cell surface human leukocyte elastase (Fig. 1 *D*). The optimal concentration of fMLP was 10^{-8} M, and this concentration was used in all subsequent experiments.

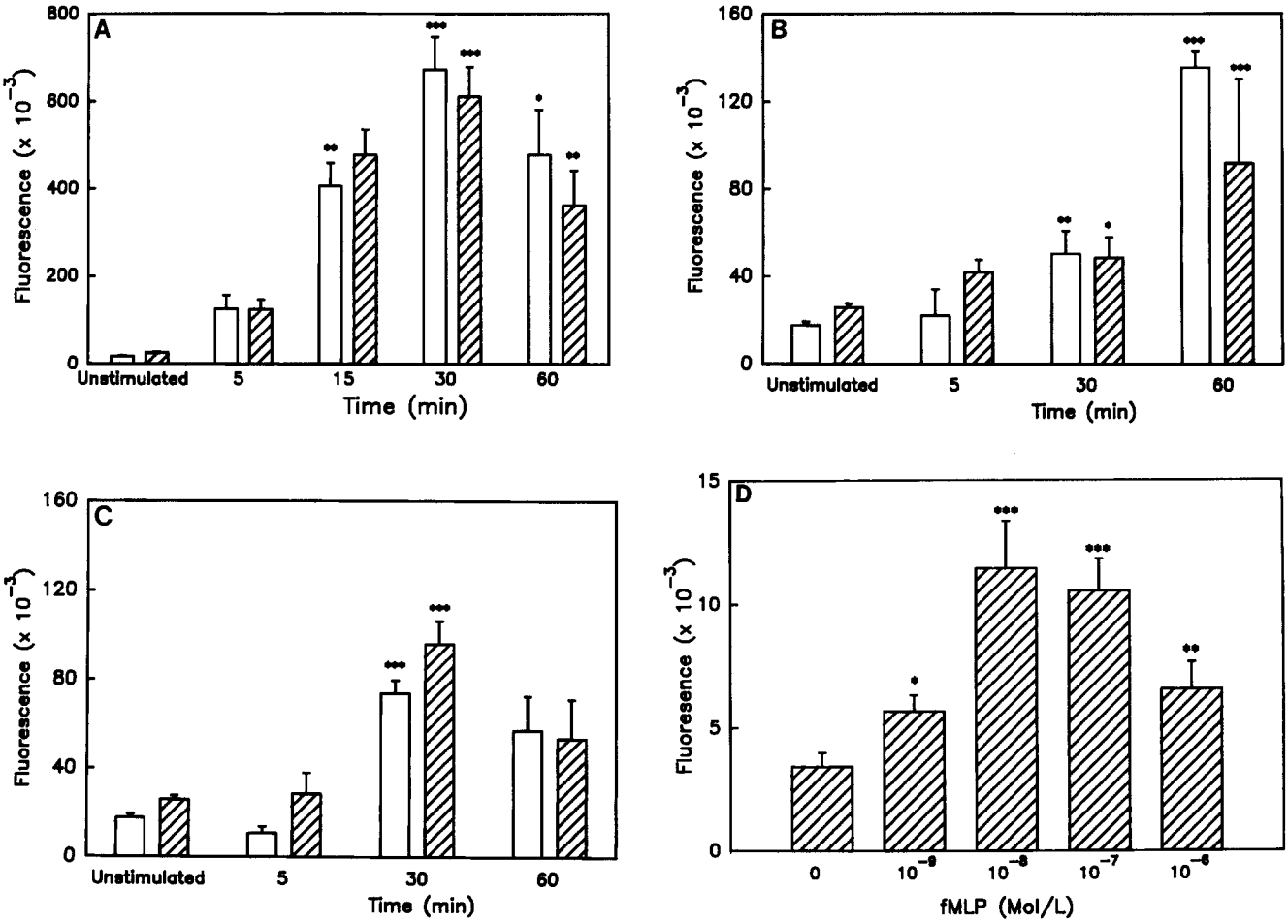

Figure 1. Effect of PMA and chemoattractants on PMN cell surface expression of serine proteinases. PMN were incubated for 5 to 60 min at 37°C with and without 50 ng/ml PMA (*A*), 10^{-8} M fMLP (*B*), and 10^{-8} M C5a (*C*), and then fixed in PBS containing 3% (wt/vol) paraformaldehyde and 0.25% (vol/vol) glutaraldehyde (pH 7.4). Cells were then stained with FITC for cell surface human leukocyte elastase (*open bars*) and cathepsin G (*hatched bars*). Fluorescence was quantified by image analysis, as described in Methods. Note that exposure of PMN to PMA induced striking increases (~30-fold) in expression of cell surface human leukocyte elastase and cathepsin G within 30 min, whereas the chemoattractants, whose effects are shown on different scales, induced modest (approximately twofold) but highly significant increases in the expression of these enzymes. Data are mean values ± SEM; n = 100 to 150 cells. *, $P < 0.05$; **, $P < 0.025$; and ***, $P < 0.001$, respectively, when compared to unstimulated cells. In *D*, PMN were incubated for 30 min at 37°C with and without varying concentrations of fMLP, and then fixed and stained with FITC for cell surface human leukocyte elastase, as described above. Note the dose-related and significant increase in cell surface serine proteinases, with a maximal effect at 10^{-8} M fMLP. *, indicates $P < 0.025$; **, $P < 0.01$; ***, $P < 0.001$ when compared to unstimulated cells.

FMLP induced a similar dose-dependent increase in cell surface expression of cathepsin G (not shown).

To exclude the possibility that any low level endotoxin contamination in our ficoll–Hypaque PMN isolation procedure could prime PMN for subsequent stimulation with chemoattractants, we compared PMN that had been isolated by the ficoll–Hypaque technique to cells isolated by the plasma–Percoll method. PMN were incubated for 30 min with fMLP, and then stained with FITC for cell surface human leukocyte elastase. The mean fluorescence signal of cells isolated by the ficoll–Hypaque technique (54,857 ± SD 10,675) was similar to that of cells isolated by the plasma–Percoll gradient method (58,338 ± 25,261).

Priming of PMN by LPS for Subsequent Stimulation by Chemoattractant

We examined the effect of LPS at concentrations ranging from 1 fg/ml to 100 ng/ml on PMN cell surface expression of serine proteinases. Exposure of PMN for 30 min to 100 ng/ml of LPS induced modest increases in cell surface expression of human leukocyte elastase (Fig. 2 *A*) and cathepsin G (Fig. 2 *B*). There was no clear relationship between LPS concentration (range 1 fg/ml to 100 ng/ml) and the quantity of surface-bound serine proteinases (not shown).

In marked contrast to the effect of LPS alone, priming of PMN for 5 min with LPS followed by stimulation for 30 min with fMLP induced striking increases in the quantity of cell surface human leukocyte elastase and cathepsin G when compared to cells that were incubated with fMLP alone (Figs. 2, *A* and *B*). Priming of PMN with 100 ng/ml of LPS induced threefold increases in cell surface expression of human leukocyte elastase and cathepsin G when compared to cells that were incubated with fMLP alone, and six- to sevenfold increases over unstimulated PMN. Higher concentrations of LPS did not further increase the quantity of cell surface–bound enzymes (data not shown). The most striking effect was observed when cells were primed with 1 to 10 pg/ml of LPS, which consistently induced three- to fourfold increases in cell surface–bound human leukocyte elastase and cathepsin G compared to cells incubated with fMLP alone, and six- to tenfold increases over unstimulated PMN.

The experiments shown in Fig. 2 used priming concentrations of LPS that have been used in other studies (31, 32, 57). However, we were impressed that even the lowest concentration tested (1 pg/ml) had a strong priming effect. Thus, we tested lower concentrations of LPS. The priming effect of LPS was still observed at a concentration of 100 fg/ml; however, the effect was lost at lower concentrations (Fig. 3, *A* and *B*).

Since stimulation of PMN in vivo occurs in a milieu containing proteinase inhibitors and a variety of other proteins, we measured cell surface–bound human leukocyte elastase on PMN that had been suspended in either buffer or buffer containing 20% autologous serum. The agonists induced striking increases in cell surface–bound enzyme whether the pericellular fluid contained serum proteins or not (Fig. 4). Interestingly, cells that were activated in the presence of serum expressed greater quantities of cell surface–bound human leukocyte elastase when compared to cells that were activated in buffer alone ($P < 0.001$). This

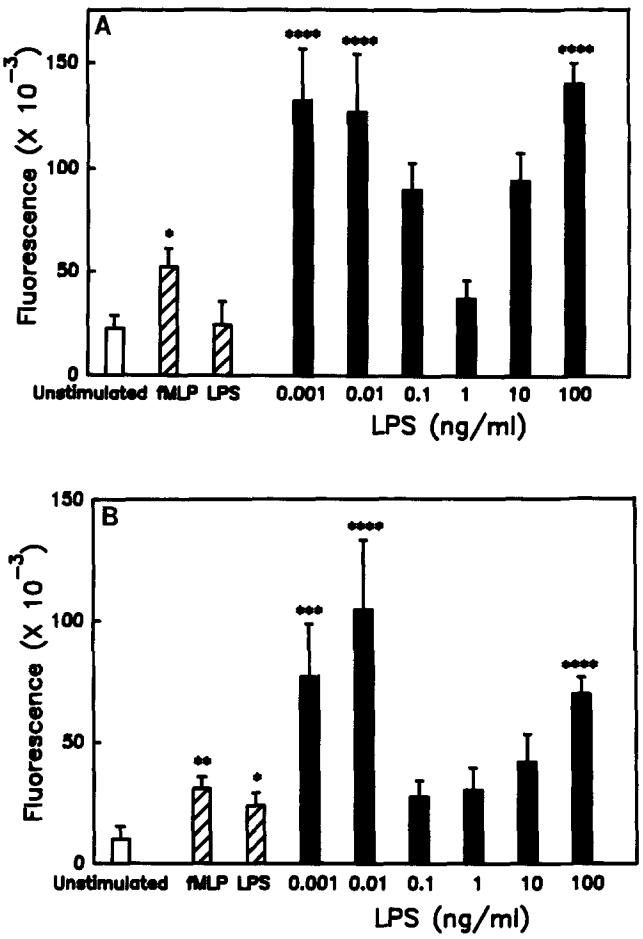

Figure 2. Effect of LPS on cell surface expression of serine proteinases by PMN. PMN were unstimulated (*open bars*) or incubated for 30 min at 37°C with 10^{-8} M fMLP or 100 ng/ml of LPS (*hatched bars*). To test the possibility that LPS primes PMN for subsequent stimulation with fMLP, cells were also incubated for 5 min at 37°C with the indicated concentrations of LPS, then stimulated with 10^{-8} M fMLP (*solid bars*). PMN were then fixed, as described in Fig. 1, and then stained with FITC for cell surface human leukocyte elastase (*A*) and cathepsin G (*B*). Note that 1–10 pg/ml and 100 ng/ml of LPS primed PMN for enhanced cell surface expression of both human leukocyte elastase and cathepsin G during subsequent fMLP exposure. This was a consistent observation in cells from four different donors. Data are mean ± SEM, n = 100 to 150 cells. *, indicates $P < 0.02$; and **, indicates $P < 0.002$ compared to unstimulated cells; ***, indicates $P < 0.03$; and ****, indicates $P < 0.001$ when LPS-primed cells are compared to PMN incubated with fMLP alone.

enhanced agonist activity may be related to the presence of LPS-binding protein in serum, which augments many of the biologic activities of LPS (66, 67, 69).

To exclude adverse effects of the above stimuli on cell viability, PMN were primed for 5 min with 1 fg/ml to 100 ng/ml LPS, then stimulated for 30 min with fMLP. Cell-free supernatant fluids were harvested, cell extracts of unstimulated PMN were prepared, and both were analyzed for lactate dehydrogenase (LDH) activity. All of the supernatant fluids contained less than 6% of the LDH activity that was contained within the corresponding PMN extracts, indicating that the stimuli did not significantly affect cell viability.

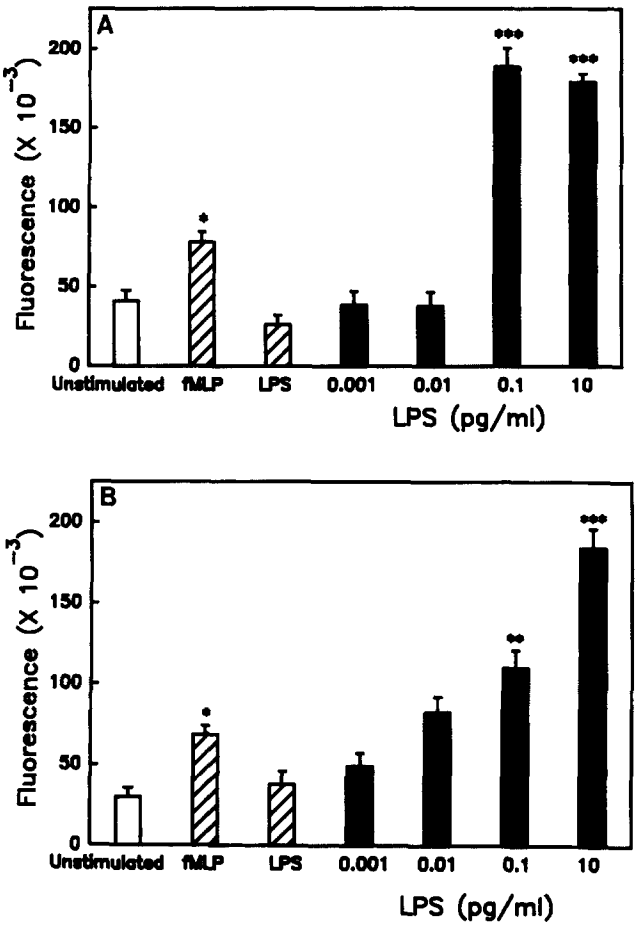

Figure 3. Optimal concentration of LPS for priming of PMN for stimulation with fMLP. PMN were unstimulated (*open bars*) or incubated at 37°C for 30 min with 10^{-8} M fMLP or 10 pg/ml LPS (*hatched bars*). Cells were also incubated at 37°C for 5 min with the indicated concentrations of LPS, then stimulated for 30 min with 10^{-8} M fMLP (*solid bars*). PMN were then fixed and stained with FITC for human leukocyte elastase (*A*) and cathepsin G (*B*). Note that the priming effect of LPS is observed at concentrations of LPS as low as 100 fg/ml. The priming effect is lost at lower concentrations. Data are mean ± SEM, n = 100 to 150 cells. *, indicates $P < 0.001$ compared to unstimulated cells; **, $P < 0.004$ and ***, $P < 0.001$ compared to cells incubated with fMLP alone.

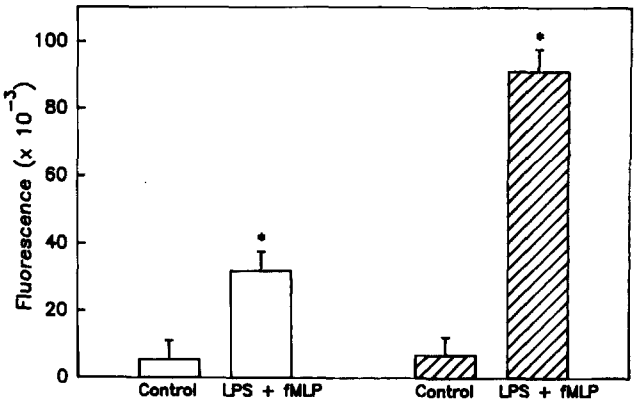

Figure 4. LPS and fMLP upregulate cell surface expression of human leukocyte elastase in the presence of serum proteins. PMN were resuspended in HBSS (*open bars*) or HBSS containing 20% autologous serum (*hatched bars*). Cells were either unstimulated or primed for 5 min at 37°C with 10 pg/ml LPS, then stimulated for 30 min at 37°C with 10^{-8} M fMLP (*LPS + fMLP*). Data were acquired as in Fig. 1. Note that the agonists induced striking increases in cell surface–bound human leukocyte elastase both in the presence and absence of serum. Data are mean ± SEM, n = 100 to 150 cells. Asterisk indicates $P < 0.001$ compared to unstimulated cells.

Immunogold Localization of Serine Proteinases to the Cell Surface of PMN

To exclude the possibility that our immunofluorescence technique was detecting intracellular antigens, we localized human leukocyte elastase and cathepsin G immunoreactivity using antibody conjugated to colloidal gold particles of 30 nm diam, which are too large to penetrate cells. There was essentially a complete lack of gold particles associated with LPS-primed and fMLP-stimulated cells that were incubated with non-specific $F(ab)_2$ (Fig. 5, *A* and *B*). Unstimulated PMN incubated with anti–human leukocyte elastase $F(ab)_2$ (Fig. 5, *C* and *D*) were not obviously different from cells that were incubated with non-specific antibody (Fig. 5 *B*). In marked contrast, LPS-primed and fMLP-stimulated cells that were incubated with anti-human leukocyte elastase $F(ab)_2$ had numerous gold particles localized to their cell surface (Fig. 5, *E* and *F*).

When unstimulated PMN were incubated with anti-cathepsin G $F(ab)_2$, there was almost a complete lack of gold particles associated with the cells (Fig. 6, *A* and *B*). In marked contrast, LPS-primed and fMLP-stimulated cells that were incubated with anti-cathepsin G $F(ab)_2$ had numerous gold particles localized to their cell surface (Fig. 6, *C* and *D*).

Confirmation of the Presence and Activity of Cell Surface Human Leukocyte Elastase and Cathepsin G by Enzyme Histochemistry

To further confirm that serine proteinases are present on the PMN cell surface and to begin to test the possibility that cell surface–bound enzymes are catalytically active, fMLP-stimulated PMN were fixed, incubated with gold sulfate and synthetic substrates that react with human leukocyte elastase and cathepsin G, and then examined by electron microscopy. Reaction product indicative of cathepsin G (Fig. 7, *A* and *B*) and human leukocyte elastase (Fig. 7 *C*) activity was located within azurophil granules. However, reaction product of both enzymes was also clearly evident on the cell surface. In sections that showed release of granules at the cell surface, intense surrounding reaction product strongly suggested local retention of enzyme on the plasma membrane. These ultrastructural studies confirm not only the cell surface localization, but also catalytic activity of the cell surface–bound enzymes. Cytochemical control preparations that contained the gold sulfate without substrate showed no reaction density (Fig. 7 *D*) that resembled reaction product (Fig. 7, *A–C*).

Exogenous Serine Proteinases Bind to PMN

To provide further assurance that serine proteinases bind

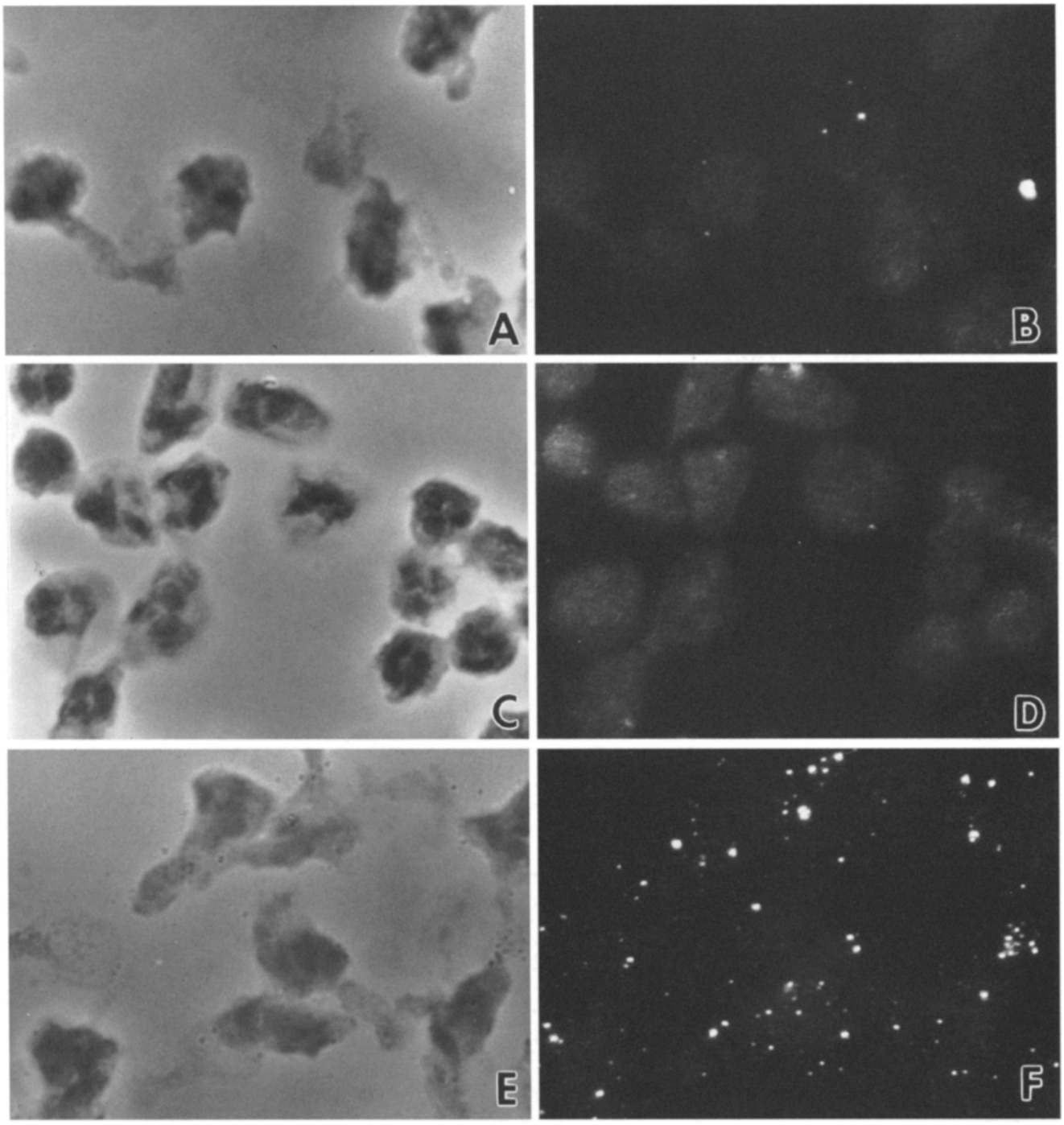

Figure 5. Immunogold microscopy of human leukocyte elastase on the cell surface of PMN. PMN were unstimulated or primed with 10 pg/ml LPS for 5 min at 37°C, and then incubated with 10^{-8} M fMLP for a further 30 min. Cells were fixed, and then incubated with anti-human leukocyte elastase $F(ab)_2$ and immunogold as described in Materials and Methods. The cells have not been permeabilized, and the gold particles cannot enter the cells. Cytocentrifuge preparations were examined both by phase-contrast and reflection polarization microscopy. (*A*, *C*, and *E*) Phase-contrast microscopy; (*B*, *D*, and *F*) reflection polarization microscopy. In *B*, note the nearly complete lack of gold particles in the preparation that was incubated with non-specific $F(ab)_2$. *D* shows that unstimulated PMN incubated with anti-human leukocyte elastase $F(ab)_2$ were not obviously different from cells incubated with non-specific $F(ab)_2$. In marked contrast, *F* shows numerous gold particles localized to the cell surface of LPS-primed and fMLP-stimulated PMN that were labeled with anti-human leukocyte elastase $F(ab)_2$ when the equatorial plane of the cells is in focus. The slight contrast of the cells is due to the polarization optics, and is present in specimens that have not been exposed to gold particles.

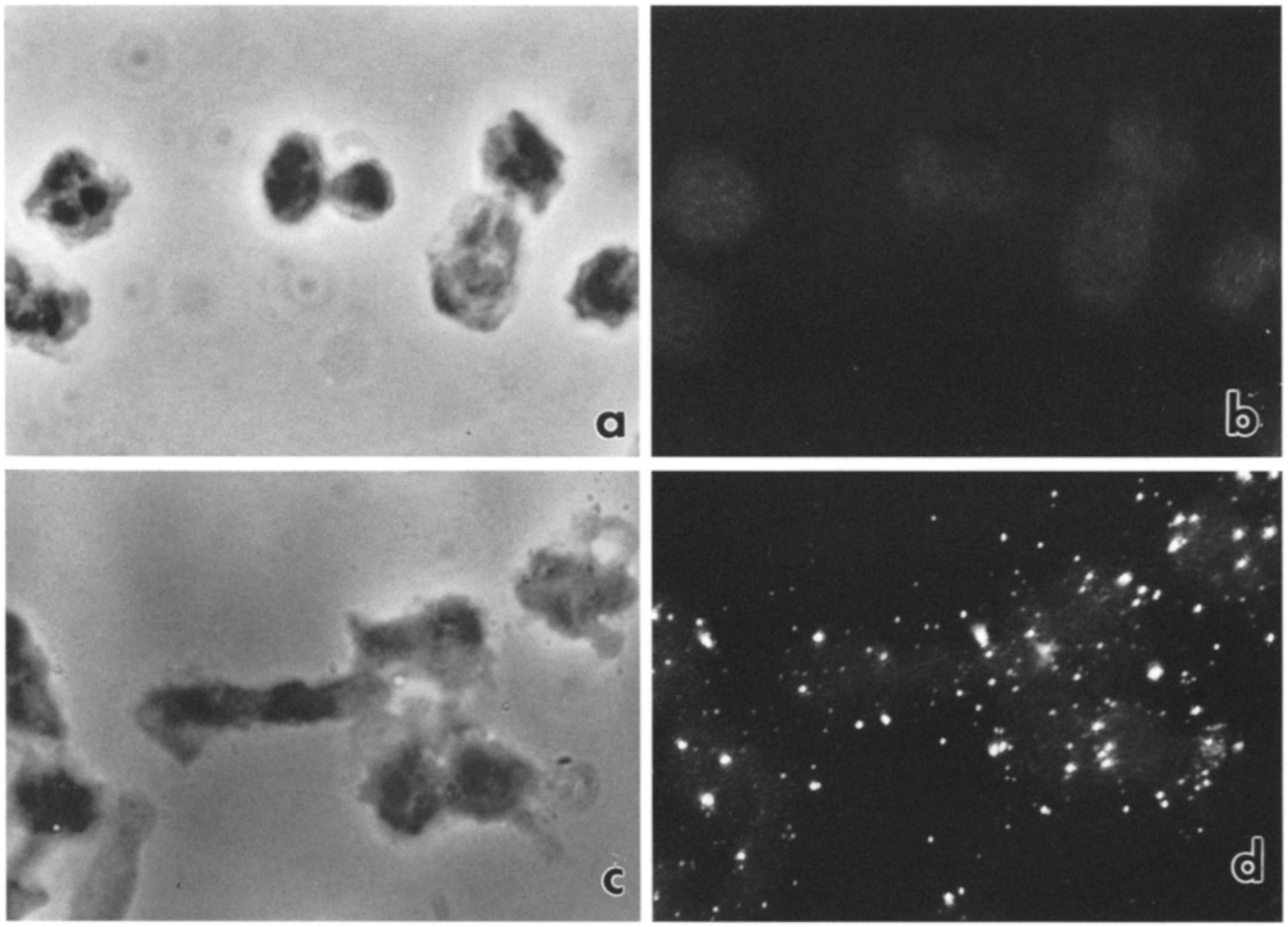

Figure 6. Immunogold microscopy of cathepsin G on the cell surface of PMN. Unstimulated or LPS-primed and fMLP-stimulated PMN were fixed, then incubated with anti-cathepsin G $F(ab)_2$ and immunogold as described in Materials and Methods. Cytocentrifuge preparations were examined both by phase-contrast and reflection polarization microscopy. (*A* and *C*) Phase–contrast microscopy; (*B* and *D*) reflection polarization microscopy. In *B* note the almost complete lack of gold particles associated with unstimulated PMN. In marked contrast, *D* shows that numerous gold particles were localized to the cell surface of LPS-primed and fMLP-stimulated PMN that were labeled with anti-cathepsin G $F(ab)_2$.

to the external plasma membrane, we tested the capacity of exogenous serine proteinases to bind to PMN. Cells that were exposed to exogenous human leukocyte elastase and cathepsin G expressed strikingly greater quantities of enzyme than cells that were incubated in the absence of exogenous serine proteinases (Fig. 8). These data indicate that serine proteinases can bind to sites on the external plasma membrane of PMN.

Cell Surface Human Leukocyte Elastase Degrades an Extracellular Matrix Protein

To determine whether cell surface–bound human leukocyte elastase has the capacity to degrade large molecular weight components of the extracellular matrix, LPS-primed and fMLP-stimulated PMN were fixed, and then incubated for 3 h with a representative soluble extracellular matrix protein (fibronectin) both in the presence and absence of HLE/CMK, a specific inhibitor of human leukocyte elastase. As a control, fibronectin was also incubated with purified human leukocyte elastase. When the cells were incubated with fibronectin, complete degradation of intact fibronectin was observed (Fig. 9 *A*, lane *2*) leaving degradation products identical to those produced by purified human leukocyte elastase (Fig. 9 *A*, lane *3*). Human leukocyte elastase activity was not detected in cell-free supernatant fluids, indicating that the proteolytic activity was cell-associated and not due to the release of intracellular human leukocyte elastase. This cell-associated proteolytic activity was completely inhibited by HLE/CMK (Fig. 9 *A*, lane *4*), but not by CG/CMK, a specific inhibitor of cathepsin G (not shown).

These data indicate that cell surface–bound human leukocyte elastase has the capacity to degrade fibronectin, yielding degradation products similar to those produced by purified human leukocyte elastase in solution.

Quantification of the Catalytic Activity of Human Leukocyte Elastase That Is Bound to the Surface of Primed and Stimulated PMN

To obtain an estimate of catalytically active human leukocyte elastase that is bound to the cell surface of primed and stimulated PMN, varying numbers of fixed cells or varying amounts of purified human leukocyte elastase were incubated for 3 h with fibronectin, and then fibronectin degra-

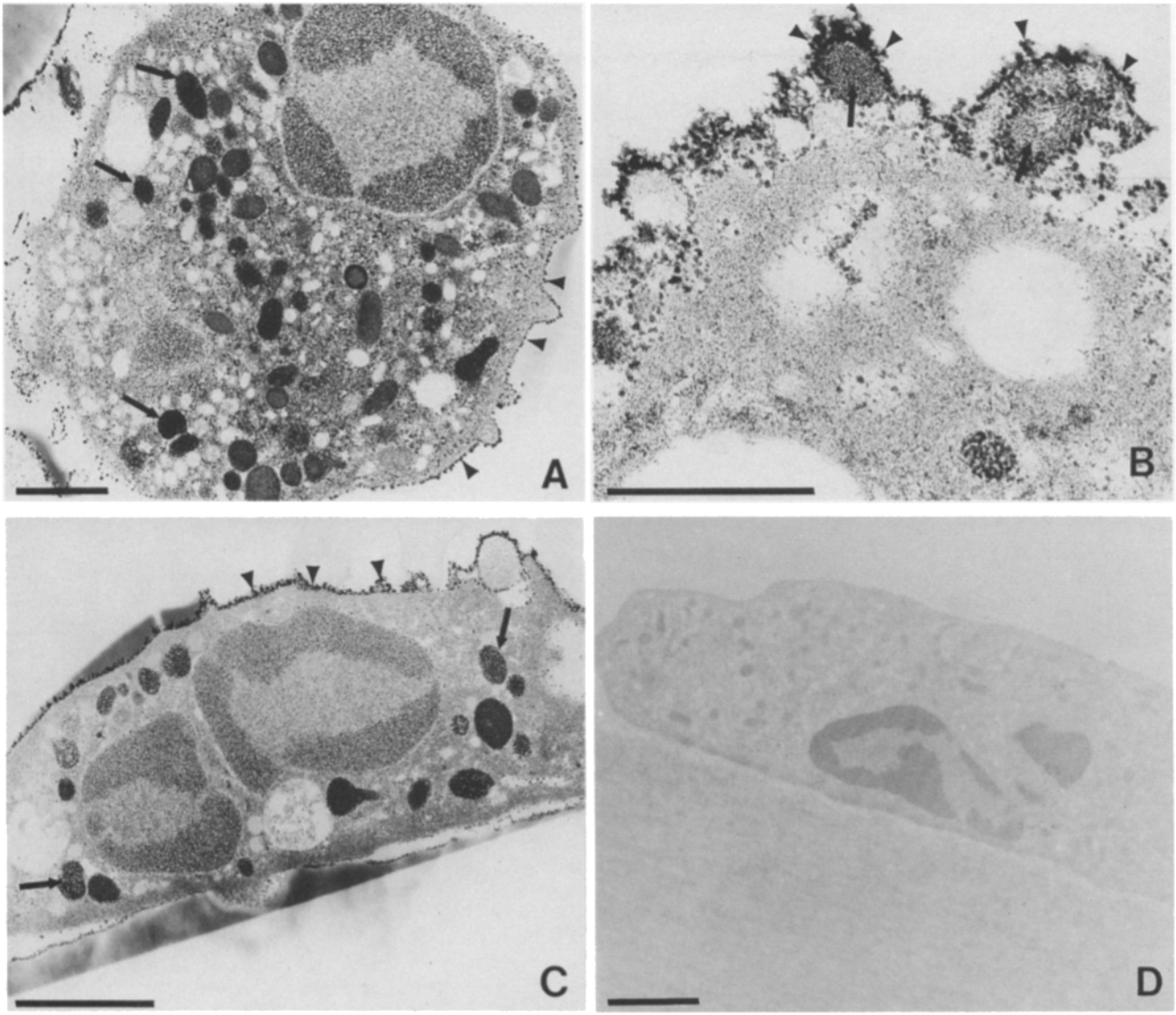

Figure 7. Serine proteinases expressed on the PMN cell surface are catalytically active. PMN were stimulated with fMLP for 20 min, and then fixed in 2% (wt/vol) glutaraldehyde and incubated for 30 min at 37°C with substrates specific for cathepsin G (*A* and *B*) or human leukocyte elastase (*C*) and in buffer containing sodium aurothiosulfate (gold sulfate), as detailed in Materials and Methods. As a control, cells were incubated with non-hydrolyzable substrate or substrate-free medium containing the same buffer and gold sulfate (*D*). Cells were washed and postfixed in osmium for 60 min and embedded in low viscosity resin. Thin sections were then prepared for electron microscopy, and were not counterstained. Electron-dense reaction product, indicative of human leukocyte elastase and cathepsin G activity, is present in azurophil granules (*arrows*, *A–C*), and on the external surface of the plasma membrane (*arrowheads*, *A–C*). *B* demonstrates granule fusion/release at the cell surface and the retention of reaction product along the external surface of the plasma membrane (*arrowheads*). Control specimens showed no visible contrasting densities (*D*). Bars, 1 μm.

dation was assessed by SDS–polyacrylamide electrophoresis of cell-free supernatant fluids (Fig. 9 *B*). One nanogram of human leukocyte elastase (lane *2*) produced no degradation of fibronectin, whereas 3 ng (lane *3*) and 5 ng (lane *4*) of human leukocyte elastase resulted in partial and substantial degradation of intact fibronectin, respectively. 50,000 fixed cells (lane *7*) produced a similar pattern of fibronectin degradation products as 5 ng of purified human leukocyte elastase. Thus, we can estimate that 10^6 primed and stimulated PMN express ~100 ng of human leukocyte elastase activity on their cell surface.

Cell-free supernatant fluids were also assayed for free human leukocyte elastase activity, as described in Materials and Methods. Human leukocyte elastase activity was not detected in cell-free supernatant fluids, which confirmed that the PMN-derived proteolytic activity was cell associated and not due to the release of intracellular enzyme.

We considered the possibility that fixation of PMN reduces the catalytic activity of cell surface–bound serine proteinases, thereby resulting in an underestimation of the quantity of cell surface–bound serine proteinases when fixed cells are studied. PMN were fixed (to prevent leakage of endogenous proteinases), incubated with exogenous human leukocyte elastase, and then incubated in the presence and absence of fixatives. Catalytic activity was

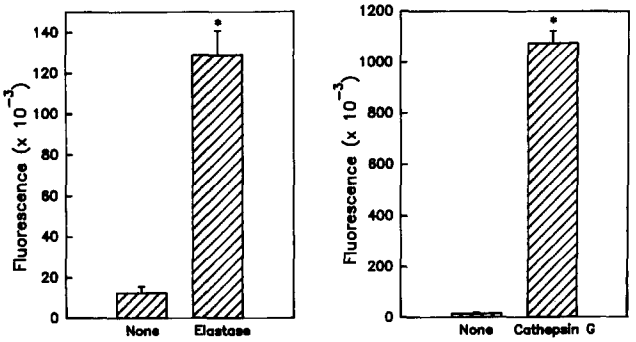

Figure 8. Exogenous serine proteinases bind to the cell surface of PMN. PMN were incubated for 30 min at 4°C in the absence (*None*) or presence of either human leukocyte elastase (*Elastase*, 1 μg/10^6 cells; *left*) or cathepsin G (1 μg/10^6 cells, *right*). Cells were washed, fixed, and then stained with FITC for human leukocyte elastase (*left*) or cathepsin G (*right*). Note that both exogenous human leukocyte elastase and cathepsin G bound readily to the cell surface of PMN. Data are mean values; error bars represent SEM; n = 100 to 150 cells. Asterisk indicates $P < 0.001$ compared to cells incubated without exogenous proteinase.

quantified using MSAPN as a substrate. Cell surface bound exogenous elastase that was not subjected to fixation expressed similar catalytic activity when compared to exogenously added elastase that was fixed onto the cells (191.6 ± SD 4.5 ng/10^6 cells and 196.5 ± SD 2.8 ng/10^6 cells, respectively). These data demonstrate that our fixation procedure has no significant effect on the catalytic activity of cell surface–bound serine proteinases.

Cell Surface Human Leukocyte Elastase and Cathepsin G Are Resistant to Inhibition by High Molecular Weight Inhibitors

To examine inhibition of cell surface–bound serine proteinases, we investigated the effect of naturally occurring and synthetic proteinase inhibitors on the activity of PMN cell surface human leukocyte elastase. To maximize cell surface enzymes, PMN were stimulated with PMA and then fixed with paraformaldehyde and glutaraldehyde. Catalytic activity of cell surface–bound human leukocyte elastase was measured with a synthetic peptide substrate (MSAPN). We compared the effect of each inhibitor on cell surface–bound human leukocyte elastase with its effect on purified human leukocyte elastase in solution and on human leukocyte elastase released into supernatant fluids from stimulated PMN. The human leukocyte elastase inhibitors PMSF, HLE/CMK, α_1-PI, eglin C, ALP, and SLPI were fully effective, at the concentrations tested, as inhibitors of purified human leukocyte elastase and human leukocyte elastase in PMN supernatant fluids (Table I). By contrast, the CG/CMK and the trypsin-inhibitory chloromethyl ketone (TLCK) had little effect.

α_1-PI, the most potent naturally occurring inhibitor of free human leukocyte elastase, was strikingly ineffective against PMN cell surface human leukocyte elastase activity (Table I). In marked contrast, the low molecular weight synthetic proteinase inhibitors, PMSF and HLE/CMK, inhibited about 85% of cell surface human leukocyte elastase activity. ALP, SLPI and eglin C, which are of intermediate

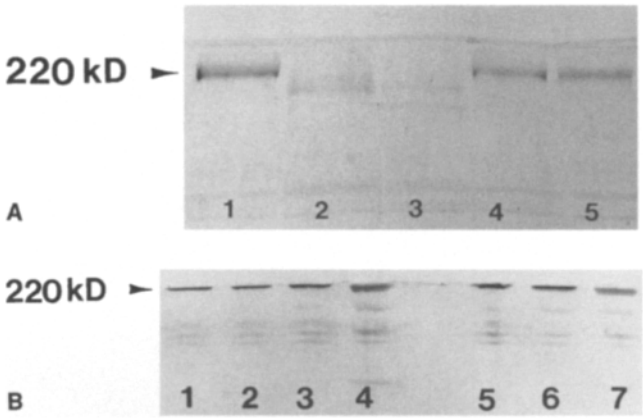

Figure 9. Fibronectin degradation by cell surface–bound human leukocyte elastase. (*A*) Fibronectin degradation and the effects of proteinase inhibitors. PMN were primed with 10 pg/ml LPS for 5 min at 37°C, incubated with 10^{-8} M fMLP for a further 30 min, and then fixed. PMN (2×10^6) or purified human leukocyte elastase (50 ng) were incubated for 3 h at 37°C with 20 μg of fibronectin both in the presence and absence of HLE/CMK (60 μM). Supernatant fluids were reduced and applied to a 7.5 to 15% SDS polyacrylamide gel. Lane *1*, fibronectin alone; lane *2*, fibronectin + cells; lane *3*, fibronectin + human leukocyte elastase; lane *4*, fibronectin + cells + HLE/CMK; lane *5*, fibronectin + human leukocyte elastase + HLE/CMK. Note that in the presence of PMN, there was complete degradation of the fibronectin substrate, and this proteolytic activity was completely inhibited by HLE/CMK. (*B*) Quantification of the amount of human leukocyte elastase that is bound to the cell surface of primed and stimulated PMN. PMN were primed with LPS, stimulated with fMLP, then fixed. PMN (5×10^3 to 5×10^4) or purified human leukocyte elastase (1–5 ng) were incubated for 3 h at 37°C with 10 μg of fibronectin. Supernatant fluids were reduced and applied to 7.5% to 15% SDS polyacrylamide gel electrophoresis. Lane *1*, fibronectin alone; lane *2*, fibronectin + 1 ng of human leukocyte elastase; lane *3*, fibronectin + 3 ng of human leukocyte elastase; lane *4*, fibronectin + 5 ng of human leukocyte elastase; lane *5*, fibronectin + 5×10^3 PMN; lane *6*, fibronectin + 10^4 PMN; lane *7*, fibronectin + 5×10^4 PMN. Note that 5×10^4 PMN express proteolytic activity against fibronectin similar to that of 5 ng of purified human leukocyte elastase.

molecular weight, inhibited ~50% of cell surface human leukocyte elastase activity. Inhibitors of cathepsin G and trypsin (CG/CMK and TLCK) had minimal effect.

We considered the possibility that exposure of PMN to fixatives reduces the effectiveness of high molecular weight proteinase inhibitors against cell surface–bound serine proteinases. PMN were fixed (to prevent leakage of endogenous proteinases), washed in HBSS, then incubated with exogenous human leukocyte elastase in the absence of fixatives. We compared the capacity of HLE/CMK and varying concentrations of α_1-proteinase inhibitor to inhibit purified human leukocyte elastase and cell surface–bound, unfixed, exogenously added human leukocyte elastase. Fig. 10 shows that while the HLE/CMK was slightly more effective against HLE in solution than against cell surface–bound enzyme, it almost completely inhibited both purified human leukocyte elastase and unfixed exogenously added enzyme that was bound to the cell surface of PMN. As expected, α_1-proteinase inhibitor was an effective inhibitor against purified human leukocyte elastase, even at

Table I. Inhibition of Free and Cell Surface-bound HLE

Inhibitor	% Inhibition (+ S.D.)		
	Fixed PMN	HLE (100 ng)	PMN supernatant*
PMSF (1mM)	85 ± 13‡	96 ± 4	100
HLE/CMK (60 αM)	86 ± 10	98 ± 4	100
CG/CMK (60 αM)	10 ± 11	18 ± 9	25 ± 3
TLCK (60 αM)	15 ± 10	5 ± 5	10 ± 14
α^1PI (25 μg/ml)	14 ± 12	98 ± 3	100
Eglin C (5 μg/ml)	45 ± 18	100	100
ALP (7.5 μg/ml)	57 ± 6	100	100
SLPI (6 μg/ml)	50 ± 18	100	100

*Cell-free supernate from phorbol-stimulated PMN.
‡n = 3–24 assays per condition tested.

a 1:1 molar ratio. In marked contrast, α_1-proteinase inhibitor was only partially effective against unfixed exogenous cell surface–bound human leukocyte elastase even when the inhibitor was added in 100-fold molar excess over the quantity of active human leukocyte elastase that was bound to the cell surface of PMN.

Together, these data indicate human leukocyte elastase that is bound to the cell surface of PMN in vivo is catalytically active, yet substantially resistant to inhibition by naturally occurring proteinase inhibitors.

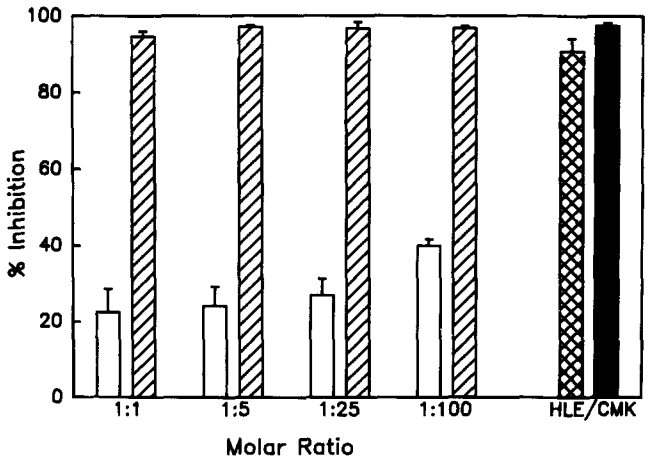

Figure 10. Susceptibility of unfixed cell surface–bound human leukocyte elastase to inhibition by proteinase inhibitors. PMN were fixed, then incubated for 30 min at 4°C with exogenous human leukocyte elastase (1 μg/10^6 cells). The quantity of human leukocyte elastase that bound to the cell surface was quantified using MSAPN, as described in Methods. These cells (5 × 10^5, *open bars*), or soluble human leukocyte elastase having equivalent catalytic activity (1.6 pmol, *hatched bars*) were then assayed in triplicate for human leukocyte elastase activity after pre-incubation with varying amounts of α_1-proteinase inhibitor ranging from equimolar to 100-fold excess. For comparison, inhibition of soluble (*solid bars*) and cell surface–bound (*cross-hatched bars*) elastase by HLE/CMK (60 μM) is also shown. Note that HLE/CMK almost completely inhibited both purified human leukocyte elastase and exogenous enzyme that was bound to the surface of PMN. α_1-proteinase inhibitor was an effective inhibitor of purified human leukocyte elastase, but was only partially effective against unfixed exogenous enzyme that was bound to the surface of PMN, even when present in substantial molar excess. Data are mean values; error bars represent SD.

Discussion

Herein, we report that human leukocyte elastase and cathepsin G are expressed on the cell surface of human PMN. In addition, we have shown that cell surface expression of elastase and cathepsin G can be strikingly upregulated by pro-inflammatory mediators. Cell surface–bound enzymes are catalytically active against a physiologic high molecular weight substrate, as well as peptide substrates. However, in marked contrast to soluble elastase and cathepsin G, cell surface–bound enzymes are remarkably resistant to inhibition by naturally occurring proteinase inhibitors. Our data indicate that binding of serine proteinases to the PMN cell surface focuses and preserves their catalytic activity even in the presence of proteinase inhibitors.

Regulation of the Expression of Cell Surface–bound Serine Proteinases

We found minimal expression of serine proteinases on unstimulated PMN; however, phorbol ester rapidly induced a 30-fold increase in PMN cell surface expression of both human leukocyte elastase and cathepsin G. Although phorbol ester induced dramatic increases in cell surface expression of human leukocyte elastase and cathepsin G, phorbol ester effects are not directly relevant to human inflammatory processes in vivo. To evaluate more biologically relevant agonists, we exposed PMN to concentrations of chemoattractants that are optimal for the chemotactic responses, as well as to LPS. FMLP induced modest but statistically significant (approximately twofold) increases in expression of these enzymes, but a wide range of concentrations of LPS (1 fg/ml to 100 ng/ml) had minimal effect when used alone.

In contrast to its minimal effect when used alone, LPS exerted a striking priming effect on PMN for subsequent responses to chemoattractants. For example, LPS-primed and fMLP-stimulated cells expressed 6- to 10-fold more human leukocyte elastase and cathepsin G on the cell surface than unstimulated cells. Moreover, these agonists induced striking upregulation of cell surface–bound human leukocyte elastase, even in the presence of naturally occurring proteinase inhibitors.

The priming effect of LPS was apparent with concentrations of LPS (10–100 ng/ml) that have been reported to prime PMN for other responses, such as release of reactive oxygen species (31) and lysosomal enzymes (32). However, the dose–response of the LPS priming effect was consistently biphasic, with a striking priming effect also apparent at concentrations of LPS in the range of 100 fg–10 pg/ml. We have also shown that priming of PMN with other pro-inflammatory mediators (platelet activating factor and tumor necrosis factor-α), followed by stimulation with fMLP, induces striking increases in cell surface serine proteinases; however, the priming effects of these agonists do not exhibit a biphasic dose-response relationship (Owen, C. A., submitted for publication).

The mechanism(s) underlying the biphasic dose response to LPS priming are unclear and are under investigation in this laboratory. However, we have been unable to find other studies that tested LPS concentrations in the lowest ranges that were effective in our hands. It is thus

possible that such low concentrations prime PMN for other responses as well.

The mechanisms underlying priming of PMN by LPS have not been completely elucidated. However, priming of PMN by LPS is mediated by binding of LPS to CD14 receptor (67–69). The exact signal transduction mechanisms for LPS priming of PMN remain unclear. Binding of LPS to PMN has been variably reported to be associated with: (*a*) increases in intracellular calcium concentrations (30, 70); (*b*) translocation of a guanine-nucleotide binding protein to the plasma membrane (68); (*c*) increased activity of intracellular kinases resulting in phosphorylation of proteins that are involved in cellular activation (25, 60); (*d*) increased activity of phospholipases (25, 29); and (*e*) upregulation of fMLP receptor number (34, 66).

Localization of Serine Proteinases to the Cell Surface of PMN

We confirmed that our quantitative immunofluorescence studies detected serine proteinases on the cell surface of PMN, rather than intracellular enzyme, by localizing human leukocyte elastase and cathepsin G antigens to the cell surface with immunogold particles that are too large to penetrate cells. Ultrastructural enzyme histochemistry demonstrated both cell surface localization and catalytic activity of human leukocyte elastase and cathepsin G in chemoattractant-stimulated cells (in addition to showing dense reaction product in azurophil granules). A previous report of ultrastructural histochemistry of elastase-like enzymes (20) studied only unstimulated PMN, and did not detect cell surface reaction product.

Mechanisms of Binding of Serine Proteinases to the Cell Surface of PMN

The histochemical studies suggested a mechanism for loading of the enzymes onto the cell membrane, in that dense reaction product decorated the cell surface regions around granules that were in the process of being released. Although we cannot exclude the possibility that a proportion of the serine proteinases are bound to the limiting membrane of the granules, which then becomes fused with the surface membrane upon granule extrusion, our working hypothesis is that high local concentrations of the enzymes load human leukocyte elastase and cathepsin G onto binding sites on the surface membrane. This possibility is supported by our observation that exogenous serine proteinase bind readily to sites on the external plasma membrane of PMN. In this regard, it is noteworthy that "receptors" for human leukocyte elastase have been reported on the cell surface of PMN (26). The reported human leukocyte elastase binding sites were numerous ($>10^6$ sites/cell) but displayed low-affinity binding characteristics (26). If confirmed, such low-affinity/high capacity binding sites would be ideally suited to allow substantial amounts of cell surface binding of human leukocyte elastase in regions surrounding the high concentrations (millimolar range of enzyme) present at sites of degranulation (8). Interestingly, binding of exogenously added human leukocyte elastase has been found to be reduced after exposure of PMN to phorbol ester (26), an effect that would be expected after occupation of cell surface binding sites by endogenous human leukocyte elastase released during degranulation of the cells.

It is possible that the mechanism of membrane binding is predominantly electrostatic, since serine proteinases have a highly positive charge at physiologic pH. This possibility is supported by our preliminary findings that exposure of PMN to other highly positively charged molecules such as lactoferrin, protamine and L-lysine almost completely inhibits the increased expression of cell surface human leukocyte elastase that is induced by LPS priming followed by fMLP stimulation, whereas negatively charged molecules are ineffective (Owen, C. A., unpublished observations).

The relationship of binding sites for human leukocyte elastase on PMN to those that we and others have described on macrophages (7, 12, 43) and monocytes (43) remains open to investigation. However, such receptors are similarly low in affinity ($2–4 \times 10^{-7}$ M), yet numerous ($54–73 \times 10^6$ sites per cell). In addition, the macrophage receptor has been shown to bind both human leukocyte elastase and cathepsin G, as well as lactoferrin, another azurophil granule constituent (7). It is also noteworthy that elastase-like proteinases have been found on the surface of peripheral blood monocytes (40, 41, 71).

Confirmation that Cell Surface–bound Serine Proteinases Are Catalytically Active

Catalytic activity of cell surface–bound human leukocyte elastase was confirmed by the synthetic substrate MSAPN. In addition, cell surface–bound human leukocyte elastase was active against a large molecular weight polypeptide substrate that is an important component of the extracellular matrix. When LPS-primed and fMLP-stimulated PMN were fixed, and then exposed to soluble fibronectin, fragments characteristic of human leukocyte elastase activity on SDS–polyacrylamide gel electrophoresis were produced. Proteolysis of fibronectin by fixed PMN was mediated by cell surface–bound human leukocyte elastase since: (*a*) it was completely inhibited by a specific inhibitor of human leukocyte elastase but not by a specific inhibitor of cathepsin G; and (*b*) it could not be attributed to free human leukocyte elastase activity released from the cells. In addition, we have demonstrated that human leukocyte elastase activity persists on the cell surface for at least 3 h when viable cells are incubated at 37°C following cellular activation (Owen, C. A., manuscript in preparation). Together, these data indicate that activated PMN express persistently active serine proteinases on their cell surface that are accessible to both natural and synthetic substrates. Moreover, we have obtained an estimate of catalytic human leukocyte elastase that is cell surface–bound by comparing the capacity of varying numbers of fixed cells or purified human leukocyte elastase to degrade fibronectin. We have estimated that 10^6 LPS-primed and fMLP-stimulated PMN express ~100 ng of human leukocyte elastase activity which is equivalent to the activity of ~10% of the total amount of enzyme that is contained within PMN. Artifacts caused by fixation of the cells were excluded, in that exogenously added enzyme, whether fixed on not, expressed similar catalytic activity.

Cell Surface–bound Human Leukocyte Elastase Is Substantially Resistant to Inhibition by Naturally Occurring Proteinase Inhibitors

Inhibitors of human leukocyte elastase that were fully effective against both purified enzyme and soluble human leukocyte elastase released from stimulated PMN were much less effective against human leukocyte elastase bound to the cell surface. The most striking example of this effect was seen with α_1-proteinase inhibitor. This serpin is the most potent known inhibitor of human leukocyte elastase (1), and α_1-proteinase inhibitor interaction with human leukocyte elastase has the highest reported association constant for an inhibitor with a proteinase ($K_{assoc} \sim 6.5 \times 10^7$); however, α_1-proteinase inhibitor was ineffective as an inhibitor of cell surface–bound human leukocyte elastase. When we tested inhibitors with a range of molecular sizes, there was an inverse relationship between the molecular size of the inhibitor and its capacity to inhibit human leukocyte elastase activity. Low molecular weight synthetic inhibitors such as PMSF and HLE/CMK substantially inhibited cell surface human leukocyte elastase activity, whereas inhibitors of intermediate size (ALP and SLPI) showed intermediate effectiveness. Because of the strong inverse relationship between molecular weight of inhibitors and their capacity to inhibit cell surface serine proteinases, it is likely that steric hindrance is the major mechanism by which cell surface–bound serine proteinases evade inhibition.

We were unable to test α_2-macroglobulin, the other major circulating inhibitor of human leukocyte elastase, because its mechanism of inhibition (entrapment of enzymes) leaves the enzymes active against low molecular weight substrates such as MSAPN (63). However, its very large size (725 kD) argues that it would be ineffective against cell surface bound enzymes.

Relationship to Other Cell Surface–bound Enzymes

Our study is the first to report that human leukocyte elastase and cathepsin G are expressed on the cell surface of PMN. In other cellular systems, surface–bound proteinases have been reported (18). For example, urokinase type plasminogen activator and plasmin are expressed on the cell surface of fibroblasts and mononuclear cells (17, 27, 48–50). It has been hypothesized that cell surface–bound u-PA and plasmin are ideally suited to facilitate cell migration through fibrin clots and tissue barriers. Priming of PMN with tumor necrosis factor-α followed by stimulation with interleukin-8 induces translocation of immunoreactive proteinase 3 from intracellular granules to the cell surface (21). Matrix metalloproteinases and cathepsin B exist in catalytically active form on cell surfaces (6, 44, 46); moreover, cell surface binding is a proposed mechanism for activation of latent metalloproteinases (44, 46).

Catalytically active alkaline phosphatase has been found to remain locally associated with the PMN cell membrane following specific granule release (33), and another granule constituent, lactoferrin, has been found on the cell surface of even unstimulated PMN (28). In addition, it has been reported that myeloperoxidase and lactoferrin appear locally on the surface of PMN as early as 5 s following phagocytic challenge (52). While these latter granule constituents are not proteinases, it is clear that they very rapidly appear, and remain, on the cell surface of PMN following degranulation.

Conclusions

We have shown that human PMN express cell surface–bound human leukocyte elastase and cathepsin G. Moreover, we have demonstrated that cell surface–bound elastase and cathepsin G are: (*a*) subject to regulation by physiologically important mediators that are released from sites of inflammation; (*b*) upregulated on the cell surface of PMN even in the presence of serum proteins; (*c*) accessible to and catalytically active against even large molecular weight substrates; and (*d*) substantially resistant to inhibition by naturally occurring proteinase inhibitors. Human leukocyte elastase, in particular, confers upon PMN the potential for powerful pro-inflammatory and matrix degrading capacity through its ability to degrade a spectrum of matrix components (2). Our evidence suggests that catalytic activity of these enzymes can be sharply focused by cell surface binding. This work also provides a novel, nonoxidative mechanism of preserving the catalytic activity of human leukocyte elastase and cathepsin G in the presence of extracellular proteinase inhibitors. Long-lived catalytically active cell surface–bound proteinases of PMN can facilitate their egress from the vasculature, facilitate penetration of tissue barriers, and/or allow local degradation of extracellular matrix proteins with minimal injury to the surrounding tissue. However, excessive upregulation of the expression of cell surface–bound serine proteinases, such as may occur during endotoxemia, could contribute to the pathogenesis of inflammatory diseases associated with considerable morbidity and mortality.

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