

Human Leukocyte Elastase Is an Endogenous Ligand for the Integrin CR3 (CD11b/CD18, Mac-1, $\alpha_M\beta_2$) and Modulates Polymorphonuclear Leukocyte Adhesion

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Summary

Integrin CR3 (CD11b/CD18, Mac-1, $\alpha_M\beta_2$) mediates the transient adhesion of polymorphonuclear leukocytes (PMN) to surfaces coated with fibrinogen, C3bi, ICAM-1, and other ligands. Recent studies (Cai, T.-Q., and S.D. Wright. 1995. *J. Biol. Chem.* 270:14358) suggest that adhesion may be favored by stimulus-dependent changes in the kinetics of ligand binding by CR3. Cell detachment, on the other hand, must occur by a different mechanism because binding kinetics cannot affect cell adhesion after binding of ligand has occurred. We have sought a mechanism that would reverse binding of ligand to CR3 and report here that lysates of PMN contain an endogenous ligand that binds CR3 and competes the binding of C3bi. Purification and sequence analysis identified the structurally homologous azurophilic granule proteins, elastase, protease 3, and azurocidin as candidates. Studies with purified elastase and azurocidin showed that each bound specifically to purified, immobilized CR3. Elastase may play a role in modulating integrin-mediated cell adhesion because it is expressed at the cell surface, and the expression level is inversely proportional to cell adhesivity. Furthermore, a monoclonal antibody against elastase prevented detachment of PMN from fibrinogen-coated surfaces and blocked chemotaxis, confirming a role for this protein in regulating integrin-mediated adhesion. These studies suggest a model for release of integrin-mediated cell adhesion in which endogenous ligands such as elastase may release adhesion by "eluting" substrate-bound ligand. A role for the proteolytic activity of elastase appears likely but is not demonstrated in this study.

PMN respond to chemoattractants with a transient increase in adhesivity (1, 2). This process results in firm attachment to endothelial cells, and the subsequent decline in adhesivity allows migration of PMN out of the vasculature. Cycles of cell adhesion (at the leading edge) and release (at the uropod) are further thought to underlie locomotion of adherent PMN. Transient adhesion of PMN is mediated by the leukocyte integrin CR3 (CD11b/CD18, Mac-1, $\alpha_M\beta_2$), which exhibits rapid changes in binding to immobilized ligands such as ICAM-1, fibrinogen, and complement protein C3bi in response to chemoattractants (3–5).

CR3 can be isolated from PMN in two forms, one that binds ligand and one that does not (6, 7). Purified, inactive CR3 becomes capable of binding ligand upon treatment with cellular lipids (7) or with the activating mAb KIM-127 (6, 8). It has thus been assumed that regulated binding is a property of the CR3 itself: an increase in the affinity of CR3 for ligand could promote adherence, and a decrease could promote detachment (9).

Recent work with purified CR3, however, has challenged the view that changes in the binding affinity of

CR3 mediate both adhesion and detachment. Transformation of purified, inactive CR3 to active CR3 was found to be isoenergetic (6). Activating stimuli thus may promote binding of ligand by altering the binding kinetics rather than the affinity. Although altering binding kinetics could readily explain enhancement of cell adhesion, it cannot explain the thermodynamically unfavorable release of ligand required for cell detachment. On a cellular level, these results suggest that changes in CR3 could initiate cell adhesion but could not drive detachment without additional cellular components. We have therefore sought evidence for a species that could reverse the binding of ligand to CR3. We discovered an abundant endogenous ligand for CR3 that appears necessary for detachment of PMN from fibrinogen-coated surfaces.

Materials and Methods

Reagents and Antibodies

Reagents. Immunopure biotin N-hydroxysuccinimido (NHS) was purchased from Pierce Chemical Co. (Rockford, IL). *N*-octyl

β -D-glucopyranoside (OG),¹ activated thiol-Sepharose, *n*-succinimidyl 3-(2-pyridithio) propionate (SPDP), diisopropyl fluorophosphate (DFP), and recombinant human complement C5a were from Sigma Chemical Co. (St. Louis, MO). GammaBind G-Sepharose was from Pharmacia Biotech AB (Uppsala, Sweden). Streptavidin-conjugated horseradish peroxidase and the ECLTM Western blot detection kit were from Amersham Corp. (Arlington Heights, IL). Dulbecco's PBS was from BioWhittaker (Walkersville, MD). A fluorogenic substrate for alkaline phosphatase, Attophos, was from JBL Scientific, Inc. (San Luis Obispo, CA). All protease inhibitors, streptavidin-conjugated alkaline phosphatase, and human plasma fibrinogen (plasminogen free) were from Calbiochem-Novabiochem Corp. (La Jolla, CA). 5- (and 6-) carboxyfluorecein diacetate, succinimidyl ester (CFSE) was from Molecular Probes, Inc. (Eugene, OR). Human serum albumin (HSA) was from Armour Pharmaceutical Co. (Kankakee, IL). Human leukocyte elastase (HLE) was purchased from Elastin Products (Owensville, MS). According to the supplier, the elastase was chromatographically purified from purulent human sputum, was >95% pure, and was free from cathepsin G, myeloperoxidase, and lysozyme. Azurocidin prepared as previously described (10) was a gift from Dr. J. Gabay (Cornell University Medical College, New York).

mAbs. IB4 (anti-CD18) (5), OKM-1 (anti-CD11b) (5), 44a (anti-CD11b) (11), 3C10 (anti-CD14) (12), and 60b (anti-CD14) (13) were as previously described and are all available from the American Type Culture Collection (Rockville, MD). KIM-127 (anti-CD18) (14) was a gift from Dr. M.K. Robinson (Celltech Ltd., Berkshire, UK). NP57 (anti-HLE) (15) was purchased from the Accurate Chemical and Scientific Corp. (Westbury, NY). no. 204 (anti-HLE) was from BIODESIGN International (Kennebunkport, ME).

Polyclonal Antibodies. Rabbit anti-HLE was from BIODESIGN International. Rabbit anti-CR3 was as previously described (16). FITC-conjugated goat anti-rabbit IgG(ab')₂ was from Boehringer Mannheim Corp. (Indianapolis, IN).

Purification of Active CR3

CR3 was purified from PMN using antibody affinity chromatography as described (6). Briefly, pellets of PMN prepared as previously described (6) were lysed with buffer containing 100 mM Tris, pH 8.0, 150 mM NaCl, 2 mM MgCl₂, 2% OG, 0.02% NaN₃, and protease inhibitors for 1 h at 4°C. The lysate was then centrifuged at 35,000 *g* for 30 min. The supernatant was collected and passed over an IB4 (anti-CD18) immunoaffinity column. After a thorough wash, CR3 was eluted with buffer containing 50 mM triethylamine, pH 11.0, 150 mM NaCl, 2 mM MgCl₂, 1% OG into tubes with neutralizing buffer (15% by volume 1 M Tris-HCl, pH 7.4). CR3 was then dialyzed against PBS, 0.02% NaN₃. CR3 purified by this method will bind its ligand and is referred to as active CR3.

Biotinylation of Soluble Proteins from PMN

Pellets of PMN, prepared as previously described (6) except that all protease inhibitors were excluded, were lysed with 100 mM sodium borate, pH 8.5, 150 mM NaCl, 2% OG, for 30 min

at 4°C with stirring. The lysate was centrifuged at 35,000 *g* for 30 min. The supernatant was collected and biotinylated by addition of 2 mg/ml of immunopure biotin NHS. After 15 min incubation on ice, an equal volume of 1 M NH₄Cl was added to stop the biotinylation. Excess biotin NHS was removed by gel filtration on a NAP-25 column (Pharmacia LKB, Uppsala, Sweden) equilibrated with PBS and 0.25% OG.

Purified proteins to be biotinylated were first dialyzed against 100 mM sodium borate buffer with 150 mM NaCl, pH 8.5. After dialysis, the proteins were biotinylated as described above.

Binding of Biotinylated Soluble Proteins to Immobilized CR3

To measure the binding of biotinylated proteins to CR3, 72-well Terasaki plates (Robbins Scientific Corp., Mountain View, CA) were coated with 10 μ l/well (~10 μ g/ml) of purified CR3 diluted in PBS and 0.02% NaN₃ for 2 h at 4°C. Wells were aspirated and then blocked with 10% dry milk dissolved in PBS for 1 h at 4°C. After four washes with PBS, 10 μ l biotinylated PMN lysate or biotinylated purified proteins diluted in PBS with 0.1% of milk were added to each well. Plates were incubated at 37°C for 1 h. After four washes with PBS, 10 μ l/well of streptavidin-conjugated alkaline phosphatase (1:1,000) were added and incubated for 45 min on ice. After four washes with PBS, 5 μ l/well of a fluorogenic substrate for alkaline phosphatase (AP) were added. The fluorescence signal generated at 21°C was detected over time with a fluorescence plate reader (CytofluorTM 2300; Millipore Corp, Bedford, MA). The amount of biotinylated protein bound to CR3-coated plates was expressed as the change in arbitrary fluorescence units over a fixed time. Each sample was assayed in triplicate.

Preparation of C3bi and a Soluble, Monomeric Probe: C3bi-AP

C3bi and a soluble, monomeric conjugate of C3bi and AP were prepared as described (6). Briefly, a sulfhydryl was first introduced into AP with the bifunctional crosslinker, SPDP. C3bi with a single free sulfhydryl was purified from normal human plasma using an activated thiol-Sepharose column and further purified with a Mono Q column. Purified C3bi was then mixed with equimolar 2-pyridyl-disulfide-AP and rotated at room temperature for 24 h. Conjugated and free proteins were separated with a Mono Q column eluted with a gradient (0–1 M) of NaCl. The resulting 1:1 conjugate of C3bi and AP was verified by electrophoresis on a native acrylamide gel (8–25%) and by immunoprecipitation using mAb against C3bi as previously described (6).

Binding of C3bi-AP to Immobilized CR3

Methods for measuring the binding of C3bi-AP to CR3 were as described (6). Briefly, plates coated with CR3 were incubated with C3bi-AP for 1 h at 37°C. After four washes with PBS, 5 μ l/well of a fluorogenic substrate for AP was added. The fluorescence signal generated at 21°C was detected over time as described above. Each sample was assayed in triplicate.

Purification of a Ligand for CR3 from PMN

Two methods were used for purifying the ligand of CR3 from PMN.

Affinity Purification Using Immobilized CR3. Terasaki plates coated with or without CR3 prepared as described above were incubated with biotinylated PMN lysate (10 μ l/well) diluted in PBS

¹Abbreviations used in this paper: AP, alkaline phosphatase; CFSE, 5- (and 6-) carboxyfluorecein diacetate succinimidyl ester; DFP, diisopropyl fluorophosphate; HLE, human leukocyte elastase; HSA, human serum albumin; NHS, N-hydroxysuccinimido; OG, *N*-octyl β -D-glucopyranoside; SPDP, *n*-succinimidyl 3-(2-pyridithio) propionate.

with 0.1% dry milk at 37°C for 1 h. After four washes with PBS, 10 μ l/well of 10 mM EDTA was added and incubated for 10 min at room temperature to elute the proteins that bound to CR3. Eluates from individual wells were pooled and concentrated using a microconcentrator (centricon-10; Amicon, Inc., Beverly, MA). Samples were analyzed by gel electrophoresis.

Ion Exchange Chromatography. CR3-binding proteins were purified by two sequential chromatographic steps on Mono Q. Biotinylated PMN lysates (from $\sim 10^9$ cells) were loaded onto a Mono Q-FPLC column (Pharmacia LKB) and washed with the starting buffer (20 mM Tris-HCl, 0.25% OG, pH 7.4). Proteins bound to the column were eluted with a gradient (0–1 M) of NaCl diluted in 20 mM of Tris-HCl, 0.25% OG, pH 7.4. All fractions were tested for the ability to bind CR3. For further purification, fractions containing CR3-binding proteins were pooled, the OG content was increased twofold, and chromatography was repeated with a more shallow salt gradient. Eluates were collected for measuring the binding to immobilized CR3 and for protein analysis using gel electrophoresis.

Immunoprecipitation of Proteins from PMN Lysates

Antibodies were coupled to GammaBind G-Sepharose by incubating the beads (15 μ l) with antibodies (35 μ g in 50 μ l PBS) for 2 h at 4°C. After washing with PBS, antibody-coupled Sepharose beads were incubated with biotinylated lysates of PMN (250 μ l) for 1 h at 37°C. After thorough washing, beads were mixed with SDS sample buffer and boiled for 6 min. Polypeptides were analyzed by SDS-PAGE (8–25%) and Western blot using the Phast Gel system (Pharmacia LKB) according to the manufacturer's instructions.

Binding of PMN to Fibrinogen-coated Surfaces

Adhesion of PMN to fibrinogen-coated surfaces was measured as described (17), except that all protease inhibitors were excluded. Briefly, freshly isolated PMN (5×10^6 cells/ml) diluted in PBS, 0.1% HSA (PBS-HSA) were fluorescently labeled with CFSE. Fluorescein-labeled PMN were added to fibrinogen-coated plates and incubated at 37°C for increasing times in the presence or absence of agonists. The fluorescence in each well was measured before and after washing with a Cytofluor™ 2300 fluorescence plate reader. Binding is expressed as the percentage of cells remaining in the well after the washing step.

Transmigration of PMN across Fibrinogen-coated Surfaces

Translucent cell culture inserts (pore size 3 μ M, Becton Dickinson Labware, Bedford, MA) were coated with purified fibrinogen (200 μ g/ml diluted in PBS, 150 μ l/insert) for 1 h at room temperature and washed with 500 μ l PBS. PMN were prepared and fluorescently labeled with CFSE as described (17) except that all protease inhibitors were excluded. Fluorescently labeled PMN (200 μ l at 5×10^6 cells/ml) and mAb (10 μ g/ml) in PBS-HSA were added to the upper compartment of each insert. Chemoattractant C5a (0.5 μ g/ml) in PBS-HSA was added to the lower compartment (300 μ l/well). Plates were incubated for 50 min at 37°C. At the end of the incubation, individual inserts were gently shaken to dislodge PMN from the lower surface of the insert. The number of transmigrated PMN in individual wells was determined by measuring the fluorescence signal using a Cytofluor™ 2300 fluorescence plate reader. Results are expressed in arbitrary fluorescence units. Each sample was assayed in triplicate.

FACS® Analysis

Freshly isolated PMN (5×10^6 cells/ml) in PBS-HSA were incubated with or without C5a (0.5 μ g/ml) for increasing times at 37°C. Cells were washed and incubated with antibodies (10 μ g/ml) for 20 min on ice, washed, and further incubated with fluorescein-conjugated secondary antibody [FITC-conjugated goat anti-rabbit IgG(ab')₂, 1:200] for 45 min on ice. Cell-associated fluorescence was analyzed on a FACScan® flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA).

Electrophoresis and Western Blot

SDS-PAGE and Western blotting were done using either the Novex system (NOVEX, San Diego, CA) or the Phast Gel system according to the manufacturers' instructions. Proteins were transferred to nitrocellulose or Immobilon membranes (Millipore, Corp.), and the membranes were blocked with 10% nonfat dry milk in PBS for 1 h at room temperature. After four washes with PBS, 0.1% Tween 20, biotinylated proteins were detected by incubating the membranes with streptavidin-conjugated horseradish peroxidase (1:300 dilution) diluted in PBS, 0.1% Tween 20, for 30 min at room temperature. Membranes were washed again, and the bound streptavidin-conjugated horseradish peroxidase was detected using an enhanced chemiluminescence kit.

Amino Acid Sequencing

After SDS-PAGE, proteins were transferred to Immobilon membranes and briefly stained with Coomassie blue. The stained bands were excised and subjected to microsequencing at the Rockefeller University protein sequencing facility.

Results

PMN Contain Ligand(s) for the Leukocyte Integrin CR3. Purified immobilized CR3 was incubated with AP-conjugated C3bi (C3bi-AP) in the presence of detergent lysates of PMN or antibodies. As expected, the binding of C3bi to CR3 was completely inhibited by mAb against the β chain (IB4) or the α chain (44a) of CR3 (Fig. 1 A), enhanced by the CR3-activating mAb KIM-127 (anti-CD18), and unaffected by a control mAb 3C10 (anti-CD14). Addition of PMN lysates also completely blocked binding of ligand C3bi (Fig. 1 A, last column on the right), suggesting the presence of a competing ligand. To confirm the presence of such a ligand, PMN lysates were biotinylated and incubated with immobilized CR3, and bound ligand was detected with AP-conjugated avidin (Fig. 1 B). Biotinylated soluble proteins from PMN bound significantly better to immobilized CR3 than to uncoated plastic, and binding was completely inhibited by a mAb against CR3 (44a), whereas a control mAb (60b, anti-CD14) was ineffective. The binding was enhanced by the CR3-activating mAb KIM-127. Additional studies showed that this binding was also inhibited by EDTA and an excess amount of soluble C3bi (not shown). These data thus establish the existence of a ligand for CR3 in PMN.

Identification of an Endogenous Ligand for CR3. Because biotinylated soluble proteins from PMN bind specifically to plates coated with CR3 (Fig. 1 B), we have adapted this binding assay for ligand identification. Biotinylated PMN

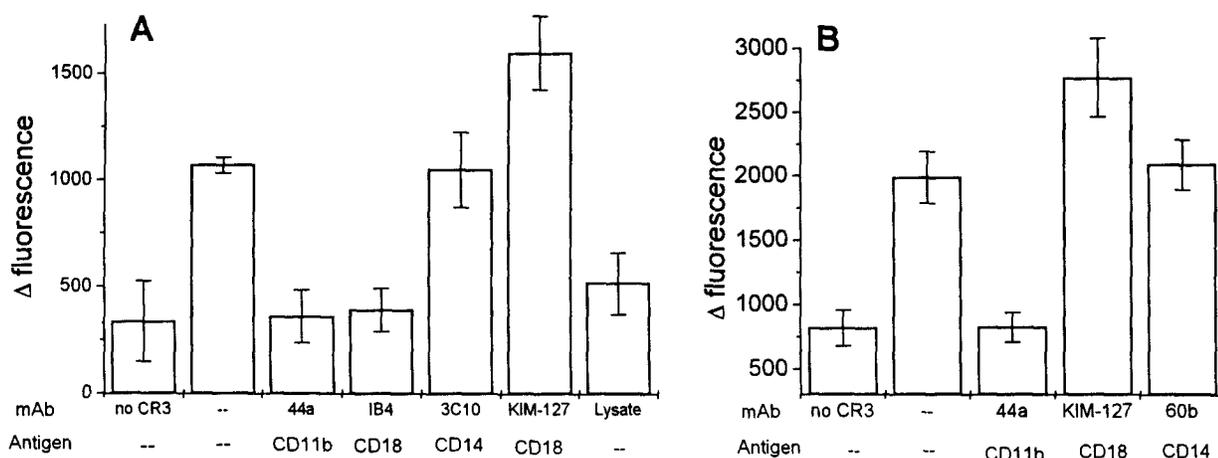


Figure 1. Soluble proteins from PMN bind specifically to immobilized CR3. Plastic plates coated with or without purified CR3 were incubated for 1 h at 37°C with (A) C3bi-AP (15 nM) in the presence of the indicated mAbs (10 μg/ml) or PMN lysates (equivalent to 10⁷ cells/ml); or with (B) biotinylated PMN lysates (equivalent to ~5.0 × 10⁶ cells/ml) in the presence of the indicated mAbs (10 μg/ml). The binding of C3bi-AP or biotinylated PMN lysate was quantitated by enzyme assay as described in Materials and Methods. The results shown are representative of three independent experiments.

lysate was incubated with plates coated with or without CR3 in the presence of a blocking (IB4) or an activating mAb (KIM-127). After thorough washing, the proteins bound to individual wells were eluted by adding 10 mM EDTA. Eluates were pooled, concentrated, and analyzed by Western blot (Fig. 2). In the presence of KIM-127, incubation of PMN lysate with CR3-coated plates resulted in a specific enrichment of a single band with a molecular mass of 28 kD. This 28-kD band was absent or present in much lower amounts on plates without CR3 coating or on plates treated with a blocking anti-CR3 mAb (IB4). These data suggest that a 28-kD protein from PMN is a candidate ligand for CR3.

To obtain sufficient material for sequencing, a biotinylated PMN lysate was subjected to chromatography, and fractions were assayed for their ability to bind CR3 (see Materials and Methods). Assays for binding to CR3-coated plates in the presence of a blocking mAb (44a) or an activating mAb (KIM-127) revealed two regions of activity with peaks at fraction numbers 27 and 41 (Fig. 3 B). Fraction 27 contained several species, including a band at 28 kD. In fraction 41, on the other hand, a single band at 28 kD predominated (Fig. 3 C). Microsequencing of the NH₂ terminus for this band revealed high similarity to a group of azurophilic granule proteins including HLE (18), protease 3 (NP3) (19), azurocidin (20), and cathepsin G (21) (Fig. 4). These proteins are characterized as neutral serine proteases (except azurocidin, which has no enzyme activity) and are normally stored at high concentration (1–2 pg/cell) in the azurophilic granules of PMN (for review see references 22–24). The sequence data lead to the hypothesis that azurophil granule proteases may interact with CR3, and this hypothesis was tested using purified proteins.

Purified HLE Binds CR3 Specifically. Although NP3 showed the best apparent match to our sequence data (Fig. 4), we focused our attention first on HLE, the best charac-

terized protein in this family. Purified biotinylated HLE bound specifically to immobilized CR3 (Fig. 5 A). The binding was inhibited by blocking mAbs against CR3 (IB4, 44a), enhanced by an activating mAb (KIM-127), and unaffected by a control mAb (3C10). Binding was also blocked by a mAb directed against HLE (NP57). NP57 appears to block binding by interacting with a specific site on HLE, because a second anti-HLE mAb, no. 204, failed to block binding to CR3 (not shown). The binding of HLE to CR3 was concentration dependent (Fig. 5 B), and the concentration of HLE required for half maximal binding was ~1 μM, suggesting that the binding was of fairly low affinity. Additional studies showed that the binding of HLE

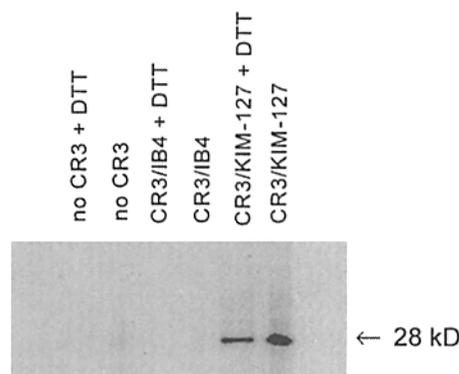


Figure 2. Identification of a ligand for CR3 from PMN. Plates coated with or without CR3 were incubated with biotinylated PMN lysate for 1 h at 37°C in the presence of buffer, mAb IB4, or KIM-127 (10 μg/ml). After thorough washing, the proteins bound to individual wells were eluted with 10 mM EDTA. Eluates from 216 Terasaki wells (10 μl/well) were pooled, concentrated, analyzed by SDS-PAGE (4–20%) under reducing (with dithiothreitol [DTT]) or nonreducing conditions, and Western blotted with streptavidin-conjugated horseradish peroxidase as described in Materials and Methods.

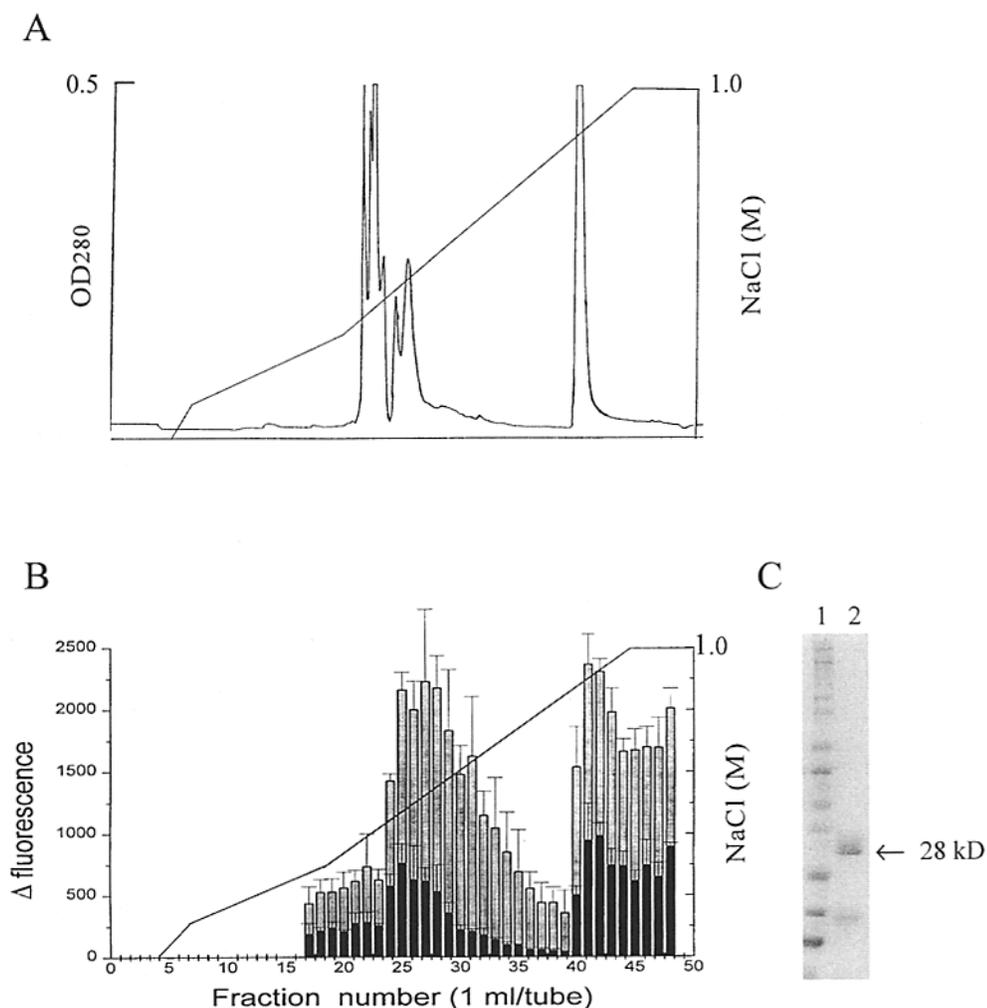


Figure 3. Purification of a ligand for CR3 from PMN. Biotinylated PMN lysates (from $\sim 10^9$ cells) were injected onto a Mono Q column and eluted with a gradient of NaCl (0–1 M) (not shown). Fractions containing CR3-binding proteins, determined by measuring the binding to immobilized CR3 as described in Materials and Methods, were pooled, the detergent (OG) content was increased twofold, and chromatography was repeated with buffer containing 0.5% OG and with a more shallow salt gradient ([A] OD₂₈₀ of eluted proteins). (B) Fractions from A were tested for their ability to bind immobilized CR3 in the presence of mAb 44a (10 μ g/ml; black bars) or KIM-127 (10 μ g/ml; gray bars). (C) Fraction number 41 from A, corresponding to 0.87 M of NaCl, was analyzed by SDS-PAGE (4–20%) and stained with Coomassie brilliant blue (Lane 1) molecular mass markers; (lane 2), fraction number 41.

to CR3 was also dependent on divalent cations and that purified HLE inhibited the binding of C3bi or biotinylated PMN lysate to CR3 in a concentration-dependent manner (data not shown). Taken together, these data indicate that HLE can bind to CR3 in vitro.

Additional studies suggest that HLE binds CR3 on PMN. Immunoprecipitation of HLE from PMN lysates with mAb no. 204, a mAb which does not block the binding of HLE to CR3, yielded not only HLE (not shown) but also CR3 (Fig. 5 C). The amount of CR3 precipitated with anti-HLE was less than the amount precipitated with a monoclonal anti-CR3, suggesting that not all of the CR3 is occupied by HLE. It is interesting to note that only the β chain of CR3 was detected in the anti-HLE immunoprecipitates. A potential explanation of these findings is proteolytic cleavage of the α chain by HLE during sample preparation.

Role of Proteolytic Activity in Binding of HLE to CR3. HLE is a potent serine protease. The presence of a catalytic triad that consists of three active site residues (His-57, Asp-102, and Ser-195) enables HLE to cleave a large variety of substrates (for review see reference 23). To determine if the

active site of HLE is involved in binding to CR3, we measured the binding of HLE to CR3 in the presence of the serine protease inhibitor DFP, which covalently modifies Ser-195. As shown in Fig. 6, addition of DFP completely blocked the binding of HLE to CR3. In additional experiments, we tested the effect of the naturally occurring HLE inhibitors, α -2-macroglobulin and α -1-antitrypsin (25), and found that both blocked the binding of HLE to CR3 (not shown). These results suggest that reactants that occupy the active site of HLE prevent binding to CR3. Con-

	I	V	X	H	E	A	Q	P	I	Q	P	Y	M	A	F	L	Q		
Sample	L			L	I		D	G	N	S	R	G		D	M	N	V		
HLE	I	V	G	G	R	R	A	R	P	H	A	W	P	F	M	V	S	L	Q
Proteinase 3	I	V	G	G	H	E	A	Q	P	H	S	R	P	Y	M	A	S	L	Q
Cathepsin G	I	I	G	G	R	E	S	R	P	H	S	R	P	Y	M	A	Y	L	Q
Azurocidin	I	V	G	G	R	K	A	R	P	R	Q	F	F	F	L	A	S	I	Q

Figure 4. Comparison of the NH₂-terminal sequences obtained from fraction 41 in Fig. 3 with those for known azurophilic granule proteins (18–21). Sequence analysis of fraction 41 resulted uncertainty with some amino acids as indicated.

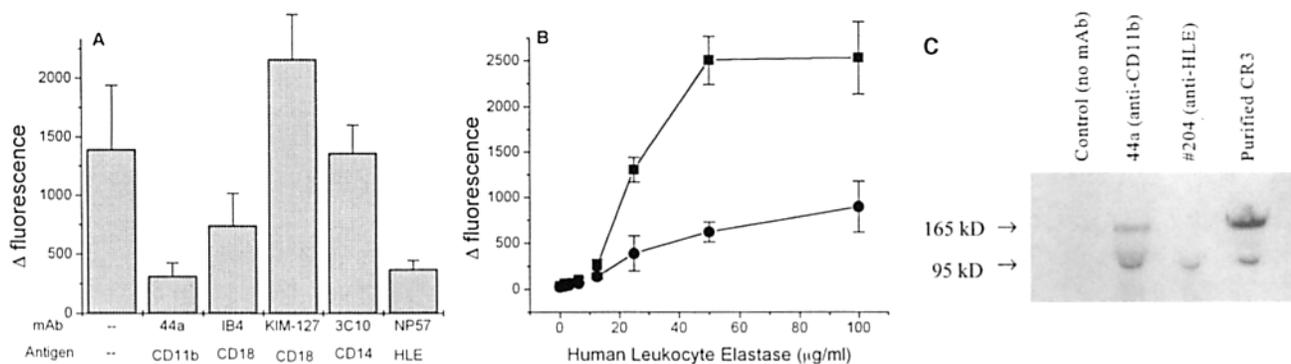


Figure 5. HLE binds specifically to CR3. Plates coated with CR3 were incubated with (A) 25 $\mu\text{g/ml}$ of biotinylated HLE in the presence of the indicated mAbs (25 $\mu\text{g/ml}$) or (B) increasing concentrations of biotinylated HLE in the presence of buffer (■) or mAb 44a (25 $\mu\text{g/ml}$; ●). Binding of biotinylated HLE was quantitated as described in Materials and Methods. (C) Protein G-Sepharose beads coated with the indicated mAbs were incubated with a biotinylated lysate of PMN for 1 h at 37°C. Proteins bound to the beads were analyzed by SDS-PAGE (8–25%) and Western blotting with anti-CR3 as described in Materials and Methods.

sistent with these findings, we found that NP57, a mAb which blocked the binding of HLE to CR3 (Fig. 5 A), also blocked the proteolytic activity of HLE (not shown).

To determine if protease activity per se is necessary for binding to CR3, we explored the binding activity of azurocidin. This protein has high sequence homology (45%) to HLE but is proteolytically inactive because two of the active site residues of the catalytic triad (His-57 and Ser-195) are altered (23, 10). We found that purified biotinylated azurocidin bound specifically (Fig. 7 A) and saturably (Fig. 7 B) to immobilized CR3. The avidity of azurocidin for CR3 (~ 250 nM for half maximal binding) appeared slightly greater than that for HLE (~ 1 μM for half-maximal binding). In keeping with its lack of proteolytic activity, DFP showed no effect on the binding of azurocidin to CR3. Specificity of the azurocidin binding was shown by inhibition with a blocking mAb against CR3 (44a) and en-

hancement with activating mAb KIM-127. These results thus indicate that neither proteolytic activity nor residues His-57 or Ser-195 of the catalytic triad are necessary for binding to CR3.

Regulation of PMN Adhesion and Chemotaxis by HLE. Previous studies have demonstrated strong expression of HLE at the surface of PMN (26) as well as the release of HLE from PMN and monocytes in response to a variety of stimuli (27). To determine if HLE could interact with cell surface CR3 in a way that affects cell adhesion, we first measured surface expression of HLE and CR3 on PMN exposed to C5a (Fig. 8). These studies used a polyclonal anti-HLE that did not block binding of HLE to purified CR3 (not shown). As expected, resting PMN expressed a high level of CR3, and stimulation of PMN with C5a increased surface level of CR3 (Fig. 8 A). We found that resting PMN also expressed a significant level of HLE (Fig. 8 B) consistent with recent findings (26). Stimulation of PMN with C5a led to an extremely rapid loss of surface HLE. PMN washed immediately after the addition of C5a showed significant loss of HLE (0 min; Fig. 8 B). This was followed by a time-dependent recovery of cell surface HLE, presumably derived from secreted granules. The rapid loss of cell surface HLE occurred in advance of enhanced CR3-mediated adhesion, and the recovery of cell surface HLE occurred in advance of cell detachment (see Fig. 9). These data are thus consistent with the hypothesis that HLE could function in the regulation of CR3-mediated cell adhesion.

To determine the role of HLE in PMN adhesion, we measured the binding of PMN to fibrinogen-coated surfaces (Fig. 9). Consistent with reported data (2), addition of C5a led first to strong PMN adhesion by 10 min and detachment by 40 min. As expected, an anti-CR3 mAb completely blocked adhesion (not shown), whereas a control mAb (anti-CD14) had no effect. Addition of a mAb against HLE (NP57) that blocks its interaction with CR3 in vitro had two effects on cell adhesion. When PMN were treated with C5a and NP57, the mAb completely blocked the detachment of PMN from the fibrinogen-coated surfaces.

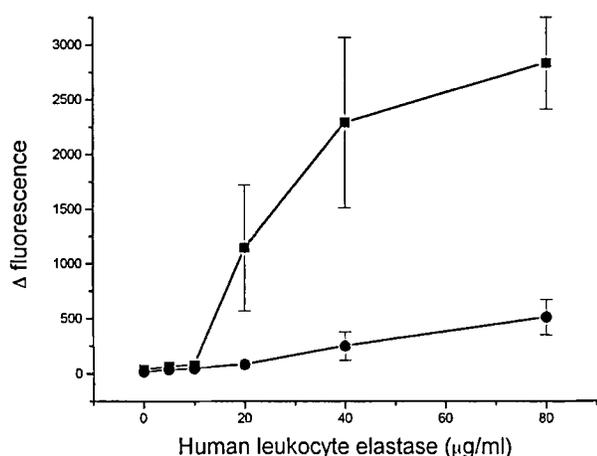


Figure 6. Effect of protease inhibitor on the binding of HLE to CR3. Plates coated with CR3 were incubated with increasing concentrations of biotinylated HLE in the presence (●) or absence (■) of protease inhibitor DFP (5 mM) for 1 h at 37°C. After thorough washing, bound biotinylated HLE was quantitated as described in Materials and Methods.

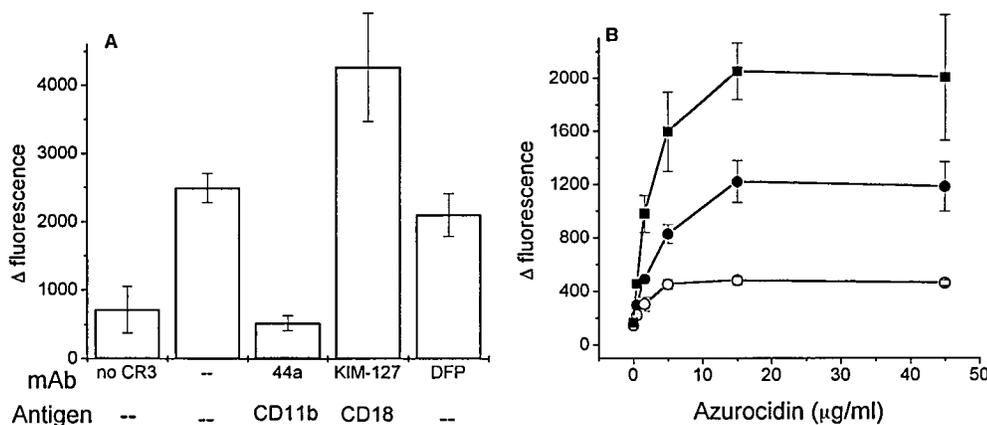


Figure 7. Azurocidin binds specifically to CR3. Plates coated with CR3 were incubated for 1 h at 37°C with (A) 15 μg/ml of biotinylated azurocidin in the presence of indicated mAbs (10 μg/ml) or protease inhibitor DFP (5 mM) or (B) increasing concentrations of biotinylated azurocidin in the presence of buffer (●), mAb IB4 (200 μg/ml; ○), or mAb KIM-127 (10 μg/ml; ■). Binding of biotinylated azurocidin to CR3 was quantitated as described in Materials and Methods.

This finding strongly implicates HLE in release of leukocyte integrins from their substrate. Second, PMN incubated with NP57 in the absence of stimulation showed a slow, spontaneous binding to fibrinogen. This finding is consistent with the observation that there is HLE on the surface of resting PMN and suggests that HLE may play a role in the control of CR3 binding activity even in resting cells. Because NP57 and CR3 compete for binding HLE, NP57 may promote the dissociation of HLE from CR3, leaving the CR3 free to bind ligand.

Movement of PMN out of the vasculature involves a firm adhesion followed by a prompt release of the adherent cells. To determine if the effect of anti-HLE antibody on PMN adhesion would also influence the migration process, we measured the transmigration of PMN across fibrinogen-coated filters (Fig. 10). In response to the chemoattractant C5a, PMN move across the membranes and into the lower chamber of the chemotaxis apparatus. As expected, migration of PMN was completely blocked by mAb anti-CR3 (IB4), because anti-CR3 prevents the initial attachment of PMN to fibrinogen-coated membranes. Addition of a mAb against HLE (NP57) decreased transmigration of PMN by 40%. No inhibition was observed when a control mAb (60b) was added. Because NP57 blocked detachment of adherent cells from fibrinogen-coated surfaces (Fig. 9), the

decrease in transmigration may derive from an absent or delayed release of the adherent PMN from the substrates.

Discussion

HLE Is an Endogenous Ligand for CR3

We have shown that PMN contain endogenous ligands for CR3 and that HLE and azurocidin are among these ligands. Both of these proteins bound specifically to purified CR3, and preliminary data suggest that NP3 may bind as well (Cai, T.-Q., and S.D. Wright, unpublished observations). HLE, NP3, and azurocidin are structural homologues and are stored together in azurophilic granules. These proteins may act as a class to ligate CR3.

We have also shown that HLE modulates leukocyte adhesion. Blockade of HLE with mAb NP57 prevented detachment of PMN from a fibrinogen-coated surface (Fig. 9) and decreased chemotactic movement of PMN across a fibrinogen-coated surface (Fig. 10). Because cell surface HLE increases before cell detachment (Fig. 8), our results are consistent with the hypothesis that HLE may normally act to weaken integrin-mediated attachment of cells to the substrate by acting as a competitive ligand. HLE may also affect adhesion through proteolytic cleavage of substrate

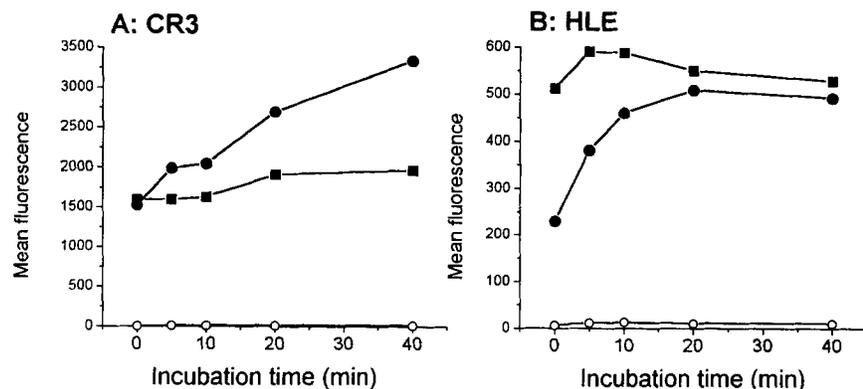


Figure 8. Expression of CR3 and HLE on PMN. PMN (5×10^6 cells/ml) were incubated with (●) or without (■) C5a (0.5 μg/ml) for the indicated amount of times at 37°C. Cells were washed and stained with no 1^{st} antibody (○), rabbit anti-CR3 (A) or anti-HLE (B) as described in Materials and Methods. Cell-associated fluorescence was analyzed on a FACScan® flow cytometer. Results were expressed by mean fluorescence intensity.

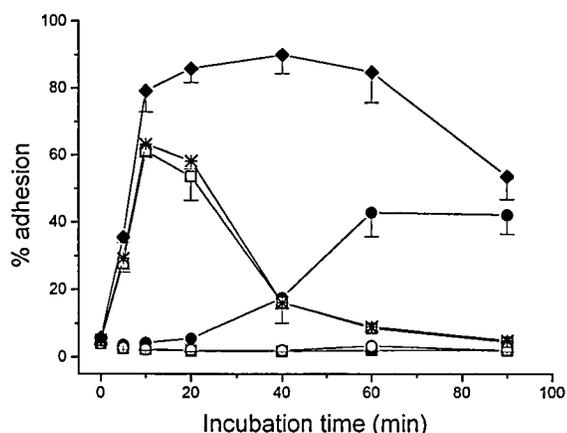


Figure 9. Effect of anti-HLE on the adhesion of PMN to fibrinogen-coated surfaces. Plates coated with fibrinogen were incubated with fluorescently labeled PMN for increasing amounts of times at 37°C in the presence of buffer (■), NP57 (10 µg/ml, ●), 60b (10 µg/ml; ○), C5a (0.5 µg/ml, □), C5a plus NP57 (◆), or C5a plus 60b (*). The percentage of PMN adhered to the plates was quantitated as described (17).

proteins. We wish to point out that NP57 reduced chemotaxis by ~40% but did not block it completely. Proteins other than HLE may thus participate in detachment of PMN from surface-bound ligands, a result consistent with a role for azurocidin, NP3, and perhaps other proteins in this function. Recent studies have suggested that heparin may serve as a ligand for CR3 (28), and release of heparin from PMN granules may further promote detachment.

Role for Proteolytic Activity of HLE

Previous authors have suggested that HLE could play a role in transmigration of PMN by digesting proteins in the cell's path (25, 29). L658,758, a specific, low-molecular-weight, irreversible, active site inhibitor of HLE, was shown

to block migration of PMN into tissues in response to PAF (30) and reperfusion (29). This inhibitor did not affect cell rolling (30), suggesting that it did not act on selectins, and it did not block binding of PMN to substrates *in vitro* (29, 30), suggesting that it did not block the binding function of integrins. Woodman et al. (30) concluded that "elastase selectively enhances or regulates CD18-dependent chemotaxis." Results reported here using the anti-HLE mAb NP57 are completely consistent with the earlier findings: blockade of HLE with this mAb impaired chemotaxis (Fig. 10) but did not block integrin-dependent cell adhesion (Fig. 9). Our findings strengthen the conclusions of prior work, and the *in vivo* studies with L-658,758 suggest an important role for HLE in modulating PMN emigration.

Our finding that HLE binds directly to CR3, the major adhesion protein of PMN, suggests a mechanism by which this protein affects PMN emigration. HLE may act not just to digest extracellular material but also to release integrins from the substrate and permit forward motion, a conclusion supported by the observation that NP57 blocks release of PMN from a fibrinogen-coated surface (Fig. 9). It is important to point out that both NP57 and active site inhibitors simultaneously block two functions of HLE: binding to CR3 and proteolytic activity. The individual contribution of these two activities thus cannot be separated.

A key question raised by our studies is whether HLE retains its proteolytic activity when bound to CR3. On one hand, Altieri and colleagues (31, 32) showed that the serine protease, Factor X, is a ligand for CR3 and that binding to CR3 modulates but does not block the proteolytic activity of Factor X. Moreover, HLE present at the PMN surface has been shown to be proteolytically active against small molecule substrates (26). On the other hand, there are many more binding sites for HLE on the PMN surface than molecules of CR3 (26, 33), and it is not clear whether the active cell surface HLE is bound to CR3 or to some

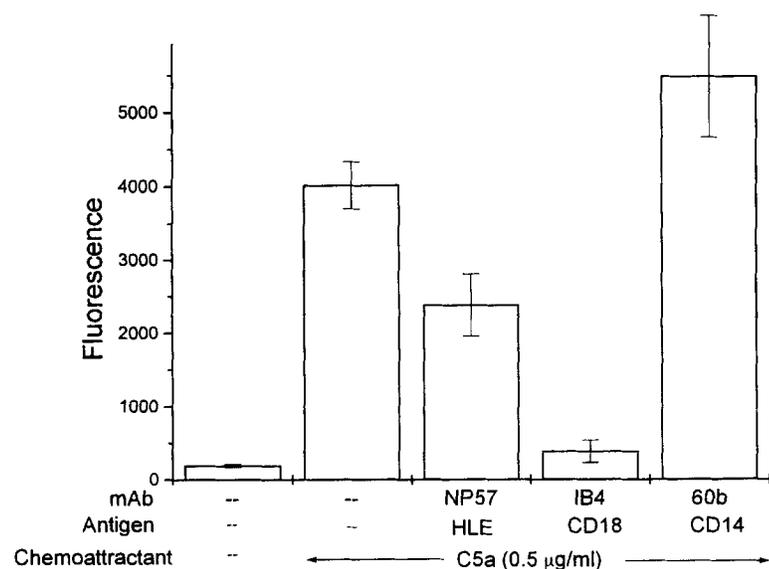


Figure 10. Effect of anti-HLE on the transmigration of PMN across fibrinogen-coated surfaces. Fluorescently labeled PMN (5×10^6 cells/ml) and the indicated mAbs (10 µg/ml) were added to the upper compartment of cell culture inserts coated with fibrinogen. Chemoattractant C5a (0.5 µg/ml) was added to individual wells of the companion plate. After a 50-min incubation at 37°C, the inserts were removed, and the number of PMN migrated through the inserts was quantitated as described. Results were expressed by arbitrary fluorescence units.

other structure. Additionally, occupation of the active site of HLE with the small molecule, DFP, completely blocked binding to CR3 (Fig. 6). This observation suggests that the active site of HLE is directly involved in interaction with CR3 and would thus be unavailable for interaction with substrates. Studies are underway to address this issue. It is noteworthy that recent work from several laboratories has described a new class of proteins that express both an integrin-binding domain and a protease domain (for review see reference 34). A clear function for this group of proteins has not been described, but the finding raises the possibility that association with a protease may be a general feature of integrins.

A New Model for Regulation of PMN Adhesion

Cell Attachment to Substrate. Stimulation of PMN adhesion clearly involves alterations in the binding properties of CR3 itself. Stimulation of cells with chemoattractants or PMA causes CR3 to exhibit neoepitopes recognized by certain mAbs (35, 36), and the anti-CR3 mAb KIM-127 causes both enhanced binding of cells to substrate (14) and enhanced binding of purified CR3 to purified ligand (6). Although the change in CR3 caused by cell stimulation has been frequently described as an enhancement in affinity, our recent work suggests that binding kinetics, not affinity, may be altered (6). Activation of purified CR3 by KIM-127 was found to be isoenergetic, a result incompatible with a significant change in binding affinity. Moreover, the activation energy for binding of C3bi to active CR3 was found to be very high (15 kcal/mol), suggesting that the kinetic barrier to ligand binding is very significant and that a relatively slight increase in this barrier could effectively halt ligand binding in the time range (minutes) available to a neutrophil on a substrate. The model that has emerged from these studies is that cell stimulation enhances the rate of ligand binding by CR3 and thereby enables binding of cells to substrate-bound ligand. Our finding of endogenous ligands for CR3 does not significantly affect this portion of

the model: whether inactive receptors are occupied by HLE or not, they are equally incapable of interacting with substrate-bound ligand because of slow binding kinetics. Upon activation of cells, the accelerated binding kinetics of CR3 should give HLE the opportunity to disassociate and/or to equilibrate with other ligands. It should be noted that the affinity of CR3 for C3bi (15 nM) is at least 50-fold greater than that for HLE. Binding of CR3 to substrate-bound C3bi may thus be favored.

Cell Detachment. In contrast with their small effect on initiating cell attachment, endogenous ligands are likely to play a large role in reversing cell adhesion. Binding of CR3 to the representative ligand, C3bi, occurs with a high affinity (15 nM). Alterations in binding kinetics can have no effect once this energetically favorable reaction is completed, and alterations in binding kinetics cannot cause cell detachment. As we have pointed out previously (6), an additional step, distinct from the energetically neutral effect on integrin activation, is required to reverse adhesion. We suggest that endogenous ligands such as HLE may accomplish this step. Azurophilic granules contain very high concentrations of HLE, NP3, and azurocidin. Fusion of granules near sites of adhesion may "elute" substrate-bound ligand from CR3 and cause detachment. Consistent with this model, our data show that blockade of HLE with a mAb blocks detachment of PMN from a fibrinogen-coated surface (Fig. 9). As discussed above, the proteolytic activity of these proteins may have an additional effect on adhesion.

In summary, these studies suggest a novel mechanism for controlling CR3-mediated adhesion. Because other integrins, such as LFA-1 of lymphocytes and gpIIb/IIIa of platelets, show behavior remarkably similar to that of CR3 (37, 38), it will be of interest to determine if endogenous ligands could play a role in regulation of these molecules. Because HLE and other granule constituents may accumulate to high levels in diseases such as cystic fibrosis (39, 40), our observations may also provide a link to the pathogenesis of some inflammatory diseases.

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