Immunology 1983 **48** 79

Isolation of two polypeptides comprising the neutrophil-immobilizing factor of human leucocytes

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Accepted for publication 15 June 1982

Summary. Human leucocyte lysosomal polypeptides of mol. wt 4000–5000, which constitute the neutrophil-immobilizing factor (NIF), were isolated from the 22,000 *g* supernate of sonicates of human neutrophils by filtration on Sephadex G-75. The larger (NIF-1) and smaller (NIF-2) of the polypeptides were resolved by filtration on Bio-Gel P6 and purified to homogeneity by sequential reverse-phase high performance liquid chromatography and paper electrophoresis. The results of analyses of amino acid composition indicated that NIF-1 and NIF-2 are distinct polypeptides composed of an apparent total of 41 and 38 amino acids, respectively. Both NIF polypeptides contain one cysteine and one methionine, lack isoleucine, tyrosine and phenylalanine, and are rich in histidine and proline. The sequence of 20 of the amino-terminal amino acids of both NIF polypeptides is identical, but NIF-2 possesses an additional alanine at the amino-terminus. Highly purified NIF-1 and NIF-2 inhibited human neutrophil random migration and chemotaxis to diverse stimuli in a concentration-dependent manner, with 50% inhibition of chemotaxis by $0{\cdot}3–1 \times 10^{-8}$ M NIF-1 and $1–3 \times 10^{-7}$ M NIF-2. Neither NIF polypeptide was cytotoxic for neutrophils, altered neutrophil phagocytosis or release of lysosomal enzymes, or inhibited mononuclear leucocyte chemotaxis. The leucocyte and functional specificity of the NIF polypeptides and the quantities released upon stimulation of human leucocytes suggest that the transition to a mononuclear leucocyte population in chronic inflammation may be attributable in part to the NIF derived from the leucocyte infiltrates of acute responses.

Abbreviations: PMN, polymorphonuclear; NIF, neutrophil-immobilizing factor; PMSF, phenylmethylsulphonyl fluoride; TLCK, N-tosyl-L-lysyl-chloromethyl ketone; HBSS-OA, Hanks's balanced salt solution containing 0·2 g/100 ml of ovalbumin and 0·005 M Tris-HCl, pH 7·4; h.p.f., high power field; E, sheep erythrocyte; A_{IgG}, rabbit IgG anti-sheep erythrocyte antibodies; OD, optical density; HBSS-HSA, Hanks's balanced salt solution containing 0·1 g/100 ml of human serum albumin; HPLC, high performance liquid chromatography; ATZ, anilinothiazolinone; PTH, phenylthiohydantoin.

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0019-2805/83/0100-0079$02.00

INTRODUCTION

Human polymorphonuclear (PMN) leucocytes and monocytes exposed to phagocytosable particles, acidic pH, or specific soluble stimuli, such as chemotactic factors or lymphokines, release a neutrophil-immobilizing factor (NIF) which non-cytotoxically inhibits PMN leucocyte random migration and chemotaxis *in vitro* (Goetzl & Austen, 1972; Goetzl, Gigli,

Wasserman & Austen, 1973; Goetzl & Rocklin, 1978). Analyses of subcellular fractions of human leucocytes suggested that NIF is contained preformed in lysosomal granules. Concentrations of partially purified NIF that maximally suppressed neutrophil and eosinophil responses to structurally diverse chemotactic factors failed to alter oxidative metabolism, phagocytosis, or adherence to surfaces, and only minimally inhibited mononuclear leucocyte chemotaxis (Goetzl & Austen, 1972; Goetzl *et al.*, 1973). NIF was inactivated in a time-dependent manner by incubation with trypsin or chymotrypsin (Goetzl & Austen, 1972). The NIF polypeptides exhibited an approximate mol. wt of 4000–5000 on Sephadex gel columns and had strongly basic properties, as assessed by ion-exchange chromatography (Goetzl, 1975). The two functionally predominant polypeptides of human neutrophil-derived NIF now have been isolated for analyses of amino acid sequence and studies of the leucocyte-directed activities of the individual constituents.

EXPERIMENTAL PROCEDURES

Materials

Hanks's balanced salt solution (M. A. Bioproducts, Walkersville, Md), ovalbumin which had been recrystallized five times (Miles Laboratories, Inc., Elkhart, Ind.), macromolecular dextran, Sephadex G-75, and Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), 5 cm × 5 cm micropolyamide sheets (Schleicher & Schuell, Inc., Keene, N.H.), synthetic N-formyl-methionyl-leucyl-phenylalanine (f-Met-Leu-Phe), zymosan, phenylmethylsulphonyl fluoride (PMSF), N-tosyl-L-lysyl-chloromethyl ketone (TLCK), and reagents for the quantification of lactate dehydrogenase, lysozyme, and β-glucuronidase (Sigma Chemical Co., St. Louis, MO.), Bio-Gel P6 (Bio-Rad Laboratories, Richmond, Calif.), dansyl chloride (Cal Biochem Co., Inc., La Jolla, Calif.), organic solvents which had been redistilled from glass (Burdick & Jackson Co., Muskegon, Mich.), and reagents for determining the amino acid composition and sequence of polypeptides (Beckman Instruments, Inc., Palo Alto, Calif. and Pierce Chemical Co., Rockford, Ill.) were obtained from the specified suppliers. Zymosan particles were opsonized in fresh human serum as described (Tauber & Goetzl, 1979). Chemotactic fragments of the fifth component of complement (C5fr) were purified from zymosan-activated human serum (Goetzl & Hoe, 1979).

Assessment of human leucocyte migration and other functions

Human leucocytes were obtained from normal subjects and resolved by centrifugation on Ficoll-Hypaque cushions into neutrophils of over 95% purity and mononuclear leucocytes consisting of 16–25% monocytes, 71–78% lymphocytes, and 4–9% neutrophils (range, $n = 10$), as described (Goetzl & Austen, 1972; Goetzl & Rocklin, 1978). The purified neutrophils and mononuclear leucocytes were washed and resuspended at concentrations of $2{\cdot}0 \pm 0{\cdot}2 \times 10^6$/ml and $4{\cdot}0 \pm 0{\cdot}4 \times 10^6$/ml, respectively, in Hanks's balanced salt solution containing 0·2 g/100 ml of ovalbumin and 0·005 M Tris-HCl, pH 7·4 (HBSS-OA). Leucocyte random and chemotactic migration were assayed by a modification (Goetzl & Austen, 1972; Goetzl & Hoe, 1979) of the Boyden micropore filter technique using disposable chambers (Adaps, Inc., Dedham, Mass.) with 3 μm pore filters for neutrophils and 8 μm pore filters for mononuclear leucocytes (Sartorius Filter, Inc., Hayward, Calif.). Leucocytes in fixed and stained filters from duplicate chambers known to lack a stimulus were counted at 10 μm levels between 60 and 120 μm from the source of leucocytes in order to determine the levels at which mean counts were in the range of 20 to 25 leucocytes/high power field (h.p.f.) for random migration and 3–5 leucocytes/h.p.f. for the chemotactic background. Random migration is expressed as leucocytes/h.p.f. and chemotaxis as net leucocytes/h.p.f., after subtraction of the corresponding background value. Leucocytes were preincubated for 10 min at 37° with dilutions of samples of neutrophil-derived polypeptides or buffer alone for the control leucocytes, before addition to the chambers, and the migration of treated leucocytes is expressed as a percentage of that of control leucocytes.

In order to quantify the phagocytosis of opsonized erythrocytes by neutrophils, sheep erythrocytes (Es) first were coated with purified rabbit IgG anti-E antibodies (A_{IgG}) (Bockenstedt & Goetzl, 1980). Duplicate portions of $1{\cdot}6 \times 10^6$ neutrophils were preincubated for 10 min at 37° in 0·5 ml of a dilution of neutrophil-derived polypeptides or buffer alone, 8×10^6 EA_{IgG} were added to each suspension of neutrophils, and the mixtures were incubated for 30 min at 37° as described (Bockenstedt & Goetzl, 1980). 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° and the optical density (OD) of the supernate was determined at 414 nm. The calculation 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 quantification of the percentage phagocytosis of EA_{IgG} (Gigli, Wintroub & Goetzl, 1976). Phagocytosis by neutrophils that were preincubated with polypeptides is expressed as a percentage of control phagocytosis by neutrophils exposed to buffer alone.

Modulation of lysosomal enzyme release was analysed by preincubating replicate samples of 4×10^6 neutrophils in 0·5 ml of Hanks's solution containing 0·1 g/100 ml of human serum albumin (HBSS-HSA) for 10 min at 37° without or with a dilution of neutrophil-derived polypeptides. A stimulus of enzyme release in 0·5 ml of HBSS-HSA containing 1 mg/100 ml of cytochalasin B or the buffer alone was added to each suspension, the incubation was continued for 20 min at 37°, and the suspensions were centrifuged at 1000 *g* for 5 min at 4°. One hundred microlitre aliquots of the 1000 *g* supernates and 50 μl aliquots of sonicates of the pellets in 1 ml of buffer were used to assay the concentrations of β-glucuronidase, lysozyme, and lactate dehydrogenase as described (Bockenstedt & Goetzl, 1980). The net percentage release of each enzyme was calculated by subtracting the release observed in buffer alone.

Determinations of statistical significance were based on the standard Student's *t* test.

Extraction, purification, and structural analysis of polypeptides from human neutrophils

Neutrophils which were purified as for the functional studies were suspended at a concentration of 1×10^8/ml in 0·05 M NaCl–0·1 M sodium acetate (pH 5·5) containing 10^{-3} M TLCK and 10^{-4} M PMSF. The suspensions were sonicated at 4° with 300 W for 3 min (Branson Sonic Power Co., Model 350, Danbury, Conn.) and centrifuged at 22,000 *g* for 30 min at 4°. The 22,000 *g* supernate was filtered on a 92 cm × 2·5 cm column of Sephadex G-75 which was equilibrated and developed with 0·1 M NH_4HCO_3 at a flow rate of 30 ml/hr at 4°. The column effluent that had NIF activity was pooled, lyophilized, redissolved in 2 ml of 1% (v:v) acetic acid, and filtered on a 120 cm × 2·5 cm column of Bio-Gel P6 in 1% acetic acid which resolved the NIF activity into two overlapping peaks. Each of the NIFs was purified separately by sequential reverse-phase high performance liquid chromatography (HPLC) and paper electrophoresis.

Reverse-phase HPLC was performed with a dual metered pump system and a 4·6 mm × 25 cm Ultrasphere 5 μm ODS column (Altex Scientific Division of Beckman Instruments, Inc., Berkeley, Calif.), the effluent from which was monitored at 230 nm in the flow cell of an Hitachi model 100-40 spectrophotometer with Hewlett-Packard Model 3380A recording integrator. The column was equilibrated in 0·04 M potassium phosphate, pH 2·8, and developed with a linear gradient to 60% acetonitrile: 40% 0·02 M H_3PO_4 in water (v:v) over 60 min at room temperature. Each of the two NIFs was recovered as a single peak of optical density and chemotactic inhibitory activity, lyophilized, redissolved in 0·1% (v:v) acetic acid, and spotted on 3 cm × 40 cm strips of paper (Schleicher & Schuell, Inc., Keene, N.H.) which were wetted with pyridine:glacial acetic acid:water (25:1:225, v:v, pH 6·4) and electrophoresed at 300 V for 3·5 hr at room temperature. A 3 mm segment was cut from the edge of each paper strip and stained with ninhydrin. The sections of the unstained paper strip corresponding to the bands of staining, as well as the other sections, were eluted separately with 0·01 M NH_4OH.

For analyses of amino acid composition, replicate portions of each purified polypeptide were lyophilized in 9 mm × 150 mm glass test tubes and resuspended in 0·3 ml of constant boiling (5·7 M) HCl. The tubes were evacuated and heated at 108° for 24–72 hr (Hare, 1975). Ten percent of two preparations of each of the two purified NIF polypeptides was oxidized with performic acid before hydrolysis in 5·7 M HCl in order to permit analyses of cysteine as cysteic acid. The amino acids in the hydrolysates were quantified with a Durrum D500 analyser, utilizing norleucine as an internal standard (Dionex Co., Sunnyvale, Calif.). Purified polypeptides were reacted with dansyl chloride and hydrolysed, and the hydrolysates were analysed by chromatography on 5 cm × 5 cm polyamide thin layer sheets as described (Watt, Takagi & Doolittle, 1979).

The amino-terminal amino acid sequence of 100–150 nmol of each of the purified NIF polypeptides was determined with a spinning cup sequenator (Model 890C modified with cold trap, Beckman Instruments, Inc., Palo Alto, Calif.). Polybrene was added to the cup and the sequenator was operated according to a standard program, using a 0·1 M Quadrol buffer system and a cup temperature of 57° (Beckman Instruments, Inc., Palo Alto, Calif.; Waterfield &

Bridgen, 1975). One-half of each amino acid residue generated by the sequenator as the anilinothiazolinone (ATZ) derivative was hydrolysed in 0·2 ml of 0·1 g $SnCl_2$ in 100 ml of 5·7 M HCl at 150° for 18 hr to generate the free amino acid, which was quantified in the amino acid analyzer. The other one-half of each amino acid residue was incubated in 0·2 ml of 25% (v:v) aqueous trifluoroacetic acid at 55° for 30 min to achieve conversion to the corresponding phenylthiohydantoin (PTH)-amino acid. The PTH-amino acids were resolved and quantified by reverse-phase HPLC, as described (Zimmerman, Appello & Pisano, 1977).

RESULTS

NIF polypeptides released by sonication of human neutrophils in the presence of proteolytic inhibitors were separated from the proteins and larger polypeptides in 22,000 *g* supernates by filtration on Sephadex G-75. Dilution-effect analyses of portions of the column effluent amounting to 1·5% of the bed volume and of the 22,000 *g* supernate of the sonicate before gel filtration indicated that 61%–73% of the inhibitory activity for neutrophil chemotaxis to C5fr (range, $n=6$) eluted at 93%–96% bed volume, which coincided with a peak of optical density at 230 nm. Filtration on Bio-Gel P6 of the pool of NIF polypeptides recovered from Sephadex G-75 resolved the neutrophil chemotactic inhibitory activity into two overlapping peaks of approximate mol. wt 4000–5000, the larger polypeptide of which was designated NIF-1 and the smaller NIF-2. Each of the NIF polypeptides was purified to homogeneity by sequential reverse-phase HPLC and paper electrophoresis. NIF-1 was eluted from the HPLC column at 22% acetonitrile and NIF-2 at 24% acetonitrile, and the overall yields based on recovery of neutrophil chemotactic inhibitory activity were 37%–43% and 32%–39%, respectively (range, $n=4$). Paper electrophoresis of the NIFs after HPLC led to the isolation of the predominant polypeptide that accounted for each activity (Fig. 1). The major peak of ninhydrin staining for NIF-1 and the only quantitatively significant peak for NIF-2 inhibited neutrophil chemotaxis to C5fr by a mean of 82% and 75%, respectively, and had substantially less effect on mononuclear leucocyte chemotaxis. In contrast, eluates of other regions of the paper strips inhibited neutrophil chemotaxis by a mean of 26% or less.

The purified NIF polypeptides were assessed for homogeneity by ion-pair reverse-phase HPLC and by

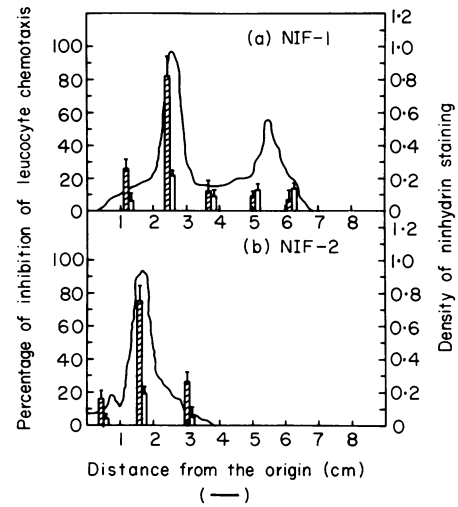

Figure 1. Purification of NIF peptides by paper electrophoresis. Each solid line depicts the pattern of density of ninhydrin staining in a 3 mm segment cut from the edge of a paper strip after the electrophoresis of approximately 30 nmol of NIF-1 (a) and NIF-2 (b). The maximum density of staining of 30 nmol of Gly-His-Leu, which had been electrophoresed concurrently, was arbitrarily set at 1·0 in order to calibrate the recorder. The cross-hatched bars and open bars represent the mean percentage inhibition ± SD of the chemotactic responses to C5fr of neutrophils and mononuclear leucocytes, respectively, which had been preincubated with 1/50 dilutions of eluates from sections of the paper strips for three different NIF preparations. Sections eluted included the paper that was 0·5–2·0, 2·0–3·0, 3·0–4·5, 4·5–5·7 and 5·7–6·7 cm from the origin for NIF-1 and 0·2–1·0, 1·0–2·3, and 2·3–3·9 cm for NIF-2. The control chemotactic responses of untreated leucocytes were 58·3 ± 14.9 net neutrophils/h.p.f. (mean ± SD, $n=3$) and 37·6 ± 11·7 net mononuclear leucocytes/h.p.f.

analysis of the amino-terminal amino acids. One-four nanomoles of purified polypeptide were applied to a 4·6 × 25 cm ODS reverse-phase HPLC column (5 μm ion-pair Ultrasphere, Altex Scientific Division of Beckman Instruments, Inc., Berkeley, Calif.) that was equilibrated and developed isocratically for 20 min in 0·01 M hexane sulphonate–0·01 M potassium phosphate (pH 2·9) in 16% acetonitrile:water (v:v), and then eluted with a linear gradient to 0·01 M hexane sulphonate–0·01 M potassium phosphase (pH 2·9) in 80% acetonitrile:water (v:v) over 40 min. NIF-1 and NIF-2 eluted as distinct symmetrical peaks of optical density at 210 nm and of chemotactic inhibitory activity at 33·7% and 41·1% acetonitrile, respectively. Chromatographic analyses of hydrolyses of dansyl-derivatives of purified NIF-1 and NIF-2 on thin-layer

polyamide sheets revealed a predominant spot corresponding to dansylalanine for both polypeptides, with far lesser quantities of dansylated valine, glycine, and aspartic acid.

Structural analysis of purified NIF peptides

Amino acid analyses of acid hydrolyses of four different preparations of each NIF peptide indicated that NIF-1 and NIF-2 apparently were composed of 41 and 38 residues, respectively (Table 1). Calculated mol. wt values were 4196 for NIF-1 and 4128 for NIF-2. Both peptides lacked Ile, Tyr, and Phe and had lower than average contents of hydrophobic amino acids and approximately twice the average number of basic amino acids. The presence of one cysteine or half-cystine in each peptide was confirmed by analyses of portions of two separate preparations of each polypeptide that had been oxidized with performic acid, which revealed 0·87 and 0·91 cysteic acid residues for NIF-1 and 0·79 and 0·95 for NIF-2. The amino terminal sequences of NIF-1 and NIF-2 confirmed the purity of the peptides, as both methods identified the same predominant amino acid for each of the positions 1–20/21, despite up to 20% carry-over of the preceding amino acid for some of the analyses (Table 2). For example, alanine represented a mean of 80% of the PTH-amino acids recovered from residue 1 of NIF-2, as assessed by HPLC, and a mean of 86% by amino acid analysis, while alanine accounted for over 95% of the amino acids from residue 1 of NIF-1 by both methods. In the first 20/21 amino acids, NIF-1 and NIF-2 differed only in the finding of an additional alanine at the amino terminus of NIF-2. The methionine, five of the histidine, two of the lysine, and two of the proline residues were found in the first 20/21 amino terminal amino acids.

Characterization of the leucocyte-directed functions of purified NIF peptides

The quantification of NIF peptides by amino acid analyses permitted an assessment of the potencies of the factors on a molar basis with respect to different

Table 1. Amino-acid composition of neutrophil-immobilizing factors

	NIF-1	NIF-2
Asx	1·96±0·15*	2·92±0·13
Thr	1·94±0·30	1·85±0·14
Ser	1·57±0·16	1·62±0·12
Glx	5·97±0·24	4·94±0·25
Pro	4·66±0·23	3·03±0·38
Gly	7·09±0·31	5·08±0·21
Ala	1·87±0·07	2·10±0·24
Cys	0·75±0·14	0·64±0·47
Val	0·89±0·07	1·08±0·15
Met	0·95±0·13	0·85±0·12
Leu	1·94±0·25	2·84±0·15
His	6·96±0·15	4·73±0·18
Lys	3·01±0·14	3·89±0·07
Arg	—	1·14±0·12
Apparent total number of amino acids	41	38
Minimum molecular weight	4196	4128
Acidic and amide amino acids†	19·5	21·1
Basic amino acids†	24·4	26·3
Hydrophobic amino acids†	9·8	13·2

* Each value is the mean±SD of the results of analyses of four different preparations of NIF-1 and NIF-2.

† Mean values for the amino acid compositions of 314 diverse proteins (Dayhoff, Hunt & Hurst-Calfrone, 1978) were 19·8% acidic and amide amino acids, 13·5% basic amino acids and 20·2% hydrophobic amino acids, where the latter group consists of Leu, Ile, Val and Met.

Table 2. Amino-terminal amino acid sequences of NIF peptides

Peptide	Amino acid sequence										
	1	2	3	4	5	6	7	8	9	10	
NIF-1	Ala*	Ser	His	Glu	Lys	Met	His	Glu	Gly	Asp	
NIF-2	Ala*	Ala	Ser	His	Glu	Lys	Met	His	Glu	Gly	
	11	12	13	14	15	16	17	18	19	20	21
NIF-1	Glu	Gly	Pro	Gly	His	His	His	Lys	Pro	Gly	
NIF-2	Asp	Glu	Gly	Pro	Gly	His	His	His	Lys	Pro	Gly

* Each amino acid tabulated represented 46%–98% of those detected by both methods for that position in two different preparations of NIF peptides.

functions. Both NIF-1 and NIF-2 inhibited significantly the chemotaxis to C5fr and the random migration of neutrophils in a concentration-dependent manner (Fig. 2). Inhibition of chemotaxis to C5fr achieved a mean level of 50% at approximately 3×10^{-9} M–10^{-8} M NIF-1 and 1–3×10^{-7} M NIF-2, respectively, while inhibition of random migration was 50% at 3×10^{-8} M NIF-1 and 3×10^{-6} M NIF-2. Inhibition of chemotaxis to 10^{-7} M f-Met-Leu-Phe was similar to that seen with C5fr, but the mean 50% level was at 3×10^{-9} M NIF-1 and 1×10^{-7} M NIF-2 ($n=3$). In contrast, the chemotactic response of mononuclear leucocytes to C5fr was inhibited by only $22 \pm 13\%$ (mean $\pm$ SD, $n=3$) with 10^{-6} M NIF-1 and $10 \pm 15\%$ with 10^{-6} M NIF-2.

In addition, neither purified NIF peptide influenced phagocytosis or release of lysosomal enzymes by neutrophils. Erythrophagocytosis by neutrophils was $39{\cdot}1 \pm 6{\cdot}1\%$ (mean $\pm$ SD, $n=3$) in buffer alone and $38{\cdot}0 \pm 4{\cdot}5\%$, $36{\cdot}1 \pm 5{\cdot}3\%$, $39{\cdot}2 \pm 7{\cdot}4\%$, and $41{\cdot}7 \pm 5{\cdot}7\%$ in the presence of 10^{-7} M NIF-1, 10^{-6} M NIF-1, 10^{-7} M NIF-2, and 10^{-6} M NIF-2, respectively. The release of

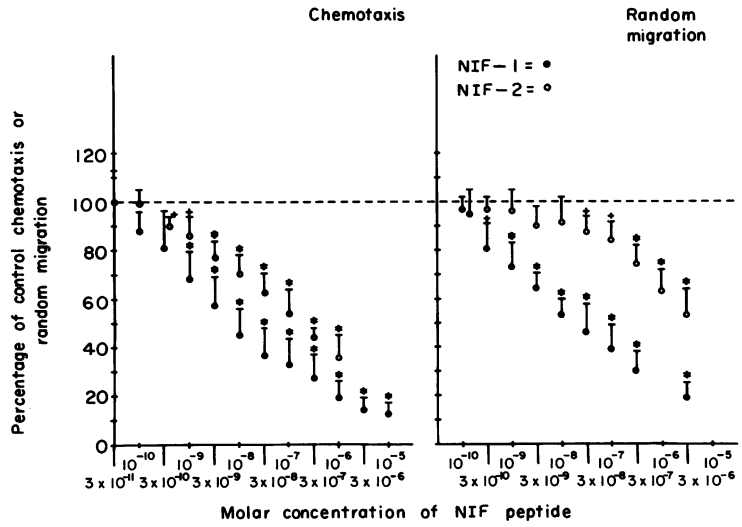

Figure 2. Inhibition of human neutrophil migration by NIF peptides. Each point and bracket depicts the mean $\pm$ SD of the values for four separate studies with neutrophils from different subjects. The control levels of random migration for untreated neutrophils ranged from 20·4 to 24·3 neutrophils/h.p.f. and of chemotaxis to C5fr from 50·6 to 69·2 net neutrophil/h.p.f. The levels of statistical significance of the inhibition are shown by symbols: $+ = P \leq 0{\cdot}05$ and $* = P \leq 0{\cdot}01$.

β-glucuronidase and lysozyme elicited by 10^{-6} M f-Met-Leu-Phe was $29{\cdot}4 \pm 3{\cdot}2\%$ (mean ± SD, $n=4$) and $46{\cdot}2 \pm 5{\cdot}8\%$, respectively, and was not altered by preincubating the neutrophils with 10^{-8} M–10^{-6} M NIF-1 or NIF-2. Neither purified NIF peptide at concentrations from 10^{-9} M to 3×10^{-6} M released lactate dehydrogenase or the lysosomal enzymes from neutrophils over 60 min at 37°.

DISCUSSION

Two human neutrophil polypeptides, which account for the bulk of the selective activity of NIF, have been resolved partially by gel filtration and purified to homogeneity by the sequential application of reverse-phase HPLC and paper electrophoresis (Fig. 1). The purity of the distinct NIF polypeptides isolated was over 85%, as assessed by both ion-pair reverse-phase HPLC and analyses of the amino-terminal amino acids. Amino acid compositions indicated that each NIF basic polypeptide is rich in histidine and proline, has one cysteine or half-cystine and one methionine, and lacks isoleucine, tyrosine and phenylalanine (Table 1). The larger polypeptide (NIF-1) apparently contains 41 amino acids, but has one fewer residue of aspartic acid/asparagine and of leucine than the polypeptide, apparently composed of 38 amino acids (NIF-2), and lacks the single arginine of NIF-2. The amino-terminal amino acid sequence confirmed the purity of both NIF-1 and NIF-2 and revealed complete homology between the first twenty amino acids of NIF-1 and amino acids two to twenty-one of NIF-2 (Table 2). NIF-2 had an additional alanine at the amino-terminus.

The availability of purified NIF polypeptides, which were quantified by amino acid analyses, permitted the evaluation of leucocyte-directed functions of precise concentrations of each active principle. The NIFs inhibited maximal chemotactic responses of human neutrophils to C5fr and f-Met-Leu-Phe in a concentration-dependent manner with a mean of 50% inhibition by 3×10^{-9} M–10^{-8} M NIF-1 and $1\text{–}3 \times 10^{-7}$ M NIF-2 (Fig. 2). Random migration of neutrophils was more resistant to NIF than chemotaxis, as 50% inhibition required three- to ten-fold higher concentrations of either NIF-1 or NIF-2. Neither purified NIF polypeptide was cytotoxic for neutrophils, as assessed by the failure of concentrations as high as 3×10^{-6} M to release significant quantities of lactate dehydrogenase. The cellular specificity of the NIF polypeptides was demonstrated by their relatively minor effect on the chemotactic responses of human mononuclear leucocytes to C5fr, which were inhibited by less than 25% at concentrations of NIF-1 or NIF-2 as high as 10^{-6} M. In addition, both NIF polypeptides exhibited functional specificity. Concentrations of NIF-1 and NIF-2 that inhibited neutrophil chemotaxis by 80% and 60%, respectively, failed to either modify the release of lysosomal enzymes by neutrophils exposed to chemotactic factors or to initiate enzyme release, and did not alter the extent or rate of phagocytosis of IgG-sensitized erythrocytes by neutrophils. While the mechanism of action of NIF polypeptides on PMN leucocyte migration has not been elucidated, the absence of cytotoxicity after prolonged exposures and the lack of effect on other functions that depend on cellular motility, such as phagocytosis, suggest a high degree of specificity for translational motion of the leucocytes. Although random migration of PMN leucocytes was less sensitive to NIF polypeptides than chemotaxis, the pronounced inhibition observed at higher concentrations mitigates against a predominant effect of NIF on receptors for chemotactic factors.

A consideration of the quantities of the NIF polypeptides released by leucocytes, in relation to their potency, implies an important role in endogenous regulation of the leucocyte components of tissue inflammatory responses. The NIF polypeptides accounted for the bulk of the chemotactic inhibitory activity in sonicates of human neutrophils. The other chemotactic inhibitory activities detected in the effluent from Sephadex G-75 columns together amounted to only 25%–35% of the total recovered from neutrophil sonicates (range, $n=6$) and were attributable to proteins of approximate mol. wt 34,000 and 180,000, which act by enhancing neutrophil adherence to surfaces and thereby depressing migration (Bockenstedt & Goetzl, 1980; Curnutte, Crowley, Rosin & Babior, 1980). Preliminary analyses of the NIF polypeptides released by human neutrophils challenged with chemotactic factors or phagocytosable particles indicate both that a substantial portion of the preformed stores are mobilized and that the functional properties of the released NIF are similar to those of the NIF obtained by sonication. Replicate suspensions of 10^8 neutrophils in 5 ml of Hanks's solution–0·1% ovalbumin were incubated 30 min at 37° with 10^{-6} M f-Met-Leu-Phe or opsonized zymosan, or were sonicated by the standard procedure. The 22,000 *g* supernates of the incubation

mixtures and the sonicates were purified by sequential filtration on Bio-Gel P6 and reverse-phase HPLC. The HPLC elution times of NIF-1 and NIF-2 were identical for the released polypeptides and those in the sonicates. The quantities of NIF-1 and NIF-2 released by f-Met-Leu-Phe and opsonized zymosan, based on the relative area of the peaks of optical density at 210 nm were 10%–14% (range, $n=2$) and 26%–33%, respectively, of the quantities in the sonicates.

The mean yield of NIF-1 from the complete purification procedure was 41·6 pmol per 10^7 neutrophils ($n=12$ sonicates). Assuming an overall recovery for NIF-1 of 20% and a mean release from stimulated neutrophils of 20%, 10^7 neutrophils would generate a concentration of approximately 10^{-8} M NIF-1 in the restricted volume of a local inflammatory response. This concentration of NIF-1 is capable of inhibiting substantially the chemotactic responses and resultant influx of up to 10^7 other neutrophils or eosinophils, without altering the functions of the PMN leuococytes already trapped at the site of inflammation and without preventing the subsequent accumulation of mononuclear leucocytes. The concentrations of NIF polypeptides that can be achieved in both acute and chronic inflammation may explain in part the transition of the cellular response from PMN leucocytes to predominantly macrophages and lymphocytes.

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