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Research Article

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CAP37, a Human Neutrophil-derived Chemotactic Factor with Monocyte Specific Activity

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Abstract

CAP37, an antimicrobial protein of human neutrophil granules, is a specific chemoattractant for monocytes. Purified to homogeneity by sequential chromatography over carboxymethyl Sephadex, G-75 Sephadex, and hydrophobic interaction HPLC, demonstratively endotoxin-free CAP37 was maximally chemotactic over a range of 1.3×10^{-9}–10^{-8} M. Thus it was active in the same molar concentrations as formyl-methionyl-leucyl-phenylalanine. CAP37 lacked chemotactic activity for neutrophils and lymphocytes. In checkerboard assays CAP37 had some chemokinetic activity as well. It was also chemotactic for rabbit mononuclear cells. Higher concentrations (2.7×10^{-8} M) were required for activity with rabbit cells than with human. Sequence analysis of the first 42 NH_2-terminal amino acid residues of CAP37 showed strong homologies with known serine proteases that mediate various functions in inflammation. However, a critical substitution of a serine for a histidine at position 41 suggested that CAP37 lacked serine protease action. This impression was supported by the failure of CAP37 to bind tritiated diisopropyl fluorophosphate. 89% of total CAP37 was released extracellularly from human neutrophils while they phagocytized *Staphylococcus aureus*. We propose that CAP37 released from neutrophils during phagocytosis and degranulation may mediate recruitment of monocytes in the second wave of inflammation. (*J. Clin. Invest.* 1990. 85:1468–1476.) cationic protein • inflammation • serine protease • antimicrobial activity • degranulation

Introduction

A second wave of monocytes soon follows the initial wave of PMN typical of the inflammatory response (1, 2). Although it is a widely held view that they do so as a result of chemotaxis (3), the specific mediator(s) responsible for the recruitment of this single cell type remains unresolved. This preferential migration of monocytes during the latter phase of inflammation indicates the requirement for a highly cell-specific chemoattractant, which has little or no effect on the migration of PMN. Accumulating evidence supports the concept that a factor(s) derived from the PMN may be responsible for the mobilization of monocytes during the second wave of inflammation (4–7).

We show here that a granule-associated cationic protein (molecular mass, 37,000 D) purified from human PMN acts as a monocyte-specific chemoattractant. This protein, CAP37 (cationic antimicrobial protein of molecular mass 37,000 D) possesses substantial antimicrobial activity against a number of gram-negative bacteria (8). Here we report on its potent monocyte-specific chemotactic activity, cellular localization, purification, NH_2-terminal amino acid sequence, incapacity to bind diisopropyl fluorophosphate (DFP),[1] and 89% release from PMN during phagocytosis of *Staphylococcus aureus*.

Methods

Source and preparation of peripheral blood PMN and monocytes. Blood was collected by venipuncture from healthy adult donors into sterile sodium-EDTA tubes. The PMN were separated from the mononuclear cells by a modification of the Boyum (9) Ficoll-Hypaque density gradient technique, followed by dextran sedimentation (3% T500 in saline; Pharmacia Fine Chemicals, Piscataway, NJ) and hypotonic lysis of contaminating red blood cells (RBC). For the immunocytochemical studies the PMN were suspended at 1×10^6 cells/ml in 10% heat-inactivated fetal bovine serum (Hyclone Laboratories Inc., Logan, UT) in 0.01 M PBS containing 0.15 M NaCl, pH 7.4. For the chemotaxis studies the PMN were suspended in Geys' buffered saline (Gibco Laboratories, Grand Island, NY) containing 2% BSA (fraction V, endotoxin free; Boehringer Mannheim Biochemicals, Indianapolis, IN) at a final concentration of 2×10^6 cells/ml.

The mononuclear band obtained in the Ficoll-Hypaque density gradient separation was further purified to separate the monocytes from the lymphocytes (PBL). The mononuclear cells were washed once in PBS and then resuspended to a volume of 5.1 ml in PBS. This cell suspension was then added to 6.7 ml of Sepracell-MN reagent (Sepratech Corporation, Oklahoma City, OK) and centrifuged (1,500 *g* for 20 min at 22°C). The cells were washed twice in PBS (150 *g* for 15 min) and resuspended in Geys' buffered saline containing 2% BSA at a final concentration of 2×10^6 cells/ml. The monocytes were > 95% pure by Wright's and nonspecific esterase staining.

Mixed mononuclear cells from peripheral blood from adult female rabbits were separated on a 61% Percoll (Pharmacia Fine Chemicals) gradient (10). This technique separated the mononuclear cells from the RBC and PMN. No further separation of monocytes from PBL was undertaken with the rabbit blood.

This work was presented in part at the 73rd Annual Meeting of the Federation of American Societies for Experimental Biology, New Orleans, LA, 19–23 March 1989, and at the 29th Interscience Conference on Antimicrobial Agents and Chemotherapy, Houston, TX, 17–20 September 1989.

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1. *Abbreviations used in this paper:* CAP, cationic antimicrobial protein; CGE, crude granule extract; CMS, carboxymethyl Sephadex; DFP, diisopropyl fluorophosphate; MPO, myeloperoxidase; RBC, red blood cell; RMCP, rat mast cell protease.

Purification of CAP37. The extraction of CAP37 from human PMN and its purification by ion exchange chromatography (carboxymethyl Sephadex, CMS C-50; Pharmacia Fine Chemicals) and molecular sieve chromatography (Sephadex G-75 Superfine; Pharmacia Fine Chemicals) have been previously described (11, 12). Briefly, PMN were obtained by leukapheresis and the cytoplasmic granules treated with DFP to inactivate serine esterases (12). After differential centrifugation, crude granule extracts (CGE) were prepared and concentrated from these granules using 0.2 M sodium acetate buffer (pH 4.0). This provided the starting material for CMS ion exchange chromatography. The fractions obtained from this column were screened using ELISA (13). Those fractions positive for CAP37 and CAP57 (cationic antimicrobial proteins of molecular mass 37,000 and 57,000 D, respectively) were pooled, concentrated, and applied to a Sephadex G-75 column. The elution profile from this column has been described previously (12). The fractions were analyzed by SDS-PAGE and ELISA and a total of 15 fractions corresponding to peak C (12) were shown to be highly enriched for CAP37. For the chemotactic assays, the CAP37-enriched fraction obtained from the molecular sieve column was further processed using hydrophobic HPLC (Biogel TSK phenyl 5PW column, 7.5 × 0.75 mm; Bio-Rad Laboratories, Richmond, CA). The proteins were eluted using a 60-min linear gradient from 1.7 to 0 M $(NH_4)_2SO_4$, which contained 0.1 M sodium phosphate, pH 7.0. The recovery was determined by the optical density of the proteins at 210 nm. The fractions obtained from the hydrophobic HPLC column were pooled independently and the $(NH_4)_2SO_4$ was dialyzed out by using a stirred cell concentrator (YM 5 membrane, molecular mass cut off 5,000 D; Amicon Corp., Danvers, MA) using 0.2 M sodium acetate buffer, pH 4.0 (12). The purity of the fractions was determined by SDS-PAGE, Western blot analysis, and ELISA.

Amino acid sequence analysis. The purified CAP37 obtained from the hydrophobic interaction HPLC was desalted by reverse-phase HPLC on a C8 Synchropak column (4.6 × 250 mm; Hewlett-Packard Co., Avondale, PA). The column was equilibrated with 0.1% trifluoroacetic acid in water and the protein eluted with a 0–60% gradient of 0.1% trifluoroacetic in acetonitrile over 60 min at a flow rate of 1 ml/min. The material in the peak at 41 min was concentrated under vacuum (Speed Vac; Savant Instruments, Inc., Hicksville, NY). The amino terminus sequence of the purified CAP37 was determined using the 477A pulse liquid protein sequencer (Applied Biosystems Inc., Foster City, CA). The PTH–amino acids were determined on-line with the PTH analyzer (Applied Biosystems Inc.) using the standard program provided by the manufacturer.

1-3-[^{3}H]DFP binding studies. CAP37, elastase, and cathepsin G were prepared from normal CGE that had not been treated with DFP or other protease inhibitors, as well as from CGE that had been treated with 5×10^{-3} M DFP (Sigma Chemical Co., St. Louis, MO), 5×10^{-5} M PMSF (Sigma Chemical Co.), 5×10^{-3} M trisodium EDTA (Sigma Chemical Co.), and 10^{-7} M pepstatin A (Sigma Chemical Co.). An aliquot of each of these samples was incubated immediately with 1-3-[^{3}H]DFP (sp act 3.5 Ci/mmol; Amersham Corp., Arlington Heights, IL) at a final concentration of 10^{-5} M. Incubations were allowed to proceed for 1 h and overnight at 37°C. The reaction was stopped with Tris-HCl (pH 8.1, at a final concentration 15 mM) (14). The samples were run on a 15% SDS gel, and after fixation (25% vol/vol isopropanol, 7% vol/vol acetic acid) the gel was treated with Amplify (Amersham Corp.) for 30 min and dried at 80°C. The autoradiograph was developed after a 10-d exposure at −70°C using X-Omat-AR film (Eastman Kodak Co., Rochester, NY).

SDS-PAGE and Western blot analysis. SDS-PAGE was performed according to the method of Laemmli (15). All protein samples were solubilized in 0.625 M Tris (pH 6.8), 4% (wt/vol) SDS, and 1% (vol/vol) beta mercaptoethanol at 100°C for 5 min and analyzed on a 12.5% gel. The gel was silver stained. After electrophoresis the proteins were transferred onto nitrocellulose membrane (Bio-Rad Laboratories) under constant current (200 mA) at 11°C for 1.5 h for Western blot analysis (TE series transphor unit; Hoefer Scientific Instruments, San Francisco, CA). The method used was essentially that of Towbin et al. (16) except that the concentration of methanol was reduced from 20 to 5% (vol/vol) and the pH raised to 9 (11). The wash buffer contained 10 U/ml heparin sulfate (Eastman Kodak Co.) (11).

Chemotaxis and chemokinetic assays. Chemotaxis was measured using the modified Boyden chamber technique (17). The leading front method was used to assess migration of monocytes and PBL through an 8-μm filter (Millipore/Continental Water Systems, Bedford, MA). PMN chemotaxis was measured using a 3-μm filter (Millipore/Continental Water Systems). All preparations of CAP37 used in chemotaxis assays were endotoxin free as determined by the Limulus amebocyte lysate assay (Whittaker M. A. Bioproducts, Walkersville, MD). The dilutions of CAP37 and FMLP were made in Geys' buffer containing 2% endotoxin-free BSA. Geys' buffer containing 2% BSA served as the negative control, while a 10^{-8} M solution of FMLP acted as the positive control. The chambers were incubated in a humidified atmosphere (6.2% CO_2) for 2 h when using the monocytes and PBL, and for 30 min when using PMN, at which time the filters were removed and processed as described (17). The cells were viewed under oil immersion and the distance migrated by the cells was determined over five different fields on the same slide. Triplicates were set up for each experimental point. Chemokinetic activity of CAP37 was determined by the checkerboard assay of Zigmond and Hirsch (18).

Preparation of antibodies in ascites fluid. An antibody to CAP37 in ascites fluid was prepared by injecting BALB/c mice (Jackson Laboratory, Bar Harbor, ME) with 250 ng of alum-adsorbed antigen (19) emulsified in an equal volume of Freund's complete and incomplete adjuvant (Difco Laboratories, Detroit, MI). The mice were injected subcutaneously with a total volume of 200 μl of this suspension. The mice were boosted intraperitoneally 3 wk later with 250 ng of alum-adsorbed CAP37 and at the same time were injected with 1 ml of pristane (2,6,10,14-tetramethyl pentadecane; Sigma Chemical Co.). 1 wk later 10^7 SP2/O mouse myeloma cells were injected intraperitoneally (20). Ascites fluid was collected and checked for specificity in ELISA (13) using CAP37, CAP57, myeloperoxidase (MPO), lactoferrin, cathepsin G, and lysozyme as the antigens. The production of antibodies to CAP57 in ascites fluid was identical to that described for CAP37. The production of the ascites fluid to human MPO was similar to that described for CAP37, except that 10 μg of purified MPO was used to prime and boost the animals.

Immunocytochemical studies. The staining was performed using the Vectastain™ avidin-biotin complex–glucose oxidase technique (Vector Laboratories, Inc., Burlingame, CA) as described previously (11). 100 μl of the cell suspension (1×10^6 cells/ml) was cytocentrifuged onto alcohol-cleaned glass slides. The cells were fixed in buffered formol acetone, pH 7.2, at 4°C for 60 s. A monospecific polyvalent mouse anti-CAP37 ascites fluid (1:100) was used to stain the cells. Normal mouse serum and PBS served as the negative controls. Ascites made against MPO served as the positive control. Color development was obtained using the nitroblue tetrazolium salt (Vectastain™ GO substrate kit 1; Vector Laboratories, Inc.) at room temperature for 30 min.

Extracellular release of CAP37 from PMN. PMN were separated from peripheral blood as described above and the concentration was adjusted to 2×10^7 cells/ml in Geys' buffered saline. The stimulus used in these experiments was *S. aureus* (ATCC 25923; American Type Culture Collection, Rockville, MD). An overnight culture of *S. aureus* was resuspended in Geys' buffer to a final concentration of 2×10^8 bacteria/ml. The test mixture contained Geys' buffer, PMN (1×10^7), *S. aureus* (1×10^8), and normal human serum at a final concentration of 3.3%. The reaction mixture was incubated at 37°C for 0, 5, 15, 30, and 60 min. At the end of each incubation time the samples were placed on ice immediately and the cells separated from the medium by centrifugation. The supernatant was aspirated and stored at 4°C until completion of all time points. Total CAP37 and CAP57 were obtained by lysing the same number of PMN in 0.02% hexadecyl trimethyl ammonium bromide in 0.1 M sodium acetate buffer (pH 4.0) (21). CAP37 and CAP57 were quantitated by competitive ELISA (13). All supernatants were adjusted accordingly with detergent and acetate

buffer before analysis by ELISA. The microtiter trays were coated with 5 μg of normal CGE. The inhibition mixture contained the supernatant and the relevant antibody at a final dilution of 1:100. A standard curve varying from 120 to 3.7 ng for each antigen was incorporated with the assay. Lactic dehydrogenase was measured spectrophotometrically by assaying the decrease in absorbance of NADH at 340 nm for 2 min using pyruvate as the substrate (22).

Results

Purification of CAP37. Purification of CAP37 proceeded as previously reported (8, 12). Mixed human PMN granules collected with differential centrifugation from cells treated with a cocktail of proteinase inhibitors and extracted with acetate buffer provided crude granule extract, the starting material for an initial separation over the CMS column. Eluted with a salt gradient (12), the effluent at 0.5 M salt solution contained a mixture of granule proteins, including CAP37, CAP57, cathepsin G, and the defensins. These pooled fractions separated on the G-75 column into five peaks, including a peak rich in CAP37 (peak C). The results obtained with CMS and G-75 chromatography were similar to those obtained before (8, 12). This CAP37-rich peak from the G-75 in turn yielded two peaks during passage through a hydrophobic interaction HPLC column.

The progress of purification was monitored with reduction and SDS-PAGE. As seen in Fig. 1, the CGE was an electrophoretically highly complex mixture (Fig. 1, lane *2*). The CAP37-rich fraction obtained by passing the crude extract over the CMS column yielded a substantially less complex but still heterogeneous CAP37-rich fraction during passage through a G-75 column (Fig. 1, lane *3*). Of the two peaks obtained by passing this material over the phenyl column (Fig. 2), the more hydrophobic one (Fig. 2, peak 2) contained protein that migrated as three closely spaced bands in SDS-PAGE (Fig. 1, lane *4*) with an apparent molecular mass of 37,000 D. This protein reacted only with antibody to CAP37 in repeated ELISA analysis.

Western blots (Fig. 3) developed with monospecific goat antibody to cathepsin G (five left lanes) and mouse antibody to CAP37 (four right lanes) showed that cathepsin G was present in the CGE (lane *2*), in the fraction from the G-75 column

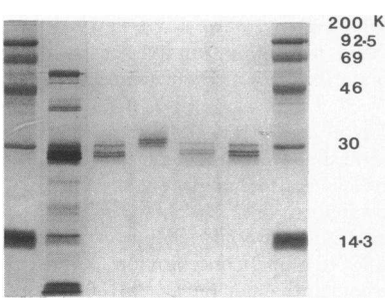

Figure 1. Silver-stained SDS-PAGE depicting the stages of purification of CAP37 from normal human PMN CGE. The following samples, 2 μg "rainbow" molecular weight markers (Amersham Corp.) (lane *1*), 5 μg CGE (lane *2*), 400 ng of peak C material obtained from the G-75 column (17) which served as the starting material for HPLC separation (lane *3*), 350 ng of peak 2 from HPLC column (lane *4*), 350 ng of peak 1 from the HPLC column (lane *5*), and 375 ng of cathepsin G obtained from Dr. J. Travis (lane *6*), were analyzed on a 12.5% SDS polyacrylamide gel. The molecular weight standards used included myosin (M_r 200,000), phosphorylase b (M_r 92,500), BSA (M_r 69,000), ovalbumin (M_r 46,000), carbonic anhydrase (M_r 30,000), trypsin inhibitor (M_r 21,500), and lysozyme (M_r 14,300).

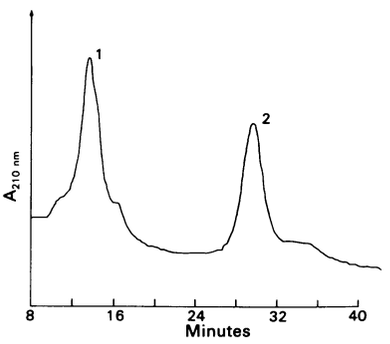

Figure 2. Elution profile of peak C (12) material from the Sephadex G-75 column on hydrophobic interaction HPLC. For this particular run 320 μg of protein was applied to the column, of which 55.2 μg of protein was recovered in peak 1 (cathepsin G), 90.6 μg of protein was recovered in peak 2 (CAP37), and 162 μg of protein was found in the fall-through. The total protein loaded apparently exceeded the capacity of the column, thus accounting for the large amount of protein that eluted in the fall-through. Total protein recovered was 112%. The total values for all the HPLC runs are given in Table I.

(lane *3*), or as purified cathepsin G (lane *5*). The protein of peak 2 from the HPLC column (Fig. 2), which was placed in lane *4* of Fig. 3, failed to bind anti-cathepsin G. In contrast, the monospecific mouse antibody to CAP37 bound to CAP37 in the CGE in lane *6*, and to CAP37 in the G-75 fraction (lane *7*). It bound very well to the homogeneous CAP37 in lane *8*, but it failed to bind at all to the purified cathepsin G in lane *9*. The CAP37 of peak 2 (Fig. 2) yielded an unambiguous NH_2-terminal sequence (see below). We believe, in light of these results, that the three bands are due to isoforms of CAP37. The less hydrophobic first peak (Fig. 2) comprised cathepsin G immunochemically (Fig. 3, lanes *2* and *3*) and compared favorably in SDS-PAGE (Fig. 1, lane *5*) with purified cathepsin G, kindly provided by Dr. James Travis of the University of Georgia, Athens GA (Fig. 1, lane *6*). Tests for endotoxin done with the limulus amebocyte assay failed to reveal contamination with endotoxin in the samples. Importantly, the CAP37 preparation was devoid of other granule proteins such as cathepsin G, the defensins, elastase, lysozyme, lactoferrin, MPO, and CAP57.

Table I depicts the typical recoveries of total protein and CAP37 obtained at the three major steps of the purification process. In step 1, 112 mg of CGE was loaded onto the CMS column. The CGE contained 5.4 mg of CAP37 (4.8%) by ELISA inhibition assay. A pool was made with the CAP37-rich

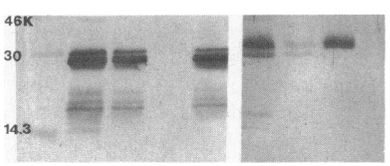

Figure 3. Western blot analysis of CGE, CAP37, and cathepsin G using antiserum to cathepsin G and CAP37. Lane *1*, molecular weight markers as described above; lanes *2* and *6*, CGE; lanes *3* and *7*, peak C from G-75 column; lanes *4* and *8*, peak 2 from HPLC column; and lanes *5* and *9*, peak 1 from HPLC column. The protein concentrations of all samples loaded onto the gel are given in Fig 1. Lanes *1–5* were probed with goat anti-human cathepsin G (1:2,000) and lanes *6–9* were probed with mouse anti-human CAP37 (1:100). The second antibody was conjugated to alkaline phosphatase (1:7,500) (Promega Biotec, Madison, WI). Enzymatic autodigestion of cathepsin G is probably responsible for the reaction seen with the goat anti-cathepsin G antiserum and the smaller molecular weight proteins.

Table I. Recoveries of Total Protein and CAP37 during the Purification of CAP37

	Total protein loaded	Total CAP37 loaded	CAP37 per milligram protein loaded	Total protein recovered	Total CAP37 recovered	Total protein in CAP37 peak	CAP37 per milligram protein in peak	Fold purification
	mg	*mg*		*%*	*mg (%)*	*mg*		
Step 1: CMS	112	5.4	0.048	102	3.8 (70)	20	0.19	3.9
Step 2: G-75	20	3.8	0.19	95.4	3.2 (85)*	6.4	0.5	10.4
Step 3: HPLC	1.4	0.71	0.58	117	0.38 (54)	0.38	1.0	20.8

* Estimated from integrated peaks achieved with HPLC analytical phenyl column.

fractions. It contained 3.8 mg of CAP37 in 20 mg total protein (ELISA inhibition assay). The recovery of CAP37 at the end of this first chromatographic step was 70%, and the CAP37 content of our sample had been enriched from 4.8 to 19%. The protein recovery at the end of the second step of the purification was 95.4%, and CAP37 was confined to peak C. Peak C had a total CAP37 content of 3.2 mg in 6.4 mg of total protein. Our HPLC profile indicated that the peak C fractions contained only two proteins at this point. At the end of the third purification step on HPLC a yield of 0.388 mg of highly homogeneous CAP37 was obtained. Overall recovery was 32%. Principal losses were experienced due to irreversible absorption of CAP37 to filters used to concentrate pooled fractions.

Amino acid sequence analysis. To assess the homogeneity of the CAP37 preparation and to begin to relate its structure to its functions, we determined the first 42 residues of its amino terminus (Fig. 4). The amino acid composition of CAP37 is published (12).

1-3-[^{3}H]DFP binding studies. Because of CAP37's strong homologies to serine proteases it was important to test its capacity to bind the serine protease inhibitor, DFP. This CAP37 was purified without any proteinase inhibitors. It was compared with uninhibited CGE, cathepsin G, and elastase, and with the same proteins pretreated with cold DFP. SDS-PAGE was performed, the gel was dried, and the autoradiograph was prepared at the end of 10 d (Fig. 5). Untreated CGE (lane *4*) and elastase (lane *8*) bound [^{3}H]DFP strongly. Uninhibited cathepsin G (lane *6*) also bound [^{3}H]DFP, but to a lesser extent. However, CAP37 (lane *2*) did not bind [^{3}H]DFP even when the incubation was allowed to proceed overnight. The results obtained after 1 h incubation were essentially the same as for the overnight incubation, except that the amount of [^{3}H]DFP bound to uninhibited CGE, elastase, and cathepsin G was relatively less. The slight reaction seen with inhibited cathepsin G and [^{3}H]DFP is probably due to incomplete inhibition of the original sample.

Chemotaxis and chemokinetic assays. With the foregoing assurances of the homogeneity and character of the CAP37 preparation, we examined its chemotactic action. Assays were performed using the modified Boyden chamber technique (17). CAP37 (Table II) was maximally chemotactic for human monocytes in the range 50–500 ng/ml (i.e., 1.3×10^{-9}–1.3×10^{-8} M) and was measurably active at 5 ng/ml (1.3×10^{-10} M). Thus CAP37 was active at the same molar concentrations as FMLP. CAP37 failed to cause PMN and PBL chemotaxis in the extensive ranges used in these experiments. CAP37 at the high concentration of 1,000 ng/ml (2.7×10^{-8} M) was chemotactic for rabbit monocytes as well. The need for more concentrated CAP37 may reflect a reduction in the number of receptors for CAP37 on rabbit monocytes when compared with the number on human monocytes or a difference in the K_a values for the two species. Rabbit PMN did not show a chemotactic response to CAP37 at the two concentrations tested.

Finally, it was important to distinguish directed movement (chemotaxis) through the filters of a Boyden chamber as opposed to merely accelerated random motion (chemokinesis). The checkerboard assay (18; Table III) demonstrated that CAP37 has some chemokinetic effect, but this failed to account for its decided chemotactic effect on monocytes.

Specificity of mouse anti-CAP37 antibodies in ascites fluid. We used ELISA and immunohistochemistry extensively in this work. The antibodies in mouse ascites fluid prepared against CAP37 reacted specifically with CAP37, as determined by ELISA (Table IV). It failed to bind other neutrophil antigens tested. We have described the mouse ascitic antibodies to MPO and CAP57 previously in detail (Pereira, H. A., J. K. Spitznagel, E. F. Winton, W. M. Shafer, L. E. Martin, G. S. Guzman, J. Pohl, R. W. Scott, M. M. Marra, and J. M. Kinkade, manuscript submitted for publication).

Immunocytochemical studies. Immunocytochemistry established that CAP37 was a component of the cytoplasmic granules of human PMN (Fig. 6). The staining in PMN was punctate. At the level of sensitivity of the immunocytochemical assay used, we were unable to detect the presence of CAP37 in eosinophils, monocytes, PBL, and RBC.

Extracellular release of CAP37 from PMN. If a chemotactic factor is to be effective it must be released from the cell of

```
      1                5                  10                  15                  20
CAP37 ILE-VAL-GLY-GLY-ARG-Lys-ALA-ARG-PRO-Arg-Gln-Phe-PRO-PHE-Leu-Ala-SER-Ile-GLN-Asn-
ELAST ILE-VAL-GLY-GLY-ARG-Arg-ALA-ARG-PRO-His-Ala-Trp-PRO-PHE-Met-Val-SER-Leu-GLN-Leu-

                      25                  30                  35                  40
CAP37 Gln-GLY-Arg-HIS-PHE-CYS-GLY-Gly-Ala-LEU-ILE-His-Ala-Arg-PHE-VAL-MET-Thr-ALA-ALA-Ser-CYS-
ELAST Arg-GLY-Gly-HIS-PHE-CYS-GLY-Ala-Thr-LEU-ILE-Ala-Pro-Asn-PHE-VAL-MET-Ser-ALA-ALA-His-CYS-
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Figure 4. The amino-terminal sequence of CAP37 compared with that of elastase. Residues that are identical in both sequences are capitalized. Ser-41 indicates the expected location of the 'his' residue, and cys 26–cys 42 indicates the probable position of the first disulfide bond.

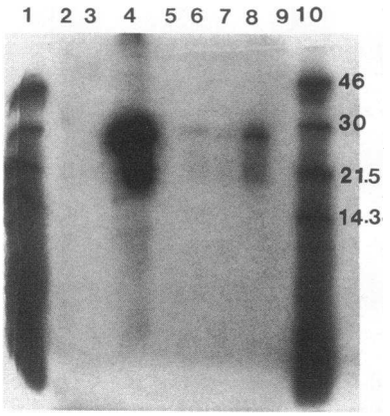

Figure 5. Autoradiograph of 1-3-[^{3}H]DFP binding to CGE, cathepsin G, elastase, and CAP37. Lanes *1* and *10*, ^{14}C-labeled molecular weight markers of 46–2.35 kD (4.5 μg; Amersham Corp.); lane *2*, 1.5 μg uninhibited CAP37; lane *3*, 1.5 μg inhibited CAP37; lane *4*, 15 μg uninhibited CGE; lane *5*, 15 μg inhibited CGE; lane *6*, 1.5 μg uninhibited cathepsin G; lane *7*, 1.5 μg inhibited cathepsin G; lane *8*, 1.5 μg uninhibited elastase; and lane *9*, 1.5 μg inhibited elastase. All of the above samples except the molecular weight standards were incubated overnight with [^{3}H]DFP (10^{-5} M final concentration) at 37°C. The molecular weight standards included ovalbumin (46 kD), carbonic anhydrase (30 kD), trypsin inhibitor (21.5 kD), lysozyme (14.3 kD), aprotinin (6.5 kD), insulin b chain (3.4 kD), and insulin a chain (2.35 kD).

origin under circumstances and at a time likely to be relevant. The kinetics of the extracellular release of CAP37 from 10^7 PMN phagocytosing opsonized *S. aureus* is depicted in Fig. 7. Unstimulated PMN released minimal amounts of CAP37. However, opsonized *S. aureus* incorporated in the assay caused rapid extracellular release of CAP37, detectable even after 5 min incubation; release increased further with the time of phagocytosis.

Quantitated by inhibition ELISA, extracellular CAP37 at zero time was 4 ng, whereas after 30 min 100 ng of CAP37 was detected. Lysis of 10^7 PMN yielded 112 ng of total CAP37. In other words, 89% of CAP37 appeared extracellularly (Fig. 8) compared with only 4% of CAP57 being detected extracellularly even after 60 min. Lactic dehydrogenase levels were < 5%.

Discussion

We have demonstrated a naturally occurring PMN cationic granule protein that selectively promotes monocyte chemotaxis. Other workers have shown the important role of PMN, particularly the secretion of the specific granule (23), in initiating and amplifying the inflammatory response through the generation of chemotactically active complement fragments (24) and monocyte chemotactic factors (4, 7). That a monocyte-specific chemoattractant may be derived from PMN was previously advocated by Ward (4), who found that rabbit PMN lysates attracted monocytes selectively. He attributed this to cationic peptides. Observations made on patients with congenital specific granule deficiency (5) suggested that the defective monocyte accumulation seen in Rebuck skin windows was either due to the failure of the patient's PMN to release monocyte chemoattractants directly, or to the failure of PMN to release factors capable of generating complement chemoattractants. Moreover, lysates of specific granules stimulated migration of monocytes. However, this effect was more a reflection of chemokinesis rather than chemotaxis (7). In an early study the absolute requirement for circulating PMN for subsequent migration of monocytes (25) was also indirect evidence that PMN provided a monocyte-specific chemoattractant. Finally, a factor from the azurophil granules has been thought to terminate the inflammatory response by inactivating C5a (24).

Table II. Effect of CAP37 on the Chemotaxis of Human Monocytes, PMN, and PBL, and on Rabbit Mononuclear Cells and PMN as Measured by the Leading Front Method

	Distance migrated as a percent of control				
Concentration of chemoattractant	Human monocytes	Human PMN	Human lymphocytes	Rabbit mononuclear cells	Rabbit PMN
ng/ml					
0 CAP37	100	100	100	100	100
0.01 CAP37	ND	ND	116.1±5.2	ND	149.1±22.5
0.1 CAP37	118.3±0.8	ND	ND	ND	ND
1 CAP37	114.9±0.3	ND	ND	100.1±0.9	ND
5 CAP37	133.3±4.0*	91.4±10.3	ND	ND	ND
10 CAP37	127.6±6.0*	ND	104.5±11.6	110.6±2.6	ND
50 CAP37	157.5±1.0*	90.6±4.4	ND	ND	ND
100 CAP37	167.2±5.8*	99.3±11.3	91.2	106.5±10.1	133.3±4.6
250 CAP37	ND	111.9±11.4	ND	ND	ND
500 CAP37	145.9±1.4*	110.5±14.6	ND	131.0±12.3‡	ND
1,000 CAP37	111.6±9.2	102.3±4.5	ND	177.1±11.3*	ND
5,000 CAP37	114.9	ND	ND	115.6±1.8	ND
4.37 FMLP (10^{-8} M)	193.8±27.5*	207.9±16.5*	ND	172.4±15.9*	210.8±4.0*

All results are presented as a percent of the control±SEM for three determinations, except the value at 5,000 ng/ml with human monocytes, which was a single reading. * Results that are significantly different from the control as determined by one-way analysis of variance followed by Dunnett's multiple comparison procedure at the $P = 0.05$ level of significance (47). ‡ Results that are significant at the $P = 0.10$ level of significance.

Table III. Determination of Chemokinetic Properties of CAP37 by the Checkerboard Assay (18) Using Human Monocytes

Concentration of CAP37 above the filter	Distance migrated as a percent of control				
	Concentration of CAP37 below the filter (nanograms/milliliter)				
	0	5	50	100	1000
ng/ml					
0	100%	108.9±11.9	110.0±11.2	193.9±2.8*	134.9±1.3*
5	101.9±6.9	119.3±10.6	100.2±8.2	124.8±2.9	109.0±5.8
50	ND	ND	131.9±8.2‡	115.1±7.6	98.2±5.8
100	108.7±10.0	95.6±9.1	110.2±3.1	114.6±11.4	124.5±6.5
1,000	ND	ND	ND	129.5±6.0‡	123.4±5.3

Determination of chemokinetic properties of CAP37 by the checkerboard assay (18) using human monocytes. The concentrations of CAP37 used above and below the filters were 0, 5, 50, 100, and 1,000 ng/ml. The results are expressed as described in Table II.

In contrast to the work described above, our experiments have isolated and purified to apparent homogeneity a chemotactic protein from the granules of PMN. CAP37 was isolated by a series of chromatographic steps which included ion exchange and molecular seive chromatography, and a final purification step on hydrophobic interaction HPLC. The final purified product appeared as a single distinct peak on reverse-phase HPLC and yielded an unambiguous amino acid sequence, affirming our belief that the functional studies were performed with a highly homogeneous preparation of CAP37.

A search of the protein sequence data base using the program FASTAMAIL through BIONET (26) showed that CAP37 had both substantial similarities with and provocative differences from the NH_2 termini of a subset of serine proteases involved in inflammation. Homology was strong with two PMN-derived granule proteins, human elastase (27) or medullasin (28) (57.5%) (which has 100% homology with elastase [29]), and human cathepsin G (45%) (30). Other homologies existed with bovine plasminogen (45%) and human complement factor D (42.5%) (31, 32), a key enzyme in the alternative complement pathway. Other serine proteases with strong homologies to CAP37 were those derived from granules of atypical rat mast cells (rat mast cell protease I [RMCP I; 38.6%] RMCP II [40%]) (33, 34) and from cytotoxic T cells (CCP I, 40% [35], and H factor, 38.6% [36]). The first 20 amino acids of CAP37 are similar to the first 20 residues of the recently published sequence of an antibacterial substance named azurocidin (37). Purified from the membranes of azurophil granules of human PMN, azurocidin had a molecular mass of 29 kD on SDS-PAGE. Despite the discrepancy in their molecular masses, CAP37 and azurocidin appear similar. We have previously described CAP37, its association with PMN granules, and its physical, chemical, and antimicrobial properties in 1984 and 1986 (8, 12).

Table IV. ELISA Demonstrating the Specificity of Antibodies to CAP37 in Mouse Ascites Fluid

Antigen (60 ng/well)	ELISA reading at A_{492}nm				
	Dilution of mouse anti CAP37 ascites			Negative control	Positive control
	1:100	1:500	1:1,000		
CAP37	1.384	0.871	0.466	0.065	—
CATG	0.024	0.0	0.001	0.037	0.991
LYS	0.030	0.008	0.001	0.042	ND
LF	0.034	0.006	0.002	0.029	1.088
MPO	0.067	0.025	0.007	0.048	0.806
CAP57	0.081	0.019	0.004	0.035	0.323

The antigens tested in the ELISA included CAP37, CATG (cathepsin G), LYS (lysozyme), LF (lactoferrin), MPO, and CAP57. The ascites fluid was tested at 1:100, 1:500, and 1:1,000 dilution with each antigen (60 ng/well). The negative control consisted of normal mouse serum at 1:100 dilution. The positive controls consisted of rabbit anti-cathepsin G (1:100), mouse anti-lactoferrin monoclonal ascites (1:1,000), mouse anti-MPO ascites (1:1,000), and mouse anti-CAP57 ascites (1:500).

It is noteworthy that most of the serine proteases with which CAP37 shares its greatest homology (elastase, cathepsin G, RMCP I and II, H factor, and CCP I) are all derived from the granules of peripheral blood cells and are proteins known to play important roles in inflammation, be it cytolysis or degradation of extracellular matrices. Two other serine proteases, bovine thrombin (38) and a trypsin-like protease in guinea pig plasma (39), have been described as exhibiting chemotactic activity.

A highly conserved feature in all mammalian serine proteases is the three disulfide bonds that characteristically occur at cys-42 to cys-58 (the 'his' loop), cys-136 to cys-201, and cys-168 to cys-182 (the 'met' loop) in chymotrypsin numbering nomenclature. In CAP37 (Fig. 4) the cysteines corresponding to cys-42 and cys-58 of the 'his' loop are conserved. This is similar to elastase, cathepsin G, and the other serine proteases discussed.

Another feature of serine proteases is the residues forming the charge relay system of the active catalytic site that occurs at his-57, asp-102, and ser-195 (chymotrypsin numbering). In Fig. 4, a serine residue replaces the expected his-41 residue in CAP37. This substitution may help to explain the failure of CAP37, unlike the other serine proteases, to bind DFP. It is conceivable that through evolution the catalytic site of primordial CAP37 was changed without affecting those domains involved in chemotaxis and bactericidal activity. In this regard two other molecules, streptokinase and staphylokinase, were noted to show sequence homology with several serine proteases, including trypsin. However, streptokinase is enzymatically inactive because his-57 of the catalytic triad is replaced by gly, whereas asp-102 and ser-195 were in the required positions

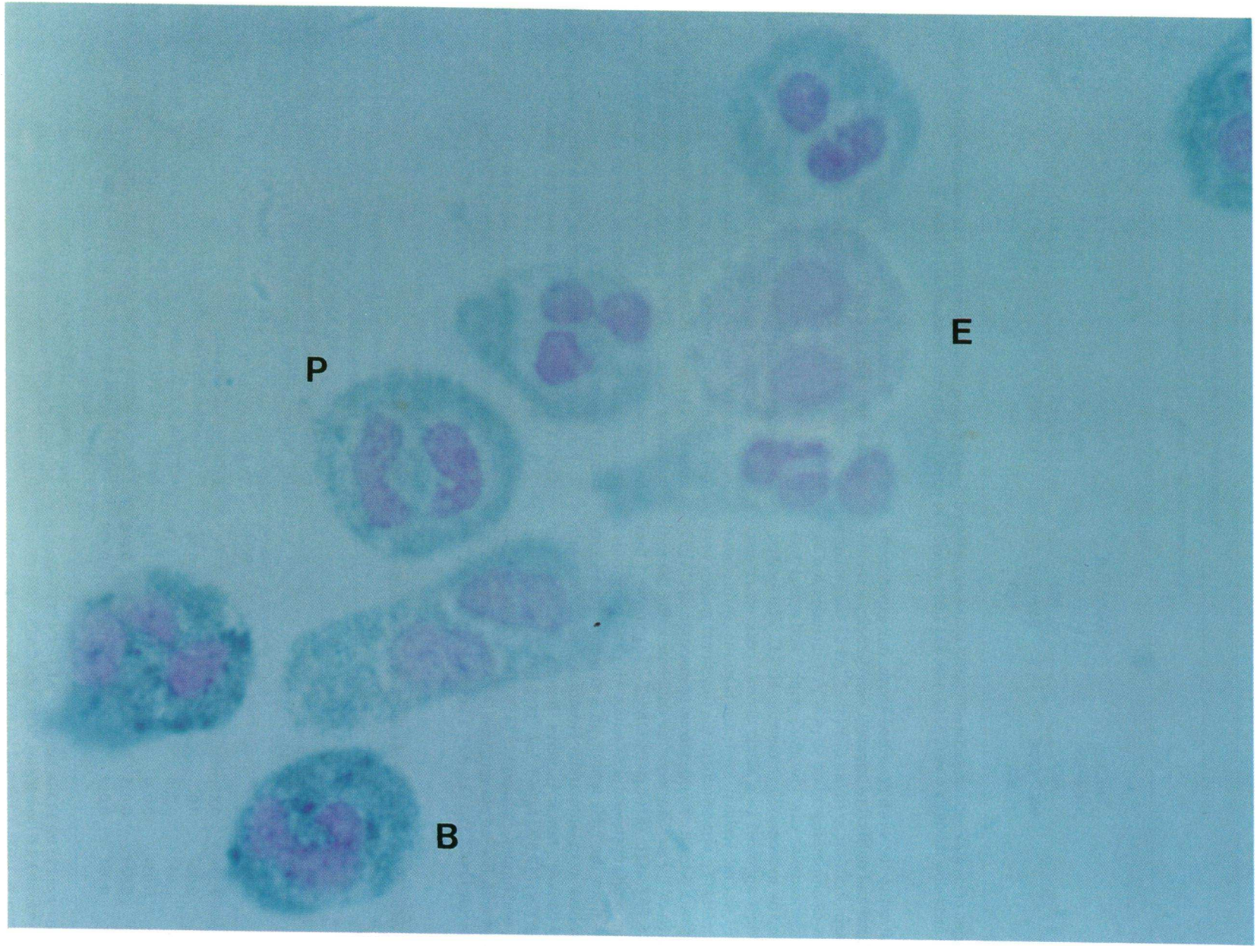

Figure 6. The immunocytochemical localization of CAP37 in human PMN using the monospecific polyclonal antibody to CAP37 and the Vectastain™ avidin-biotin complex–glucose oxidase technique. The cells are counterstained with lithium carmine. *P*, PMN; *E*, eosinophil; *B*, band.

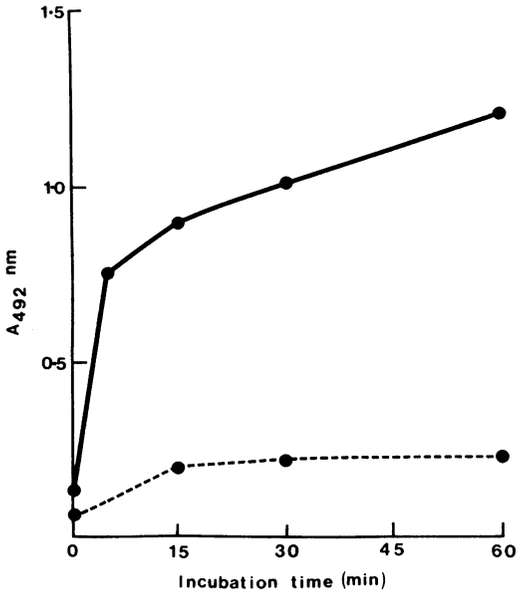

Figure 7. Time course of the extracellular release of CAP37 from PMN. The release of CAP37 extracellularly into the medium from unstimulated PMN (-- ● --), and from PMN allowed to phagocytose opsonized *S. aureus* (— ● —) as measured by ELISA.

(40). It has been suggested that this molecule arose by gene duplication and fusion (40).

For CAP37 to be a chemotactic factor in vivo it would need to be released either as a result of degranulation or during cell death. In this respect, we have evidence that CAP37 is liberated into the extracellular milieu when PMN are stimulated with *S. aureus*. Almost 90% of the total CAP37 was released extracellularly after 30 min exposure to the opsonized microorganisms, far less than azurophil granule markers such as CAP57, bactericidal/permeability-increasing protein (41), MPO, and the defensins (21). Extracellular release of lactoferrin has ranged from 40 to 80% depending on the stimulus and whether the cells were treated with cytochalasin B (42–44). A component of the tertiary granules, gelatinase, is known to be released in greater amounts than azurophil granule markers (45). The tertiary granules have been shown to degranulate in response to very mild stimuli, which normally do not result in the release of lactoferrin (45). We are currently conducting studies using percoll density gradients (46) to determine the exact subcellular localization of CAP37.

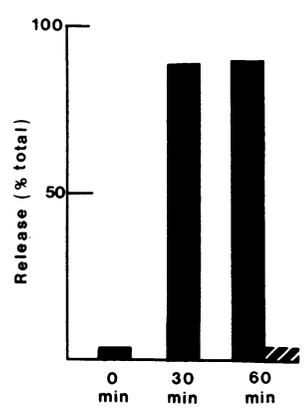

Figure 8. Quantitation of the total release of CAP37 by inhibition ELISA. The percent total release of CAP37 from 10^7 PMN after phagocytosis of opsonized *S. aureus* at 0, 30, and 60 min incubation time is indicated by solid bars, and the percent total release of CAP57 from 10^7 PMN after phagocytosis of *S. aureus* at 60 min by the hatched bar.

The hypothesis we have proposed in this report is that CAP37 when liberated from PMN at the site of inflammation is able to specifically attract monocytes and could thus be one of the factors responsible for the influx of monocytes in the second wave of inflammation. We do not claim that CAP37 is the sole mediator for this recruitment of monocytes in the second wave of inflammation. However, to our knowledge this is the first report of the isolation, characterization, and purification to homogeneity of a PMN granule-associated protein that has monocyte-specific chemotactic activity. It is particularly noteworthy that in addition to its described chemotactic activity, CAP37 is capable of bactericidal activity and thus may perform two very important functional roles in vivo.

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