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PURIFICATION AND IDENTIFICATION AS ACIDIC PROTEINS OF THE SPECIFIC GRANULES

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ABSTRACT The endogenous constituents of human neutrophils that enhance the adherence of the neutrophils to surfaces have been isolated from sonicates of purified neutrophils. The predominant adherence-enhancing activity in the neutrophil sonicates cofiltered on Sephadex G-75 with a major peak of chemotactic inhibitory activity and exhibited ~30,000 mol wt. Sequential isoelectric focusing and electrophoresis in glycerol gradients of the 30,000-mol wt activities resolved two distinct acidic protein with isoelectric points of 3.6–3.8 and 3.3–3.4 that were designated the neutrophil adherence factor (NAF) I and II, respectively. Glutamic acid and aspartic acid together accounted for a total of 18 and 19% of the amino acids in purified preparations of NAF I and NAF II, respectively, whereas the basic amino acids lysine, arginine, and histidine represented <2 and 3% of the total residues. The preincubation of portions of 2×10^6 neutrophils with as little as 6 pmol of NAF I or 9 pmol of NAF II enhanced adherence to plastic petri dishes and inhibited chemotactic migration to a maximal extent, with comparable dose-response relationships for the two effects. Neither of the NAF was cytotoxic, exhibited substantial neutrophil chemotactic or chemokinetic activity, or influenced the phagocytosis of sheep erythrocytes sensitized with immunoglobulin (Ig)G. Analyses of subcellular fractions of neutrophils indicated that the NAF are contained predominantly in the specific granules. These distinctive acidic proteins of the specific granules of human neutrophils represent a new class of endogenous constituents that may regulate the involvement of neutrophils in inflammation.

INTRODUCTION

Several basic proteins and peptides derived from human polymorphonuclear leukocytes are capable of altering neutrophil migration in vitro (1–3). The neutrophil-immobilizing factor (NIF)[1] is a 4,000–5,000-mol wt basic polypeptide that is released from the granules of human neutrophils engaged in the phagocytosis of opsonized particles, exposed to acid pH, or incubated with endotoxin or lymphokine preparations containing the leukocyte inhibitory factor (1, 4). NIF inhibits the random and chemotactic migration of neutrophils and eosinophils without affecting the migration of monocytes (5). Cationic proteins of 20,000–30,000 mol wt, which possess proteolytic activity analogous to that of chymotrypsin, are released from the granules of human neutrophils and eosinophils in the course of chemotaxis in modified Boyden chambers (2, 6). The purified cationic proteins enhance the random and chemotactic migration of neutrophils at concentrations of 128–256 μg/ml that are achieved early in the chemotactic response, and inhibit chemotaxis at concentrations of 1 μg/ml or higher. Lysozyme, a 15,000-mol wt basic protein, is released from neutrophil granules by chemotactic factors (7, 8) and irreversibly suppresses the chemotactic response of neutrophils to products of the complement sequence and extracts of *Escherichia coli* (3). The capacities of the various cationic protein constituents of the granules to enhance or inhibit neutrophil migration are not attributable to cytotoxicity, effects on oxidative metabolism, or alterations in adherence to surfaces.

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[1] *Abbreviations used in this paper*: HBSS, Hanks' balanced salt solution; 12-L-HETE, 12-L-hydroxy-eicosa-5,8,10,14-tetranenoic acid; HPF, high power field; NAF, neutrophil adherence factor; NIF, neutrophil-immobilizing factor.

The filtration on Sephadex G-75 of sonicates of subcellular fractions from human neutrophils led to the recognition of acidic protein constituents of the specific granules that noncytotoxically inhibit neutrophil chemotactic responses to diverse factors. The ability of highly purified preparations of the acidic proteins to augment the adherence of neutrophils to surfaces, with a dose-response relationship comparable to that observed for the inhibition of chemotaxis, suggests that change in adherence may be the mechanism by which these endogenous principles regulate neutrophil chemotaxis.

METHODS

Materials

Polystyrene disposable chemotactic chambers (ADAPS, Inc., Dedham, Mass.), filters with 3-μm pores (Sartorius, Göttingen, West Germany), Hanks' balanced salt solution with or without phenol red (Microbiological Associates, Inc., Walkersville, Md.), five times recrystallized ovalbumin (Miles Laboratories, Inc., Research Products Div., Elkart, Ind.), dextran, superfine Sephadex G-75, Ficoll-Hypaque (Pharmacia Fine Chemicals, Div. Pharmacia Inc., Piscataway, N. J.), trypan blue dye (Allied Chemical Corp., Specialty Chemicals Div., N. Y.), calf thymus DNA (Worthington Biochemical Corp., Freehold, N. J.), perchloric acid (Mallinckrodt Inc., St. Louis, Mo.), sodium lauryl sulfate, Wright-Giemsa stain (Fisher Scientific Co., Pittsburgh, Pa.), 3,5-diaminobenzoic acid (Aldrich Chemical Co., Inc., Milwaukee, Wisc.), 35 × 10 mm plastic petri dishes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.), arachidonic acid, bis-*p*-nitrophenol phosphate, *Micrococcus lysodeikticus*, reagent kits for assaying lactic acid dehydrogenase and β-glucuronidase, zymosan A (Sigma Chemical Co., St. Louis, Mo.), the synthetic chromogenic substrates H-D-Phe-Pip-Arg-NH-phenyl-NO_2·2HCl and H-D-Pro-Phe-Arg-NH-phenyl-NO_2·2HCl (Kabi Diagnostica, Stockholm, Sweden), the human complement components C1, C4, C2, and C3, immunoglobulin (Ig)G- and IgM-enriched preparations of rabbit antisheep erythrocyte antibodies (Cordis Laboratories, Inc., Miami, Fla.), heat-inactivated fetal calf serum (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.), Amicon ultrafiltration chambers and UM-10 membranes (Amicon Corp., Lexington, Mass.), and pH 3–10 and 3–5 ampholytes (Bio-Rad Laboratories, Richmond, Calif.) were obtained as noted. Purified synthetic formyl-methionyl-leucyl-alanyl-phenylalanine (f-Met-Leu-Ala-Phe) was supplied by Dr. R. J. Freer (Medical College of Virginia, Richmond, Va.). Chemotactic fragments of the fifth component of human complement (C5fr) were prepared by filtration on Sephadex G-75 of portions of human sera that had been incubated with zymosan as described (4). 12-L-hydroxy-eicosa-5,8,10,14-tetraenoic acid (12-L-HETE) was generated by incubating arachidonic acid with human platelet lipoxygenase and purified as described (4). Statistical analyses were based on the standard *t* test.

Assessment of neutrophil random migration, chemotaxis, and chemokinesis

Human neutrophils were obtained from normal subjects (1) and purified to over 96% (4, 5, 9) as described, and were washed and resuspended at a concentration of $2.0 \pm 0.2 \times 10^6$ neutrophils/ml in Hanks' balanced salt solution (HBSS) containing 0.4 g/100 ml of ovalbumin and 0.005 M Tris-HCl, pH 7.4. Neutrophil random and chemotactic migration was assayed by a modification (4, 5, 10) of the Boyden chamber micropore filter technique (11). Chemotaxis was evoked by the addition of varying dilutions of the stimulus to the lower compartment alone and chemokinesis was elicited by using an equal concentration of the stimulus in the lower compartment and in the neutrophil well. Neutrophils in filters from duplicate chambers known to lack a stimulus were counted at several levels between 80–120 μm from the cell source to determine the level where the mean cell count was in the range of 7–12 neutrophils/high power field (hpf). The cell count at this level served both as the background for chemotactic responses and as the control value for random migration, and filters from experimental and additional control chambers were counted at the same level. Random migration was expressed as neutrophils/hpf and chemotaxis and chemokinesis as net neutrophils/hpf, after subtraction of the corresponding value for random migration. The alteration in neutrophil migration that resulted from the addition of a test sample to the neutrophils was expressed as the percentage of inhibition (+%) or enhancement (−%) of the corresponding control response of neutrophils in buffer alone.

Measurement of adherence of neutrophils to plastic petri dishes

Each portion of 5×10^6 neutrophils was preincubated in 1 ml of HBSS containing 0.2 g of ovalbumin/100 ml (pH 7.4) for 30 min at 37°C without or with varying concentrations of test samples. The neutrophils then were washed once, resuspended in 2 ml of the same buffer, and incubated for 45 min at 37°C in a 35-mm Diam plastic petri dish. The adherent neutrophils were washed twice with 2-ml portions of HBSS without ovalbumin and were dissolved in 1 ml of 3 g/100 ml sodium lauryl sulfate in distilled water as described (5). The content of DNA in duplicate 50-μl portions of each solution and in 5×10^6 neutrophils of each preparation was determined by the diaminobenzoic acid method (12) using a standard curve prepared from 0.01–0.5 μg/ml of calf thymus DNA. The adherence of control neutrophils in buffer alone was expressed as a percentage of the total number of neutrophils added to each dish and the change in adherence of neutrophils that was induced by preincubation with a test sample, was expressed as a percentage of the adherence of the control neutrophils.

Purification of the factors in neutrophil sonicates that enhance adherence to surfaces and inhibit chemotaxis

Sephadex G-75 gel filtration. 1×10^9 purified neutrophils in 2 ml of HBSS were ultrasonicated at 200 W for 10 min at 4°C (model 350, Branson Sonic Power Co., Danbury, Conn.). After the sonicate was centrifuged at 100 *g* for 10 min at 4°C, the supernate was transferred to a clean test tube and the pH was adjusted to 6.0 with 1 M acetic acid. The acidified sonicate was centrifuged at 1,000 *g* for 10 min at 4°C, and the supernate was transferred to a clean test tube. The 1,000 *g* supernate was filtered on a 1.5 × 68-cm column of superfine Sephadex G-75 that was equilibrated and developed at a flow rate of 14 ml/h with 0.05 M ammonium acetate, pH 6.0, at 4°C. Each fraction contained 2% of the bed volume.

Isoelectric focusing. The fractions from Sephadex G-75 which contained the activities of ~30,000 mol wt were pooled and concentrated to 1 ml, and the concentrate was mixed

with 0.25 ml of 100% glycerol and 25 mg of pH 3–5 ampholytes immediately before isoelectric focusing. Isoelectric focusing was performed in a vertical column (Model 212, ISCO, Instrumentation Specialties Co., Lincoln, Neb.) that held a 30-ml linear gradient of 10–50 g/100 ml of glycerol in distilled water with 2 g/100 ml of pH 3–5 ampholytes (13). The cathodic reservoir was filled with 0.1 M sodium acetate solution (pH 6.3) and the anodic reservoir contained a 100% glycerol:0.1 M phosphoric acid solution (60:40, vol:vol) (pH 2.2). The column was prefocused at 900 V for 16 h at 6°C, the sample was applied and the focusing was continued at 900 V for an additional 24 h at 6°C. The column was divided into 1-ml fractions that were dialyzed for 16 h against HBSS at 4°C.

Electrophoresis in a glycerol gradient. The isoelectric focusing fractions that contained the two acidic activities were pooled separately, concentrated to 1 ml each, and mixed with 0.25 ml of 100% glycerol. Each sample was electrophoresed at a constant 8 mA for 7 h at 6°C in a vertical column containing a 30-ml gradient of 10–50 g/100 ml of glycerol in 0.03 M sodium acetate (pH 5.0). The gradient was recovered in 1-ml fractions that were dialyzed against HBSS for 16 h at 4°C.

Amino acid analysis

Protein samples were lyophilized in 15 × 75-mm test tubes and resuspended in 0.4 ml of 6 M HCl containing 0.001 M phenol. The tubes were evacuated and heated at 110°C for 24 h (14). Alkaline hydrolyses for determinations of the content of tryptophan, glutamine, and asparagine were carried out in 4.2 M NaOH as described (15). The amino acids in the hydrolysates were quantitated on a Durrum D-500 analyzer (Dionex Inc., Sunnyvale, Calif.).

Localization in neutrophil subcellular fractions of factors that enhance adherence to surfaces and inhibit chemotaxis

1×10^9 purified neutrophils were washed twice in Ca^{++}- and Mg^{++}-free HBSS containing 2 mM EDTA and 0.005 M Tris-HCl (pH 7.4). The washed neutrophils were resuspended and homogenized at 4°C in 5 ml of 0.34 M sucrose with 2 mM EDTA, 20 U of DNase/ml, and 0.005 M Tris-HCl (pH 7.0). The homogenate was centrifuged at 100 *g* for 20 min at 4°C and the supernate was centrifuged at 400 *g* for 10 min at 4°C. The 400 *g* supernate was apportioned between two 10-ml gradients of 10–50 g/100 ml of sucrose containing 2 mM EDTA and 0.005 M Tris-HCl (pH 7.0). The gradients were centrifuged at 20,000 *g* for 45 min at 4°C and divided into 1-ml fractions. After ultrasonication and dialysis of each sample (13), duplicate 50-μl portions were assayed for the lysosomal markers β-glucuronidase (16) and lysozyme (17), the cytoplasmic marker lactic acid dehydrogenase (18), and the plasma membrane marker alkaline paranitrophenylphosphatase (19). 1 U of activity of each enzyme has been defined (13).

Assessment of other neutrophil functions

Phagocytosis of erythrocytes bearing antibodies of the IgG class (EA_{IgG}). EA_{IgG} were prepared by sensitizing sheep erythrocytes (E) with purified rabbit anti-E antibodies of the IgG class (AIgG) (20). Duplicate portions of 1.6×10^6 neutrophils were preincubated for 10 min at room temperature in 0.5 ml of varying concentrations of a purified neutrophil-derived factor or in buffer alone, 8×10^6 EA_{IgG} were added to each suspension of neutrophils, and the mixtures were incubated for 30 min at 37°C as described (20). Then each mixture and separate tubes containing the same number of EA_{IgG} or neutrophils as the mixture, received 2.5 ml of 0.84% NH_4Cl, which lyses only the uningested erythrocytes. Each tube was centrifuged at 100 *g* for 5 min at 4°C and the optical density of the supernates was determined at 414 nm. The calculations of the number of ingested EA_{IgG} from the difference between the $OD_{414\ nm}$ of a lysate of the total number of EA_{IgG} and the $OD_{414\ nm}$ of the lysate of the uningested EA_{IgG} permitted the quantitation of the percentage of phagocytosis of EA_{IgG} (20). Phagocytosis by neutrophils that were preincubated with a purified factor was expressed as a percentage of control phagocytosis by neutrophils exposed to buffer alone.

Formation of rosettes with $EA_{IgM}C43b$. Sheep erythrocytes were sensitized with rabbit antisheep erythrocyte antibodies of the IgM class and reacted sequentially with human C1, C4, C2, and C3 as described (20–22). Duplicate portions of 1.6×10^6 neutrophils were preincubated for 10 min at room temperature in 0.4 ml of varying concentrations of a purified factor or in buffer alone, 8×10^6 $EA_{IgM}C43b$ were added to each suspension of neutrophils, and the mixtures were centrifuged at 50 *g* for 2 min at room temperature and incubated for 30 min at 37°C (22). Each cell pellet was resuspended in 50 μl of heat-inactivated fetal calf serum. 300 neutrophils were counted in the stained smear of each suspension and the neutrophils to which three or more $EA_{IgM}C43b$ had adhered were considered to constitute a rosette (22). The formation of rosettes by neutrophils in the presence of a purified neutrophil-derived factor was expressed as a percentage of the rosetting observed for control neutrophils exposed to buffer alone.

RESULTS

Identification of inhibitors of chemotaxis and enhancers of adherence in sonicates of human neutrophils

To define further the neutrophil-derived chemotactic inhibitory activity that had been recognized in the exclusion volume of Sephadex G-25 columns (1), sonicates of 1×10^9 neutrophils were filtered on Sephadex G-75 (Fig. 1). Neutrophil chemotactic inhibitory activity was found in two distinct peaks, at 44–56% bed volume and 72–76% bed volume, respectively (Fig. 1, top frame). The larger of the chemotactic inhibitory activities eluted between the ovalbumin and chymotrypsinogen A markers which permitted the assignment of ~30,000 mol wt, while the molecular weight of the smaller inhibitory activity is ~4,000–5,000. Neutrophil adherence to plastic petri dishes was enhanced by neutrophil-derived principles that filtered at 40–64% bed volume and the predominant peak of enhancing activity was at 44–52% bed volume, coinciding with the position of elution of the higher molecular weight chemotactic inhibitor (Fig. 1, lower frame).

Purification of the principles in neutrophil sonicates that enhance adherence and inhibit chemotaxis

Because the activities that eluted from Sephadex G-75 with an apparent 30,000 mol wt only appeared in

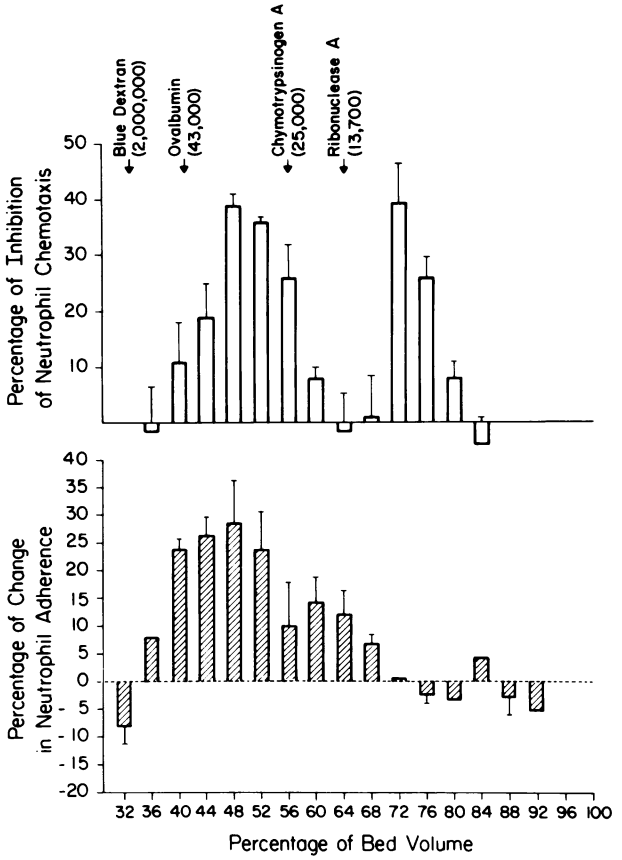

FIGURE 1 Sephadex G-75 filtration of sonicates of human neutrophils. (Top) Each bar and bracket represent the mean ±0.5 the range of the inhibition of neutrophil chemotaxis by 50 μl portions of the fractions from two preparations of 10^9 sonicated neutrophils. The chemotactic response to C5fr of control neutrophils exposed to buffer alone (0% inhibition) was 54 and 66 net neutrophils/hpf, respectively, in the two assays. (Bottom) Each bar and bracket represent the mean ±0.5 the range of the change in neutrophil adherence induced by 40-μl portions of the fractions from two preparations of 10^9 sonicated neutrophils. The extent of adherence of control neutrophils exposed to buffer alone (0% change) was 36 and 53% of the total neutrophils, respectively, in the two assays.

acidic fractions when subjected to isoelectric focusing in a glycerol density gradient containing pH 3–10 ampholytes, the preparative isoelectric focusing procedures were carried out with ampholytes of pH 3–5. With pH 3–5 ampholytes, the chemotactic inhibitors of 30,000 mol wt were focused within the pH range of 3.3 to 3.8 (Fig. 2). Two peaks of inhibition of neutrophil chemotaxis to C5fr were recognized that encompassed fractions of pH 3.3–3.4 and of pH 3.6–3.8. The peaks were pooled separately and concentrated to 1 ml. 20 μl each of the more acidic and less acidic pools of chemotactic inhibitors from one of the isoelectric focusing procedures enhanced neutrophil adherence by a mean of 36 and 48%, respectively, whereas 20 μl from 1-ml pools of fractions 1–5 (pH 4.3–5.1) and 24–29 (pH 3.0–3.2) inhibited adherence by 6% and enhanced adherence by 11%, respectively.

The two pools of active acidic principles were electrophoresed separately for 7 h in glycerol density gradients buffered with 0.03 M sodium acetate (pH 5.0). Inhibition of neutrophil chemotaxis to C5fr of >30% was found in electrophoresis fractions 1, 7–15, and 19–23 for the less acidic peak (Fig. 3, upper frame) and in electrophoresis fractions 1, 5–9, 13–15, and 19–21 for the more acidic peak (Fig. 3, lower frame) from isoelectric focusing. The inhibitory fractions noted were pooled separately and each of the seven pools was concentrated for ~0.1 absorbancy units at 280 nm/ml and assessed for inhibition of neutrophil chemotaxis in a dose-response fashion. At a concentration of 50 μl/ml, the pool of fractions 7–15 from the less acidic peak and the pool of fractions 5–9 from the more acidic peak inhibited by 60% or more the comparable neutrophil chemotactic responses to a 1:80 dilution of C5fr, 50 nM f-Met-Leu-Ala-Phe and 4 μg/ml of 12-L-HETE, whereas the same concentration of the other five pools inhibited chemotaxis by <33%. Further, only the two predominant pools inhibited chemotaxis in a dose-response manner. For two of the preparations, concentrations of 20 μl, 40 μl, and 80 μl/ml of fractions 7–15 from the less acidic pool inhibited chemotaxis by a mean of 36, 66, and 82%, whereas the same concentrations of fractions 5–9 of the more acidic pool showed a mean inhibition of 30, 54, and 71%, respectively. For one of the preparations, 40 and 80 μl of the same two pools increased the adherence of neutrophils to plastic petri dishes by 90 and 128% and by 76 and 97%,

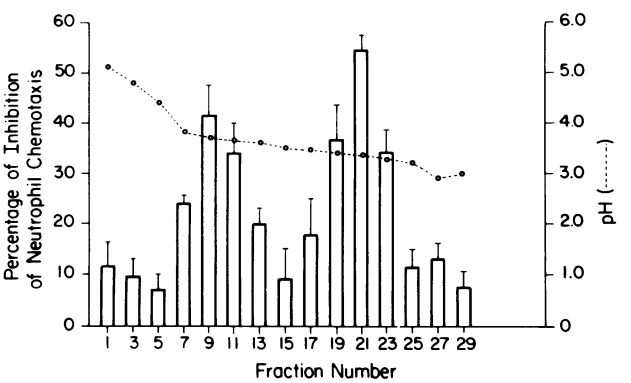

FIGURE 2 Isoelectric focusing of the 30,000-mol wt peak of neutrophil chemotactic inhibitory activity and adherence enhancing activity from Sephadex G-75. Each bar depicts the mean±SD of the inhibition of neutrophil chemotaxis by 50-μl portions of fractions from three different preparations. The chemotactic responses to C5fr of control neutrophils in buffer alone (0% inhibition) ranged from 45 to 65 net neutrophils/hpf.

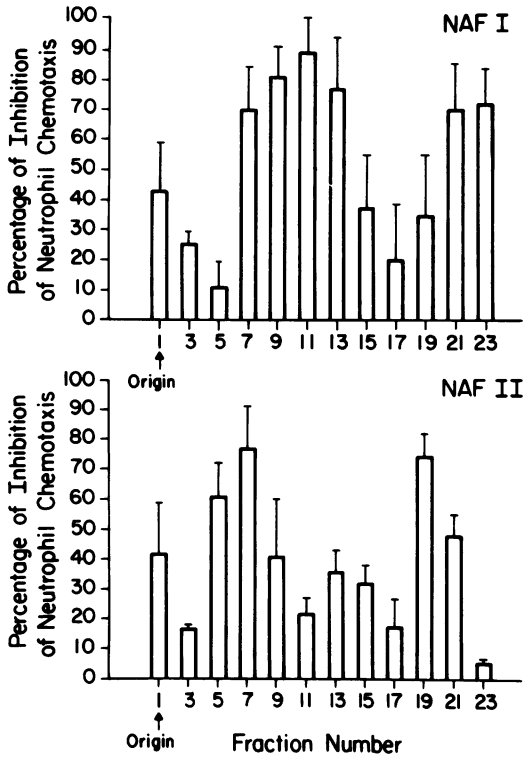

FIGURE 3 Electrophoresis of the peaks of neutrophil chemotactic inhibitory activity from isoelectric focusing. Each bar represents the mean±SD of the inhibition of neutrophil chemotaxis by 50-μl portions of fractions from three electrophoretic analyses of the peaks derived from isoelectric focusing. The chemotactic response to C5fr of control neutrophils in buffer alone (0% inhibition) ranged from 37 to 60 net neutrophils/hpf. (Top) Peak at pH 3.6–3.8 from Fig. 2. (Bottom) Peak at pH 3.3–3.4 from Fig. 2.

respectively. As the distinctive functional capability of the isolated material was the enhancement of neutrophil adherence to a surface, each of the electrophoretically purified principles thus was designated a neutrophil adherence factor (NAF); the pool of fractions 7–15 of the less acidic peak was termed NAF I and the pool of fractions 5–9 of the more acidic peak was termed NAF II.

The delineation of dose-response relationships at several stages in the preparation of the factors led to an estimation of their purity at each stage (Table I). An examination of the ratios of the fraction of each pool required to achieve the same effect to the total absorbancy units at 280 nm in each pool suggests that the specific activity of the NAF was increased approximately 10- to 40-fold by the purification sequence. Further, 20% of the total NAF I and 33% of the total NAF II purified from two sonicates of 10^9 neutrophils contained no detectable lysozyme activity (17) and did not cleave the synthetic esterase substrates H-D-Phe-Pip-Arg-NH-phenyl-NO_2·2HCl or H-D-Pro-Phe-Arg-NH-phenyl-NO_2·2HCl at pH 7.0–8.6. However, the maintenance or increase in the total activity expressed with each purification step, suggesting the removal of inhibitors, and the inability to assess the additive effects of the two NAF in the initial Sephadex G-75 peak precluded a definitive calculation of yield.

Amino acid composition of NAF I and NAF II

The amino acid composition determined from 6 N HCl hydrolysates of the purified preparations permitted the calculation of 33,980 mol wt for NAF I and of 33,090 for NAF II (Table II). Both proteins contained a high percentage of aspartic acid or asparagine and glutamic acid or glutamine. Alkaline hydrolyses of one of the preparations of each factor, which yields asparagine and glutamine without significant conversion to the corresponding dicarboxylic acids, revealed that 6 of the asparaginyl residues in NAF I and 7 in NAF II were

TABLE I
*Purification of NAF Activities from Sonicates of Human Neutrophils**

Purification procedure	Total absorbancy units at 280 nm in pool of activity	Fraction of pool of activity required for:‡ 40% inhibition of chemotaxis	50% increase in adherence
Sephadex G-75 filtration	2.42§	0.014	0.031
Isoelectric focusing			
pH 3.6–3.8	0.76‖	0.016	—
pH 3.3–3.4	0.66‖	0.012	—
Electrophoresis			
NAF I	0.39‖	0.010	0.009
NAF II	0.26‖	0.012	0.016

* Values shown are for one representative purification sequence of a sonicate of 1×10^9 neutrophils.
‡ Values were obtained from plots of dose-response relationships.
§ Total volume = 4 ml.
‖ Total volume = 3 ml.

TABLE II

Amino Acid Compositions of Purified Preparations of Neutrophil Adherence Factor

	Asx	Thr	Ser	Glx	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg
NAF I	25.1 ±1.9*	15.9 ±0.9	48.7 ±3.6	41.3 ±1.8	1.2 ±0.3	45.3 ±2.2	26.2 ±2.3	6.9 ±0.8	3.3 ±0.6	8.8 ±0.9	14.9 ±1.1	11.9 ±1.0	6.3 ±0.7	8.2 ±0.8	12.2 ±1.0	7.1 ±0.2
NAF II	27.2 ±1.6*	13.9 ±1.1	51.8 ±3.7	48.5 ±3.3	1.3 ±0.2	37.1 ±3.5	18.7 ±0.9	13.0 ±0.9	2.6 ±0.3	7.6 ±0.7	10.8 ±1.1	9.8 ±0.7	7.4 ±0.8	6.0 ±0.6	7.8 ±0.5	7.3 ±0.7

* Each value is the mean±SD of the number of nanomoles of amino acid per nanomole of protein for three preparations. Hydrolyses of 150–500 pmol of NAF I and 100–300 pmol of NAF II were used for the amino acid analyses.

asparagine and 10 of the glutamyl residues in NAF I and 18 in NAF II were glutamine. This permitted the calculation of the actual content of aspartic and glutamic acids in each factor and indicated that ~18% of the amino acids in NAF I and 19% of those in NAF II are acidic residues. Substantial differences existed between the composition of the two NAF, with more glycine, alanine, leucine, and lysine in NAF I, and more glutamine and valine in NAF II.

Effects of purified NAF on neutrophil function

NAF I and NAF II were examined initially for possible neutrophil cytotoxic effects by incubating 2–100 μl of the purified preparations with portions of 2×10^6 neutrophils in 1 ml HBSS for 30 min at 37°C. At all concentrations of NAF, the net release of lactic acid dehydrogenase was <7% of the total with a spontaneous release of 3% from control neutrophils. In addition, 92% or more of the treated neutrophils excluded trypan blue as compared with 92–97% of control neutrophils.

Neutrophil migration. To assess the mechanism of the inhibition of neutrophil random migration and chemotaxis by purified preparations of NAF I and NAF II, quantities of the preparations ranging from 20 to 80 μl initially were added to the neutrophil well alone. Chemotaxis to C5fr was inhibited 23±2% (mean±SD, $n = 3$) by 20 μl of NAF I and 25±7% by 20 μl of NAF II. The extent of inhibition increased in a dose-related manner and achieved a maximal level of 73±6% inhibition for NAF I and 54±5% inhibition for NAF II at a concentration of 60 μl/ml. At a concentration of 60 μl/ml, NAF I and NAF II inhibited chemotaxis to 20 nM f-Met-Leu-Ala-Phe by a mean of 78 and 62% ($n = 2$), respectively, and to 4 μg/ml of 12-L-HETE by a mean of 81 and 65%. Random migration was inhibited less than chemotaxis and the mean maximal inhibition of random migration was 27 and 26%, respectively, at concentrations of 80 μl/ml of NAF I and 60 μl/ml of NAF II. Both preparations exhibited only marginal chemotactic and chemokinetic activity when added to the stimulus compartment alone and to both the neutrophil well and stimulus compartment, respectively. Mean chemotactic activity ($n = 3$) reached a peak of 4.0 net neutrophils/hpf for 20 μl of NAF I and 4.6 net neutrophils/hpf for 20 μl of NAF II, as contrasted with a mean of 53 net neutrophils/hpf for a 1:80 dilution of C5fr and 42 net neutrophils/hpf for 20 nM f-Met-Leu-Ala-Phe. Mean chemokinetic activity ($n = 2$) reached a peak of 6.8 net neutrophils/hpf for 40 μl of NAF I and 2.4 net neutrophils/hpf for 40 μl of NAF II, as contrasted with a mean of 16.2 net neutrophils/hpf for 1 μg/ml of 12-L-HETE. That the inhibitory effect of NAF I and NAF II on neutrophil chemotaxis was principally a function of an action on the neutrophils rather than on the chemotactic factors was demonstrated by adding 20–80 μl of the purified principles only to the stimulus compartment with the chemotactic factors in some of the chambers in two experiments. In each instance, the mean inhibition achieved by adding NAF I and NAF II to the stimulus compartment was <25% of that observed when the inhibitors were added to the neutrophil well. The reversibility of the chemotactic inhibitory effects of NAF I and NAF II was assessed by preincubating quadruplicate portions of 2×10^6 neutrophils with several concentrations of the factors for 15 min at room temperature. One set of duplicate portions was washed twice and resuspended in 1 ml of buffer and the other set was assayed without washing. The mean responses ($n = 2$) of the washed and unwashed neutrophils, respectively, were 38 and 42% of the response of control neutrophils to C5fr, which demonstrated the irreversibility of the inhibitory effects.

Neutrophil adherence to surfaces. Preincubation of neutrophils with varying concentrations of the purified preparations of NAF I and NAF II, followed by washing the neutrophils, enhanced their adherence and concomitantly decreased their chemotactic response to C5fr (Fig. 4). The dose-response relationships of the two effects were similar and both reached peak levels at the same concentration of each principle. The increase in adherence and the inhibition of chemotaxis reached mean maximum levels of 139% enhancement and 78% inhibition, respectively, at a ratio of 60 μl of NAF I:2 $\times 10^6$ neutrophils (Fig. 4, top frame). An analogous relationship existed between the increase in adherence and the inhibition of chemotaxis induced

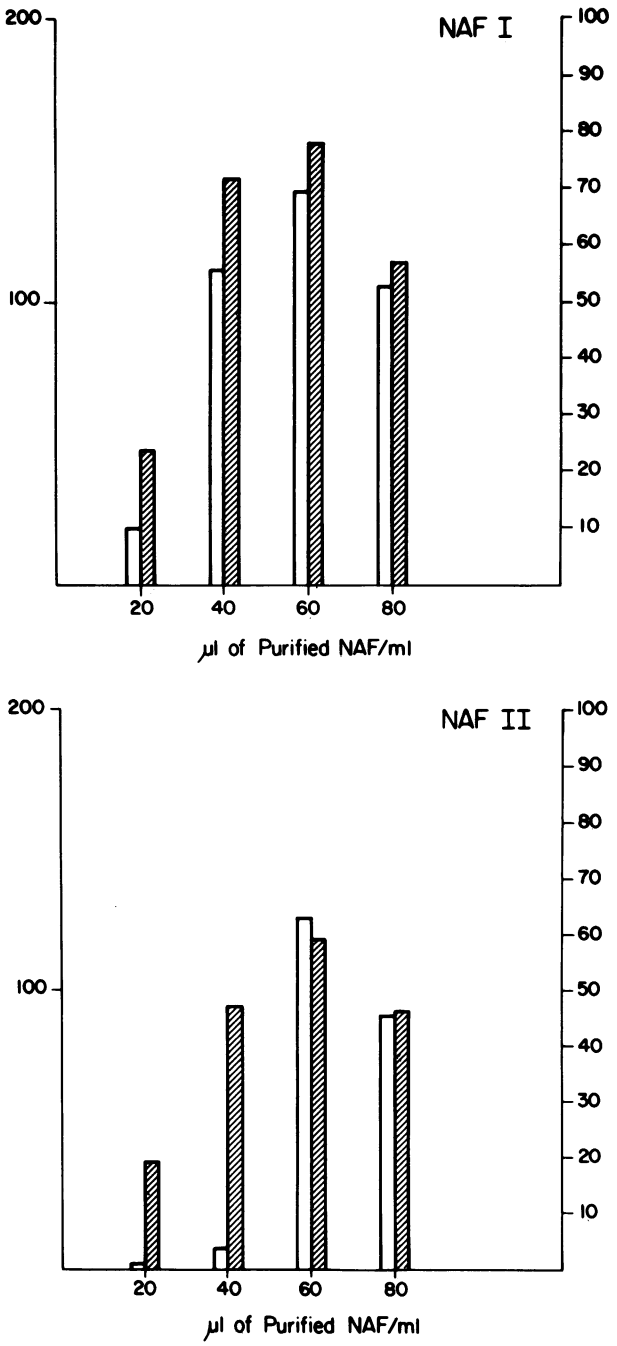

FIGURE 4 Dose-response analyses of the effects of NAF I and NAF II on neutrophil adherence to surfaces and neutrophil chemotaxis. □ represents the mean increase in adherence evoked by various concentrations of NAF I and NAF II in two experiments, which is expressed as a percentage of the adherence of control neutrophils (0% increase). The adherence of control neutrophils was 25 and 32% in the two experiments. ▨ represents the mean percentage inhibition of chemotaxis of neutrophils from the same two donors by the identical concentrations of NAF. The chemotactic responses to C5fr of control neutrophils (0% inhibition) was 43 and 52 net neutrophils/hpf in the two experiments.

by NAF II, which attained maximal levels of 126% enhancement and 59% inhibition, respectively, at a ratio of 60 μl:2 × 10^6 neutrophils (Fig. 4, bottom frame).

$EA_{IgM}C43b$ rosette formation and EA_{IgG} erythrophagocytosis. The incubation of neutrophils with $EA_{IgM}C43b$ in buffer alone led to rosette formation by 32±3% (mean±SD, n = 4) of the neutrophils. Preincubation of neutrophils for 10 min at room temperature with 40 and 80 μl/ml of purified preparations of NAF increased the extent of neutrophil rosette formation to 46±7% (mean±SD, n = 4, $P < 0.05$) and 41±7% ($P > 0.05$), respectively, for NAF I, and 55±6% ($P < 0.05$) and 44±8% ($P > 0.05$) for NAF II. Neutrophils incubated with EA_{IgG} in buffer alone ingested 24±4% (mean ±SD, n = 3) of the EA_{IgG} in 30 min at 37°C. Preincubation of neutrophils for 10 min at room temperature with 40 and 80 μl/ml of the purified preparations of NAF insignificantly increased the phagocytosis of EA_{IgG} to 31±6% ($P > 0.05$) and 28±5% ($P > 0.05$), respectively, for NAF I, and insignificantly decreased phagocytosis to 22±4% ($P > 0.05$) and 20±7% ($P > 0.05$) for NAF II. In parallel experiments, the same concentrations inhibited neutrophil chemotaxis to C5fr by 23 and 58% for NAF I and 25 and 48% for NAF II.

Subcellular localization of NAF I and NAF II

Subcellular fractions from 1 × 10^9 purified neutrophils were sonicated and dialyzed and portions of each were analyzed for enzymatic markers of subcellular constituents and for effects on neutrophil adherence. Half of each of the subcellular fractions were pooled as indicated (Table III) and the pools were filtered on Sephadex G-75 to resolve the NAF from other inhibitors of chemotaxis before an assessment of this activity. Duplicate 50-μl portions of the fractions from the Sephadex G-75 chromatograms were assayed for chemotactic inhibitory activity and the fractions from 44 to 56% bed volume that contained NAF (Fig. 1) were pooled and concentrated to 3 ml. At a concentration of 40 μl/ml, a mean inhibition of 20% or more was found only in gradient fractions 1–2 and 6–9. Assays of the enhancement of adherence by 40 μl/ml of each fraction indicated that mean increases >25% were evoked only by fractions 2 and 6–8. Fractions 6–9 contained the specific lysosomal granules, based on the presence of lysozyme, but not β-glucuronidase (Table III). Thus both the chemotactic inhibitory activity and the adherence enhancing activity of NAF were localized predominantly in the specific granules of the neutrophil. A substantial portion of the NAF activities were recovered in the lighter fractions 1 and 2 from the sucrose gradients along with 66% of the cytoplasmic enzyme lactic acid dehydrogenase, 35% of the plasma membrane marker alkaline p-nitrophenylphosphatase,

TABLE III
Subcellular Localization of NAF

	100 g pellet	400 g pellet	Sucrose density gradient fractions											
			1	2	3	4	5	6	7	8	9	10	11	12
Percentage inhibition of chemotaxis*	11	−5	45			16		51		46			18	
Percentage increase in adherence‡	21	14	21	31	−1	19	24	42	55	39	12	−2	13	0
Enzymatic markers (percentage of total activity)§														
Lysozyme	4	1	3	5	4	2	1	4	13	12	4	7	13	27
β-glucuronidase	6	9	8	5	1	1	2	1	2	6	9	10	15	25
Lactic acid dehydrogenase	1	1	35	31	17	3	2	0	1	3	0	3	2	1
Alkaline *p*-nitrophenylphosphatase	29	6	26	9	4	1	0	2	2	0	2	2	6	11

* One half of fractions 1–2, 3–5, 6–7, 8–9, and 10–12 were pooled as noted and the pools were filtered on Sephadex G-75. Each value is the mean chemotactic inhibition achieved with duplicate 40-μl portions of the 3-ml pools from Sephadex G-75 filtration of pooled fractions from gradients of two separate neutrophil preparations. The responses of control neutrophils (0% inhibition) to C5fr were 53 and 41 net neutrophils/hpf.

‡ The effect on adherence of 40 μl of each dialyzed fraction was assessed in two experiments and the mean values are presented. The adherence of control neutrophils was 30 and 36%, respectively.

§ The total activity recovered (100%) per 10^8 neutrophils was 490 U of lysozyme, 17.2 U of β-glucuronidase, 1.8 U of lactic acid dehydrogenase, and 235 U of alkaline *p*-nitrophenylphosphatase.

and 8% of the lysozyme which apparently had been released from damaged specific granules.

DISCUSSION

A high molecular weight inhibitor of neutrophil chemotaxis that had been recognized in neutrophil sonicates and supernates of phagocytosing neutrophils (1) had been purified in quantities sufficient for structural characterization and elucidation of the mechanism of chemotactic inhibition. Filtration on Sephadex G-75 of sonicates of 1×10^9 neutrophils resolved an ~30,000-mol wt chemotactic inhibitory activity from the 4,000–5,000-mol wt NIF (1, 5) (Fig. 1). Concurrent analyses of the capacity of the Sephadex G-75 fractions to alter the adherence of neutrophils to surfaces revealed that the predominant peak of adherence enhancing activity filtered in the same position as the 30,000-mol wt chemotactic inhibitory activity. Isoelectric focusing of the peak of activities from Sephadex G-75 separated two acidic principles with isoelectric points of 3.3–3.4 and 3.6–3.8, both of which inhibited chemotaxis (Fig. 2) and enhanced adherence. Electrophoresis in glycerol gradients of the two acidic principles from isoelectric focusing defined a difference in their electrophoretic mobility that was consistent with their distinct isoelectric points (Fig. 3). The principles were termed NAF according to their unique functional capability; the less acidic factor was designated NAF I and the more acidic factor, NAF II.

The yield of NAF and the quantity of each NAF in the neutrophils could not be accurately assessed because it was not possible to analyze functionally the contribution of each NAF to the effects observed with the mixture of the two in the Sephadex G-75 peak. Nonetheless, the fraction of each preparation of NAF I and NAF II that was required to achieve 40% inhibition of neutrophil chemotaxis and 50% enhancement of adherence was constant or decreased after each purification procedure, suggesting the removal of inhibitors (Table I). Purified preparations of NAF I and NAF II were hydrolyzed for determinations of their amino acid composition (Table II). The amino acid analyses indicated that the quantity of NAF I recovered was approximately twice that of NAF II and the calculated molecular weight of the principles were ~34,000 and 33,000, respectively. Glutamic acid and aspartic acid together accounted for a mean of 18% of the amino acids in NAF I and 19% of the amino acids in NAF II, whereas the basic amino acids lysine, arginine, and histidine represented less than a mean of 2 and 3% for NAF I and NAF II, respectively. The two principles differed substantially in their content of six other amino acids.

The ability of purified preparations of NAF I and NAF II to influence neutrophil migration, adherence to surfaces, erythrophagocytosis, and the expression of C3b receptors was analyzed in dose-response studies. NAF I and NAF II inhibited neutrophil chemotaxis to C5fr, f-Met-Leu-Ala-Phe, and 12-L-HETE in a dose-response manner that reached maximal levels of 73 and 55%, 78 and 62%, and 81 and 65% inhibition, respectively. There was less inhibition of random migration than chemotaxis and neither principle possessed significant chemotactic or chemokinetic activity at concentrations that gave maximal inhibition of chemotaxis. Both NAF I and NAF II

substantially augmented the adherence of neutrophils to plastic petri dishes, eliciting maximal increases of 139 and 126%, respectively, and the effects on adherence and chemotaxis exhibited comparable dose-response relationships (Fig. 4). Similar quantities of NAF increased the expression of C3b receptors on neutrophils, but did not significantly affect the phagocytosis of opsonized erythrocytes by neutrophils.

The subcellular localization of the NAF was determined by analyses of the adherence enhancing and chemotactic inhibitory activities of sonicates of defined subcellular fractions and of the 30,000-mol wt regions from Sephadex G-75 chromatograms of the sonicates (Table III). The NAF were localized predominantly in the specific granules, whereas a smaller quantity was found in the lighter fractions of the gradients that contained the cytosol, a portion of the plasma membranes, and a minor fraction of the granule constituents. In contrast, no NAF was identified with the azurophilic granules, aggregated membranes or nuclei. NAF I and NAF II are distinguished from the previously described endogenous constituents of neutrophil granules that inhibit chemotaxis based on both structural characteristics and subcellular localization. Lysosomal cationic proteins with protease activity analogous to chymotrypsin (2), lysozyme (3), and NIF (1, 5) are basic polypeptides and proteins that inhibit chemotaxis and, to a lesser extent, random migration. The apparent molecular weight of NAF I and NAF II is ~30,000–34,000 as contrasted with 4,000–5,000 for NIF, 15,000 for lysozyme, and 20,000–30,000 for the cationic proteins (1–3). NAF I and NAF II possess isoelectric points of pH 3.6–3.8 and pH 3.3–3.4, whereas all of the other inhibitors are highly basic. NAF I and NAF II reside largely in the specific granules of the neutrophils, whereas lysozyme, the cationic proteins, and NIF are contained in both the specific and azurophilic granules. The amino acid compositions of NAF I and NAF II indicate that the standard pools of material purified from 10^9 neutrophils by sequential Sephadex G-75 filtration, isoelectric focusing, and electrophoresis contained 0.3–1.0 and 0.2–0.6 nmol of protein, respectively. Thus, as small a quantity as 6 pmol of NAF I or 9 pmol of NAF II is capable of inhibiting chemotaxis to a maximal extent. In contrast, the maximal chemotactic inhibitory effects of lysozyme and the cationic proteins were achieved with quantities of 30–35 pmol and 20–30 pmol, respectively. Thus, the potency of the NAF as chemotactic inhibitors is at least three to five times that of the previously described endogenous inhibitors of neutrophil chemotaxis.

The capacity of diverse chemotactic factors to increase the adherence of human neutrophils to surfaces in vitro has been correlated with the reduced chemotactic responses of comparably treated neutrophils (23, 24). The preincubation of neutrophils with most types of chemotactic factors induces a state of unresponsiveness to chemotactic stimulation that is termed chemotactic deactivation (25, 26). The mechanism of chemotactic deactivation of human neutrophils has not been elucidated and may vary for different chemotactic factors because the ratio of the optimal deactivating concentration to the maximal chemotactic concentration is a distinct property of each factor. The results of dose-response studies demonstrated that optimal increases in neutrophil adherence and maximal chemotactic deactivation were achieved by the same high concentrations of some chemotactic factors (23, 24). However, it could not be established that the increased adherence of the neutrophils induced by the chemotactic factors was directly inhibiting neutrophil chemotaxis, because the concentrations of the stimuli that were used concomitantly suppressed random migration and stimulated both hexose-monophosphate shunt activity and lysosomal enzyme release (24). In contrast, nanogram quantities of NAF I and NAF II suppressed neutrophil chemotaxis and enhanced neutrophil adherence to surfaces with comparable dose-response relationships for the two effects and without significantly suppressing other neutrophil functions. The selective localization of NAF I and NAF II in the specific granules of neutrophils raises the possibility that the concomitant suppression of chemotaxis and enhancement of adherence by chemotactic factors and other neutrophil-directed stimuli is mediated by the release of the endogenous NAF. In two experiments, the incubation of portions of 2×10^7 neutrophils in 1 ml of HBSS with 3 mg/ml of opsonized zymosan (13) and 10 μM f-Met-Leu-Ala-Phe released a mean of 38 and 24%, respectively, of the adherence enhancing activity of the NAF, as assessed by dose-response analyses of Sephadex G-75 fractions from supernates and sonicates of the challenged neutrophils. A definitive analysis of the relevance of NAF release will require detailed studies of the time-course of appearance of NAF in the extracellular fluid, in relation to the initial, accelerated, and plateau phases of chemotactic migration.

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