Human leukocyte elastase, cathepsin G, and lactoferrin: Family of neutrophil granule glycoproteins that bind to an alveolar macrophage receptor

(mononuclear phagocytes/proteinases/inflammation/pulmonary emphysema)

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ABSTRACT Interactions between polymorphonuclear neutrophils and mononuclear phagocytes are potentially of great importance in a variety of inflammatory processes. As part of a continuing effort to elucidate the physiologic importance of human alveolar macrophage receptor-mediated binding of neutrophil (leukocyte) elastase, I have studied the binding of leukocyte elastase and two other neutrophil granule glycoproteins, cathepsin G and lactoferrin, to human alveolar macrophages. Saturable binding of all three ligands at 0°C was observed, with equilibrium dissociation constants of 4.0×10^{-7} , 2.0×10^{-7} , and 1.7×10^{-6} M, respectively. All bound to a similar number ($54\text{--}73 \times 10^6$) of sites per cell. Binding of all three ligands was inhibited by the polysaccharide fucoidin, and extensive cross-inhibition of their binding to macrophages was observed. The results indicate that alveolar macrophages possess a relatively low-affinity, high-volume receptor for a family of neutrophil granule glycoproteins, which would be ideally suited for clearing released neutrophil granule contents from the extracellular space in inflamed tissues.

Human polymorphonuclear neutrophils (PMN) release human leukocyte elastase (HLE) and other granule constituents in response to a variety of stimuli (1–6). Removal of HLE and other proteinases from the extracellular space depends upon receptor-mediated clearance by the reticuloendothelial system (7–18). Alveolar macrophages are capable of binding and internalizing HLE by two mechanisms: (i) binding of HLE- α_2 macroglobulin complexes (14–18), which requires that the HLE first be bound to this circulating proteinase inhibitor; and (ii) binding of HLE without a requirement for previous interaction with a proteinase inhibitor (18). I have recently reviewed these two pathways for proteinase metabolism and their possible importance to lung defenses against proteolytic injury (18).

Because another PMN granule glycoprotein, lactoferrin, also binds to a specific macrophage receptor (19–21), I hypothesized that HLE and lactoferrin bind to the same macrophage receptor and that additional PMN granule glycoproteins might also bind to macrophages. The present work provides evidence to support that hypothesis. These findings are important to an understanding of PMN-macrophage interactions at inflammatory foci and suggest that cross-inhibition of macrophage binding of PMN granule glycoproteins *in vivo* may produce effects that would not be expected from *in vitro* studies utilizing single purified proteins.

METHODS

Reagents and Proteins. Fucoidin was obtained from K & K. Versilube F₅₀ is a product of Harwick (Akron, OH). Succinyl-L-alanyl-L-alanyl-L-alanyl-L-alanyl-p-nitroanilide was purchased from

Bachem (Torrance, CA). Na¹²⁵I (11–17 mCi/ μ g; 1 Ci = 3.7×10^{10} becquerels) was obtained from Amersham. Trasylol was a gift from Bayer (Wuppertal, Federal Republic of Germany). Phenylmethylsulfonyl fluoride (PhMeSO₂F), N-acetyl-DL-phenylalanine β -naphthyl ester, human transferrin, porcine pancreatic trypsin, and bovine serum albumin were purchased from Sigma. Fast scarlet diazonium salt GGN was obtained from GAF (New York).

HLE and the PMN chymotrypsin-like enzyme cathepsin G were purified from PMN obtained either from plasmapheresis of a normal volunteer (22) or from purulent sputum (23). Our purified HLE has been described (18). The purified cathepsin G migrated as a single broad band on 7.5% polyacrylamide disc gels run at pH 4 (24) and as a single band of apparent M_r 30,000 on NaDodSO₄/7.5% polyacrylamide slab gels. It readily hydrolyzed the chymotrypsin substrate N-acetyl-DL-phenylalanine β -naphthyl ester (25) but was free of HLE as judged by polyacrylamide gel electrophoresis and by nearly complete lack of activity against the synthetic HLE substrate succinyl-L-alanyl-L-alanyl-L-alanyl-p-nitroanilide (26). Lactoferrin was purified from human breast milk (27) and was fully saturated with iron. The purified lactoferrin migrated as a single band of apparent M_r 75,000 on NaDodSO₄/7.5% polyacrylamide slab gels. In agreement with the findings of others (28, 29), rabbit antibody raised in this laboratory against purified lactoferrin yielded a line of complete identity with PMN extract by double immunodiffusion analysis. The purified proteins were lyophilized and stored at -20°C until needed.

For experiments requiring inactivated enzymes, HLE and cathepsin G were treated with PhMeSO₂F as described (18). The PhMeSO₂F-inactivated enzymes were devoid of activity against succinyl-L-alanyl-L-alanyl-L-alanyl-p-nitroanilide and N-acetyl-DL-phenylalanine β -naphthyl ester, respectively.

Radiolabeling of Proteins. ¹²⁵I-Radiolabeling of HLE and cathepsin G was achieved by using the lactoperoxidase method described by Marchalonis (30). Lactoferrin was iodinated by the use of Enzymobeads (Bio-Rad). Separation of bound from free ¹²⁵I was achieved by affinity chromatography (22) with immobilized Trasylol (HLE and cathepsin G) or by gel filtration with Sephadex G-25 (lactoferrin). Greater than 95% of the ¹²⁵I in the labeled proteins was precipitated by 5% trichloroacetic acid. The labeled proteins were stored at 4°C and were used within 2 wk of iodination.

Alveolar Macrophages. Alveolar macrophages were obtained from healthy volunteers by transbronchoscopic lavage of a basilar segment of the right lower lobe as described (18). To ensure high macrophage yields, only cigarette smokers were selected for lavage. After collection, the chilled cells were cen-

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Abbreviations: HLE, human leukocyte elastase; PMN, human polymorphonuclear neutrophils; PhMeSO₂F, phenylmethylsulfonyl fluoride.

trifuged at $500 \times g$ at 4°C for 10 min and then washed three times with cold medium (Neuman & Tytell, GIBCO). The final cell suspension was held on melting ice for immediate use. Viability of the freshly harvested cells, estimated by trypan blue dye exclusion, was greater than 95%. Differential counts of Wright-stained cells deposited on a slide with a cytocentrifuge (Shandon Southern Instrument, Sewickley, PA) revealed >95% macrophages and <1% PMN.

Cell Binding of Radiolabeled Proteins. All binding assays were performed in triplicate, in 400- μl Microfuge tubes (Beckman) containing 50 μl of an inert silicone-based oil, Versilube F₅₀. To eliminate the possibility of receptor turnover and internalization, assays were carried out at 0°C . Meticulous care was necessary to achieve adequate temperature control; the assays and all reagents were held in melting ice in a walk-in cold room.

The radioiodinated proteins were adjusted to the specific activity described below by addition of unlabeled protein. Various concentrations of the three labeled proteins in phosphate-buffered saline (50 mM phosphate/0.9% NaCl, pH 7.4) were placed in Microfuge tubes, and then 10^5 chilled macrophages were added (total volume, 100 μl). The aqueous phase of the mixture was thoroughly mixed. For convenience, the binding reaction was allowed to proceed to equilibrium overnight. The tubes were then centrifuged (Microfuge B; Beckman). This caused the cells to sediment through the oil, leaving the unbound radiolabel in the aqueous phase. The tips of the tubes, containing the cell pellets, were then cut off with a scalpel and assayed for radioactivity (Gamma 8000; Beckman). The tubes were washed exhaustively with phosphate-buffered saline after the tips had been removed and then counted to determine the amount of radiolabel that was nonspecifically bound to the tubes. Free ligand was that which was not bound to either the cell pellet or the washed tubes.

All results presented have been corrected for nonspecific binding. Companion assays to determine the amount of nonspecific binding were performed, also in triplicate, for each experimental condition. Either a 100-fold excess of unlabeled ligand or the polysaccharide fucoidin (12.5 mg/ml) was added to the assay mixture before addition of the cells for these assays.

Preliminary work revealed that, in the presence of either an excess of unlabeled ligand or the polysaccharide fucoidin, binding of the ^{125}I -labeled ligands was linearly related to the concentration of labeled ligand as would be expected for nonspecific binding. Fucoidin was not toxic to the cells and did not alter the binding of labeled HLE when an excess of unlabeled HLE was present. When macrophages were exposed to fucoidin (12.5 mg/ml) at 0°C , thoroughly washed, and then exposed to labeled HLE, the HLE binding was the same as that observed when fucoidin and HLE were added simultaneously. Thus, fucoidin interacted with the cells to completely inhibit receptor-mediated binding, and binding in the presence of fucoidin provided a valid estimate of nonspecific binding.

Inhibition of Receptor-Mediated Binding. The binding assay (total volume, 100 μl) was performed as described above except that the concentration of the labeled proteins was constant at 2 $\mu\text{g}/\text{ml}$ and a 250-fold molar excess of various putative inhibitors of binding was added to the assay mixture prior to addition of the cells. In several experiments the nonbound material at the termination of the assay was applied to NaDodSO₄/7.5% polyacrylamide slab gels to exclude the possibility that proteolysis of the radiolabeled ligand might occur in the presence of an excess of HLE or cathepsin G. The gel slab was stained for protein with Coomassie blue dye and then dried. An autoradiograph was prepared by exposing Kodak X-Omat film to the gel for 24 hr.

In further experiments, various concentrations of unlabeled HLE and cathepsin G were tested for the ability to inhibit macrophage binding of a saturating concentration (6.67 μM) of lactoferrin.

RESULTS

Macrophage Binding Characteristics of Leukocyte Granule Glycoproteins. Saturable binding of all three labeled proteins was observed (Figs. 1A and 2A). Expression of these data in the form of Scatchard plots (31) allowed the binding affinities and the number of receptor binding sites for each to be estimated (Figs. 1B and 2B). The three ligands bound to a similar number of sites per cell. The binding affinities of HLE and cathepsin G were similar and were an order of magnitude greater than was observed for lactoferrin.

Cross-Inhibition of Macrophage Binding by Leukocyte Granule Glycoproteins. Complete inhibition of lactoferrin binding was produced by HLE and cathepsin G, but no significant binding inhibition was produced by transferrin, trypsin,

FIG. 1. Binding of HLE (●) and leukocyte cathepsin G (○) to alveolar macrophages at 0°C . (A) Receptor-mediated binding as a function of concentration of radiolabeled protein. Values on the ordinate were calculated from the specific activity of the radiolabeled protein and Avogadro's number. (B) Data expressed as a Scatchard plot (31), with linear regression lines determined by the data. $\bar{v}$, Molecules bound per cell; c , concentration of free labeled ligand. Specific activities of the radioiodinated HLE and cathepsin G were 290 cpm/pmol and 67 cpm/pmol, respectively; the ratios of receptor-bound to free ligand varied from 0.03 to 0.23 and 0.1 to 0.52 for HLE and cathepsin G respectively. Equilibrium dissociation constants of 4.0×10^{-7} and 2.0×10^{-7} M, with 66×10^6 and 73×10^6 binding sites per cell, were determined by linear estimates for HLE and cathepsin G, respectively.

FIG. 2. Binding of lactoferrin to alveolar macrophages. (A) Amount of receptor-mediated binding as a function of lactoferrin concentration. (B) Data expressed as a Scatchard plot (see Fig. 1). Specific activity of the radioiodinated lactoferrin was 486 cpm/pmol; the ratio of receptor-bound to free ligand varied from 0.008 to 0.043. Linear estimates gave a K_d of 1.7×10^{-6} M, with 54×10^6 binding sites per cell.

or bovine serum albumin (Fig. 3). Substantial binding inhibition was also produced by PhMeSO₂F-inactivated HLE and cathepsin G.

When HLE was used as the labeled ligand, complete binding inhibition was produced by cathepsin G and by PhMeSO₂F-inactivated HLE and cathepsin G (Fig. 4). Although lactoferrin was a poor binding inhibitor at the concentration shown, >50% inhibition of HLE binding was reproducibly observed when the lactoferrin concentration was increased by an order of magnitude. No significant binding inhibition was produced by the three control proteins.

Cathepsin G binding (Fig. 5) was substantially inhibited by HLE, by PhMeSO₂F-inactivated HLE and cathepsin G, and by lactoferrin. Minimal binding inhibition was produced by the three control proteins.

Radioautographs prepared from NaDodSO₄/7.5% polyacrylamide slab gels of the nonbound material at the termination of the assay revealed the labeled proteins that did not bind to the macrophages to be intact.

For further evaluation of binding inhibition, various concentrations of unlabeled HLE and cathepsin G were assayed for the ability to inhibit binding of a saturating concentration (6.67 μ M) of ¹²⁵I-labeled lactoferrin (the ligand with the lowest binding affinity). In preliminary experiments (data not shown), various concentrations of unlabeled lactoferrin were tested for the ability to inhibit ¹²⁵I-labeled lactoferrin binding in this assay. A

FIG. 3. Inhibition of macrophage receptor-mediated binding of lactoferrin. Binding in the absence of inhibitor = 0% inhibition, whereas binding in the presence of an excess of unlabeled lactoferrin (nonspecific binding only) = 100% inhibition. PMSF-HLE, PhMeSO₂F-inactivated HLE; CG, cathepsin G; PMSF-CG, PhMeSO₂F-inactivated cathepsin G; TF, transferrin; BSA, bovine serum albumin. Numbers near the top of the bars are the number of triplicate experiments performed. The vertical bars represent standard deviations.

smooth increase in binding inhibition was produced by increasing concentrations of unlabeled lactoferrin, which at 3.7 μ M produced 50% inhibition of binding of ¹²⁵I-labeled lactoferrin. The inhibitor concentration producing half-maximal inhibition, IC₅₀, was converted to K_i according to the equation $K_i = IC_{50}/(1 + c/K_d)$, where c is the concentration of the radiolabeled ligand and K_d is its dissociation constant (32). The calculated K_i for lactoferrin self-inhibition was 7.5×10^{-7} M, in good agreement with the previously determined K_d . These data indicated that the binding inhibition assay was valid and acceptably accurate. When HLE and cathepsin G were used as inhibitors in this assay (Fig. 6), increasing inhibition was again observed with increasing inhibitor concentration. The IC₅₀s of HLE and cathepsin G were 4.0 μ M and 1.4 μ M, respectively. The calculated inhibition constants, 8.1×10^{-7} and 2.9×10^{-7} M, respectively, were in good agreement with the previously calculated K_d s.

DISCUSSION

These results clearly demonstrate specific, saturable binding of three PMN granule glycoproteins to an alveolar macrophage

FIG. 4. Inhibition of macrophage receptor-mediated binding of HLE. LF, lactoferrin. See text and Fig. 3 for details and abbreviations.

FIG. 5. Inhibition of macrophage receptor-mediated binding of cathepsin G. See text and Fig. 3 for details and abbreviations.

surface receptor, with equilibrium dissociation constants of 4.0×10^{-7} , 2.0×10^{-7} , and 1.7×10^{-6} M for HLE, cathepsin G, and lactoferrin, respectively. All three ligands bind to the same receptor, as indicated by their extensive cross-inhibition of binding. Additional supportive data for a single binding site were (i) the inhibition of binding of all three ligands by the polysaccharide fucoidin and (ii) their binding to a similar number of sites ($54\text{--}73 \times 10^6$) per cell. All mononuclear phagocytes studied, including alveolar macrophages from nonsmokers (18), human monocytes, cells of the U-937 monocyte-like cell line, and alveolar macrophages from rats, mice, rabbits, and guinea pigs bind HLE. Thus, it is likely that this receptor is widely distributed among the cells of human and animal mononuclear phagocytes.

It is likely that receptor recognition occurs through carbohydrate moieties. It has been shown that lactoferrin clearance from the circulation by the liver occurs through recognition of terminal fucose residues in $\alpha(1\rightarrow3)$ linkage to *N*-acetylglucosamine (33); it is possible that macrophage binding of the ligands studied here occurs by the same mechanism. In our experiments, binding of HLE and cathepsin G to alveolar macrophages was inhibited by lactoferrin but not by transferrin, which differs from lactoferrin only by its lack of terminal fucose residues in $\alpha(1\rightarrow3)$ linkage to *N*-acetylglucosamine (33). Binding of these ligands clearly occurs through a different receptor from that which recognizes the neoglycoprotein fucose-bovine serum albumin and glycoproteins with mannose-terminal carbohydrate residues (34); in contrast to ligand binding to the mac-

rophage mannose receptor, binding of HLE is not inhibited by yeast mannan (18).

The number of binding sites per cell for HLE at 0°C is lower than that found at 37°C (18) but still is very large in comparison with the number of binding sites for proteinase- α_2 macroglobulin complexes (16). Directly comparable studies of lactoferrin binding to human alveolar macrophages have not been reported, but binding studies utilizing murine macrophages and human mononuclear cells performed at 37°C also have shown a very large number of sites per cell (19, 35). Aggregation of the ligands might yield a falsely high estimate of binding sites per cell, but the reproducibility of these data and similar results obtained by others lead me to believe that this estimate of the number of sites is correct. Although lactoferrin binding to human macrophages has not been reported previously, it is noteworthy that half-saturation of adherent mononuclear cells obtained from human blood was observed with $2.67 \mu\text{M}$ lactoferrin (35), in excellent agreement with the K_d of $1.7 \mu\text{M}$ reported here.

Binding of lactoferrin to macrophages has been shown to allow transfer of its iron to ferritin by the cells (20) and may inhibit macrophage release of a lymphocyte-activating factor involved in regulation of granulopoiesis (36). Lactoferrin bound and internalized by macrophages may also enhance bactericidal potency of these cells by depleting bacterial iron (37) and promoting hydroxyl radical formation (38). Direct binding of PMN proteinases to macrophages may provide an important means for their removal from the extracellular space, especially in the lower respiratory tract and the interstitial space, where α_2 macroglobulin may be absent or present in low concentrations (39). A high-volume receptor with relatively low binding affinity (in comparison to hormone receptors), such as that described here, would seem to be ideally suited to such a purpose. Recent work has shown that α_1 -proteinase inhibitor is the major HLE inhibitor in the lower respiratory tract (39), and that smokers have oxidatively inactivated inhibitor in broncho-alveolar linking fluid (40), resulting in a decrease in its functional activity (41). In smokers, macrophages thus may be important in limiting lung connective tissue injury due to released PMN proteinases. Our results also indicate that studies of macrophage binding of single purified proteins may not accurately reflect *in vivo* events.

The end result of macrophage binding of PMN proteinases depends upon the fate of internalized proteinases. This may be a very complex issue because preliminary studies indicate that HLE may have a relatively long intracellular life (42) and that macrophage injury results in release of previously internalized HLE into the extracellular space *in vitro* (43).

Because macrophage binding of lactoferrin may be important both in transmitting information (36) and in allowing ligand internalization (20), lactoferrin binding resists classification by the system outlined by Kaplan (44). Similarly, HLE binding both allows internalization of the ligand (18) and promotes release of a chemotactic factor for PMN (45). The observation that HLE binding is not dependent upon divalent cation (18) suggests that the receptor system described here may more closely resemble a "class I" receptor, but further study is needed to clarify the relationship of the receptor for PMN glycoproteins to other described receptors.

The studies described in this report suggest new PMN-macrophage interactions at inflammatory foci. It already has been demonstrated that macrophages release a chemotactic factor for PMN (46–48), which not only attracts PMN but also causes PMN degranulation. The present studies indicate that macrophages are capable of confining released PMN granule contents to a localized area by binding at least three PMN-de-

FIG. 6. Inhibition of binding of lactoferrin by various concentrations of HLE (●) and cathepsin G (○). Radiolabeled lactoferrin was at $500 \mu\text{g/ml}$ ($6.67 \mu\text{M}$). Each point represents a single assay result.

rived glycoproteins. Of additional interest in this regard would be macrophage binding of PMN collagenase and myeloperoxidase. Because myeloperoxidase is capable of oxidatively inactivating α_1 -proteinase inhibitor (49–51), it is possible that macrophages might reduce PMN-induced proteolytic tissue injury by binding not only HLE but also an oxidant that is capable of inactivating a major HLE inhibitor.

In summary, this work delineates a family of PMN granule glycoproteins that bind to a macrophage surface receptor and provides further insight into the complexity of PMN-macrophage interactions. Further progress in understanding the traffic of released PMN proteinases will be essential to the development of effective strategies for limitation of PMN proteolytic injury in diseases such as pulmonary emphysema.

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