A NEUTROPHIL-IMMOBILIZING FACTOR DERIVED FROM HUMAN LEUKOCYTES

I. Generation and Partial Characterization*

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The capacity of humoral factors to influence polymorphonuclear leukocyte function has been recognized by in vitro studies. Neutrophil phagocytosis is enhanced by the presence of a fragment of the third component of complement, C3b, on a target cell or complex (1). Neutrophils respond chemotactically in vitro to by-products of the complement reaction sequence such as the cleavage product of the fifth complement component, C5a, or the trimolecular complex of the fifth, sixth, and seventh components of complement, $C\overline{567}$, and to components of the kinin-generating system such as kallikrein (2–4). The inhibitor of the activated first component of complement ($C\overline{1}INH$) blocks the neutrophil response to kallikrein.[1] Other factors which suppress chemotaxis in vitro are found in the sera of some patients (5–7). These factors have not been isolated, and in most instances it has not been definitively determined whether such inhibitors function by blocking the stimulus or by affecting the neutrophils directly. In addition, as chemotactic principles can deactivate their target cells, specific inhibitors must be separated from contaminating chemotactic activity.

An inhibitor of chemotaxis is released from human polymorphonuclear or mononuclear leukocytes during phagocytosis or exposure to mildly acid pH or endotoxin. This inhibitor, designated the neutrophil-immobilizing factor (NIF),[2] is not chemotactic and acts directly and irreversibly on human neutrophils to prevent their response to a variety of different chemotactic principles without impairment of their viability. This finding that the leukocytes themselves can modulate their response to humoral chemotactic factors represents an additional link in the inflammatory reaction sequence.

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[2] *Abbreviations used in this paper:* ANIF, neutrophil-immobilizing factor released by leukocyte acid incubation; ENIF, neutrophil-immobilizing factor generated from leukocytes by endotoxin treatment; NIF, neutrophil-immobilizing factor; PhNIF, neutrophil-immobilizing factor found in leukocyte phagocytosis supernatants.

Materials and Methods

Polystyrene disposable chemotactic chambers (Adaps Inc., Dedham, Mass.) and micropore filters with a 3 μ or an 8 μ pore size (Millipore Corporation, Bedford, Mass.) were assembled as previously described (3). Hanks' solution and Medium 199 with or without phenol red (Microbiological Associates, Inc., Bethesda, Md.), ovalbumin five times recrystallized (Pentex Biochemical, Kankakee, Ill.), dextran and Ficoll (Pharmacia Fine Chemicals Inc., Piscataway, N.J.), *Escherichia coli* 026:B6 lipopolysaccharide β endotoxin (Difco Laboratories, Detroit, Mich.), trypan blue dye (Allied Chemical Corp., New York), sodium diatrizoate (Hypaque, Winthrop Laboratories, New York), insolubilized chymotrypsin and trypsin (Miles-Yeda, Ltd., Rehovoth, Israel), blue dextran 2000 and RNase (Pharmacia), glucagon (Mann Research Labs., Inc., New York), vitamin B12 (Eli Lilly and Company, Indianapolis, Ind.), and copper sulfate (Fisher Scientific Co., Medford, Mass.) were all purchased and used without further purification. Rice starch (Whittaker, Clark & Daniels, Inc., Plainfield, N.J.) was washed five times in distilled water before use.

Ultrasonic fragmentation of leukocytes was performed using probe sonication (Ultrasonics Instruments International, Inc., Farmingdale, N.Y.). Leukocyte supernatants and chromatographic fractions were concentrated using positive pressure chambers and UM-2 membranes from Amicon Corporation, Lexington, Mass. Protein concentration was measured by optical density at 280 nm or by an adaptation of the Folin technique (8). Immunoelectrophoresis and radial immunodiffusion were performed by standard methods (9, 10).

Measurement of Chemotaxis.—Chemotaxis of human neutrophils was quantitatively assayed by a previously described modification (3, 11) of the Boyden micropore filter technique (12). Blood, collected from normal subjects in plastic syringes, was added in 9-ml portions to 14-ml plastic tubes containing 1 ml of 6% dextran in saline and 1 ml of 0.15 M citrate anticoagulant solution (pH 5.2). After the red blood cells had settled for 1 hr at 37°C, the leukocyte-rich supernatant was removed by aspiration and centrifuged at 200 *g* for 5 min. The cells were washed twice with Hanks' solution and resuspended in Medium 199, which had been made 0.5% in ovalbumin and adjusted to pH 7.4 by dropwise addition of 2% $NaHCO_3$. For each experiment, the initial cell count was adjusted to 1.3–1.6 $\times$ 10^6 leukocytes/ml. Chemotactic agents were also diluted in Medium 199, 0.5% ovalbumin, pH 7.4. Millipore filter pore size was 3 μ for neutrophil chemotaxis. The interaction of leukocytes and chemotactic factors was carried out at 37°C in moist chambers for 2–2½ hr so that the mean background neutrophil counts were 0–6 per high power field (hpf). Each interaction was examined in duplicate chambers and the counts expressed as the mean of 10 hpf, 5 from each of the duplicate filters.

Leukocyte and Platelet Purification.—Red blood cells in citrated human blood were allowed to settle without dextran for 90 min at 37°C. The leukocyte and platelet-rich supernatant was removed by aspiration and spun for 10 min at 100 *g* to leave only platelets in the supernatant and bring mixed leukocytes into the pellet. After centrifugation of this supernatant at 400 *g* for 15 min, the platelets were recovered with fewer than 1 leukocyte per 500 platelets. The platelets were then washed twice and suspended in either Medium 199 or 0.06 M phosphate buffer 0.06 N in NaCl with 0.25 g/100 ml sucrose and 0.25 g/100 ml gelatin at pH 7.0. Less platelet aggregation was seen with the latter buffer.

Mononuclear and polymorphonuclear leukocytes were separated from the leukocyte pellet on a Ficoll-Hypaque cushion by a previously described method (13). 4–6 $\times$ 10^7 leukocytes in 3 ml of Medium 199 were layered on top of 3.5 ml of a solution containing 9.57 g of Ficoll in 120 ml distilled water and 30 ml of 50% Hypaque. After centrifugation for 30 min at 400 *g*, the mononuclear leukocyte layer above the density interface was removed by aspiration and placed in a separate tube. The fluid layers were decanted from the polymorphonuclear leukocyte pellet. Both the mononuclear and the polymorphonuclear leukocytes were washed three times with Hanks' solution. Differential cell counts of leukocyte smears stained with Wright-Giemsa stain revealed 90% mononuclear cells and 96–99% polymorphonuclear cells in the

respective fractions. The mononuclear population consisted of 20–30% monocytes, 5–10% polymorphonuclear leukocytes, and 60–75% lymphocytes.

Soluble extracts were prepared from mixed leukocytes and purified polymorphonuclear and mononuclear leukocytes. Suspensions of 2×10^7 leukocytes in 2 ml of distilled water were exposed to probe ultrasound treatment for 5 min at room temperature. After addition of 0.22 ml of 2 N H_2SO_4, the mixtures were shaken for 1 hr at 25°C. This supernatant was removed, the faint viscous precipitate was extracted with another 1 ml of 0.2 N H_2SO_4, and the supernatants were pooled. The pH was adjusted to 6.0 by dropwise addition of 1 N NaOH and the soluble extract was separated from the precipitate that formed at 4°C overnight by centrifugation at 1000 *g* for 20 min.

Preparation of Chemotactic Principles.—Purified human C5 was prepared by previously published methods (14). There was no detectable C3 activity (15) in C5 fractions obtained at the final purification step on hydroxylapatite. These same fractions showed a single band on immunoelectrophoresis with a rabbit anti-human C5 antiserum kindly supplied by Dr. H. J. Müller-Eberhard. C5 concentration in the pooled column fractions, 50–60 μg/ml, was determined by radial immunodiffusion assay using this same antiserum. C5a was generated from these pools by 5% w/w trypsin digestion at 37°C for 30 min in 0.05 M Tris HCl buffer, pH 7.8 as previously described (2). This product served as the stock solution of C5a. Human kallikrein capable of generating 50–150 ng of bradykinin from 0.2 ml of heat-inactivated human plasma/10 μl of kallikrein solution was formed by incubating portions of a stock solution of prekallikrein with Hageman factor fragments (4). The Hageman factor fragments and prekallikrein were highly purified; the fragments were free of contaminating proteins, while the prekallikrein showed only one contaminating band on disc-gel electrophoresis at pH 9.3, which was IgG (4, 16). Neither component had chemotactic activity alone. 100 μl of stock solutions of human C5a or kallikrein in neutral buffer were diluted to 1 ml with suspending medium before being pipetted into the stimulus side of chemotactic chambers. Autologous serum clotted in glass for 30 min at 37°C was used as a chemotactic stimulus after 20-fold dilution in suspending medium.

Assessment of Chemotactic Inhibition.—The degree of chemotactic inhibition was assessed by adding a small volume of inhibitory supernatant to a sample of human neutrophils; the mixture was preincubated at 25°C for 10 min and then pipetted into duplicate chemotactic chambers. Control supernatants, obtained from leukocyte suspensions never exposed to a specific release factor, were mixed with an equal number of human neutrophils and this mixture was tested against an identical chemotactic stimulus. The response in the presence of the control supernatants was arbitrarily assigned a value of 100% and the effect of inhibitory supernatants, reflected as a decrease from this value, was expressed as per cent residual chemotactic activity. Inhibitory and control supernatants and column fractions derived from these supernatants were always adjusted to pH 7.4 and diluted at least fourfold with Medium 199 before being mixed with an equal volume of neutrophils suspended in Medium 199 with 1% ovalbumin. The test neutrophils were therefore always in a buffer identical in final composition with that on the stimulus side of the chamber. In addition, control mixtures were set up alternately with inhibitor mixtures to minimize slight variations in the preincubation time and the neutrophil chemotactic response time. All control and inhibitor supernatants were sterilized by filtration through 0.45 μ micropore filters and were either fresh or from storage at 4°C.

RESULTS

Production of Chemotactic Inhibitors.—Three procedures were used to generate substances from human leukocytes which function as neutrophil-active chemotactic inhibitors. These inhibitors are given the general designation neutrophil-immobilizing factor (NIF). The NIF generated from leukocytes by endotoxin

treatment is referred to as ENIF, that released by leukocyte acidic incubation as ANIF, and that found in leukocyte phagocytosis supernatants as PhNIF.

Leukocyte incubation at acidic pH: Suspensions of 2×10^7 leukocytes in 2 ml of Hanks' balanced salt solution previously adjusted to pH 6.0, 5.0, or 4.0 with 1 N HCl were incubated for up to 4 hr at 37°C. The cells were sedimented by centrifugation and the supernatant was adjusted to pH 7.0 with 2% $NaHCO_3$. After 4–8 hr storage at 4°C the supernatant was recentrifuged to remove the slight precipitate that always formed. Fig. 1 A illustrates the time course of appearance of ANIF activity in the leukocyte supernatant during incubation at pH 5.0. ANIF activity is near maximal by 1 hr. At 1 hr, the viability of pH 5.0-incubated leukocytes was the same as control cells incubated at neutral pH as assessed by trypan blue exclusion, while at 2 hr their viability was reduced to 70% relative to 85% for control leukocytes. There were separate ionicity

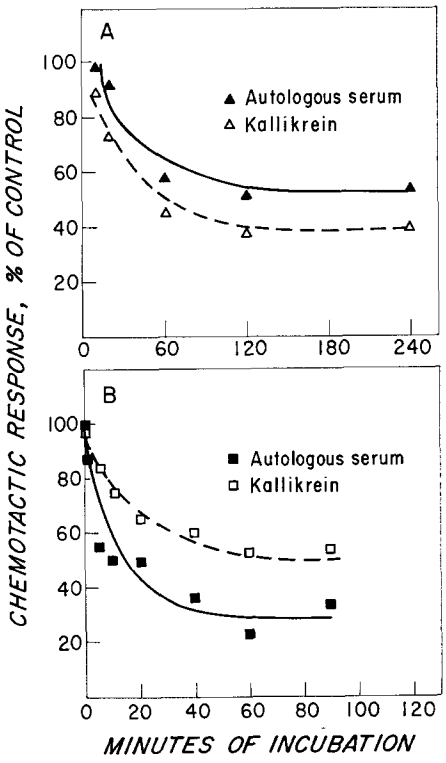

FIG. 1. The time course of generation of NIF. Each point represents the chemotactic response of test neutrophils suspended in ⅛ dilution of a NIF preparation relative to the response of neutrophils in the same dilution of a control supernatant. Chemotactic stimuli were kallikrein and autologous serum. (*A*) The time course of generation of ANIF at pH 5.0. (*B*) The time course of generation of PhNIF.

controls for each pH level, and under these circumstances the results at pH 4 and 6 were similar.

Leukocyte phagocytosis of starch particles: Suspensions of 2×10^7 leukocytes were mixed with four starch particles per leukocyte in 2 ml of buffer and incubated at 37°C for up to 1½ hr. The starch had been preincubated with autologous serum for 30 min at 37°C, and then washed three times in 0.15 N NaCl. The buffer was two parts Hanks' solution added to one part 0.30 N dextrose in distilled water and made 0.5% in ovalbumin with pH 7.4. Fig. 1 B shows the time course of appearance of PhNIF in the supernatants after centrifugation to sediment the leukocytes and starch. The plateau of PhNIF activity at 40–60 min correlated with the time of maximum leukocyte phagocytosis of starch granules assessed by counting the mean number of intracellular granules per leukocyte under phase-contrast microscopy. Fig. 2 A shows a plot of PhNIF activity generated in 1 hr by 2×10^7 leukocytes at 37°C in relation

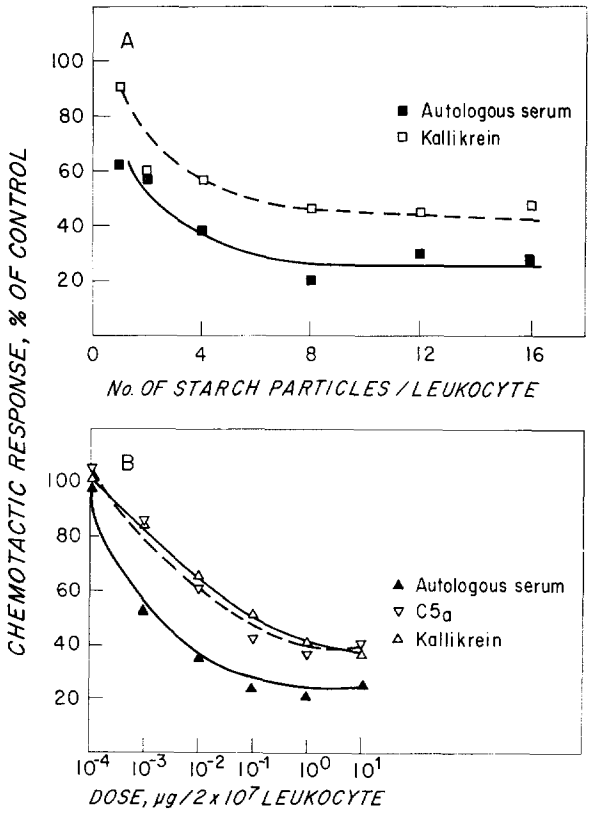

FIG. 2. Dose-response relationships in the generation of NIF. The calculation of response was as in Fig. 1. The chemotactic stimuli were C5a and those used in Fig. 1. (*A*) The relationship of particle dose to release of PhNIF. (*B*) The relationship of endotoxin dose to release of ENIF.

to the number of starch granules per leukocyte in the starting mixture; the cell concentration and buffer were as above. PhNIF activity reached a plateau at approximately six particles per leukocyte in the initial mixture. Leukocyte viability assessed by trypan blue exclusion at 1 hr after the onset of phagocytosis was 85% for both controls and cells engaged in phagocytosis.

Leukocyte exposure to endotoxin: Suspensions of 2×10^7 leukocytes in 2 ml of Hanks' solution were incubated with 0.4 μg of endotoxin for up to 4 hr at 37°C. The leukocytes were sedimented by centrifugation for 5 min at 200 *g* and supernatants were stored at 4°C. The leukocytes were then washed twice, and reincubated for up to 4 hr at 37°C in low-potassium medium which was 0.15 N NaCl brought to pH 7.4 with 2% $NaHCO_3$ solution. The leukocytes were sedimented by centrifugation and the supernatants harvested as a source of ENIF. The effects of varying the time period of endotoxin exposure and the time of incubation in low-potassium medium on release of ENIF from leukocytes were studied separately. As shown in Fig. 3 A, the optimal endotoxin activation time of leukocytes was 1–2 hr as assessed by release of ENIF activ-

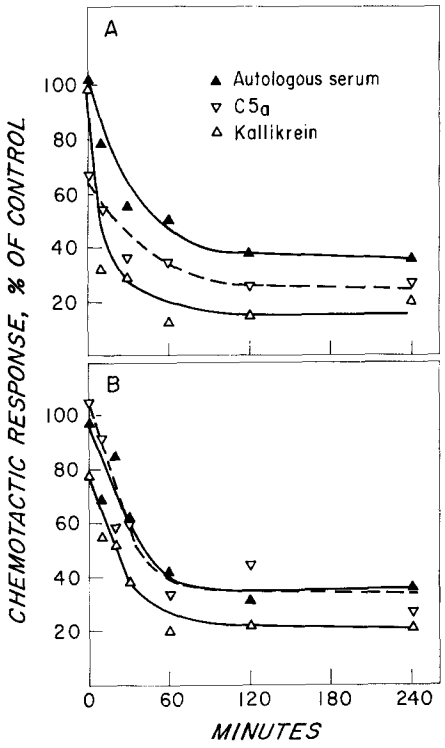

FIG. 3. The time course of generation of ENIF. The chemotactic stimuli and the calculation of neutrophil response are the same as in Fig. 2. (*A*) Relationship between the endotoxin activation time and the release of ENIF. (*B*) Relationship between the low-potassium medium incubation time and the release of ENIF.

ity into the supernatant when the activated leukocytes were subsequently incubated in low-potassium medium for 4 hr. Release of ENIF into the endotoxin-free low-potassium medium by leukocytes optimally activated by a previous 4 hr exposure to endotoxin reached a plateau after 1 hr of incubation (Fig. 3 B). The ENIF was active in inhibiting the response to all three chemotactic stimuli. Low-potassium saline was not an absolute requirement for ENIF generation, but ENIF activity in low-potassium medium supernatants was at least 50% greater than when the potassium concentration during leukocyte incubation was 4.0 meq/liter. The supernatants obtained after a 1–2 hr activation time and before low-potassium medium incubation exhibited little inhibitory activity. Investigation of the relationship between the endotoxin dose to which leukocytes were exposed for 1 hr at 37°C and the ENIF activity which they released into the low-potassium medium during a subsequent 2 hr incubation at 37°C revealed maximal endotoxin activation with 0.1 μg of endotoxin per 2×10^7 leukocytes (Fig. 2 B). Leukocyte viability after 2 hr of endotoxin activation was always within control limits by trypan blue exclusion. After a subsequent 1 hr incubation in low-potassium medium, viability dropped slightly to a mean of 80% as compared with 85% for the controls.

Separation of NIF from Chemotactic Stimuli.—Each of the NIF preparations had definite chemotactic activity when used as the stimulus in chemotactic chambers (Fig. 4). When compared with the standard kallikrein stimulus, the relative activity was 12% for PhNIF, 29% for ANIF, and 65% for ENIF. Nonetheless, NIF supernatants slightly inhibited chemotaxis when mixed with kallikrein on the stimulus side of a chamber and when placed on the cell side gave inhibition that did not correlate with their chemotactic activity (Fig. 4).

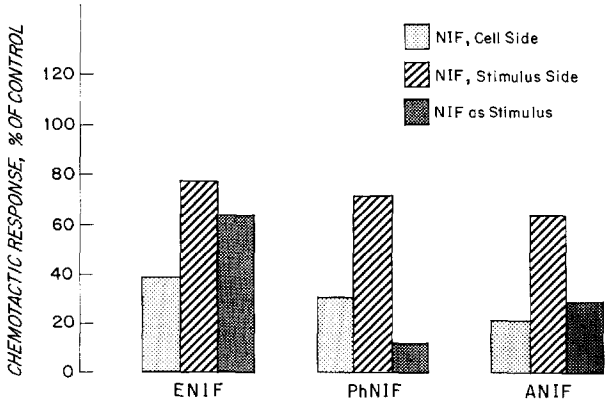

FIG. 4. Inhibitory and chemotactic activity of NIF supernatants. Either the test neutrophils or the kallikrein standard stimulus was incubated in $\frac{1}{16}$ dilution of NIF before being loaded into chemotactic chambers. The chemotactic response was expressed relative to the response with control supernatants added to neutrophils or stimulus. The direct chemotactic activity of a $\frac{1}{16}$ dilution of each inhibitory supernatant was also tested and expressed relative to the standard kallikrein response.

As the chemotactic activity of the supernatants did not account for the NIF inhibitory activity, these two properties were next separated chromatographically.

Fig. 5 shows the results of fractionating NIF preparations and leukocyte ex-

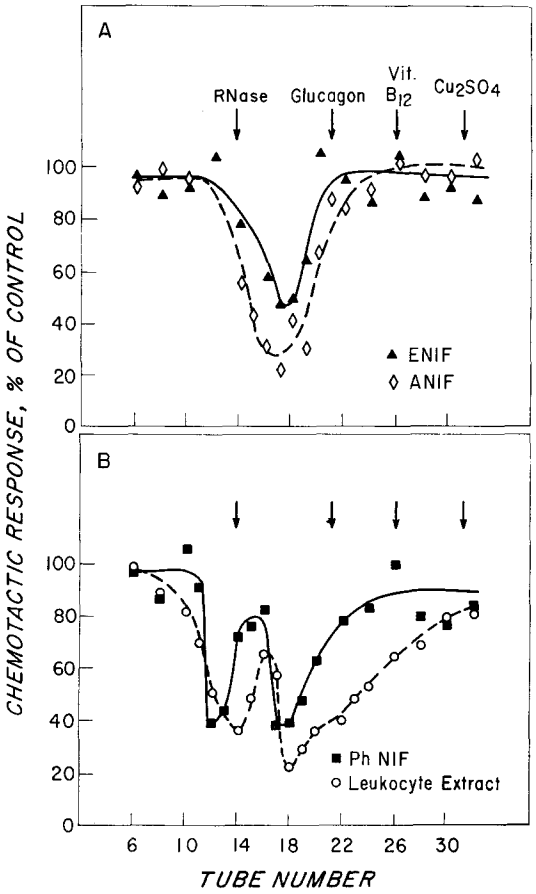

FIG. 5. G-25 Sephadex fractionation of ENIF and ANIF preparations (*A*) and PhNIF and a leukocyte extract (*B*). G-25 Superfine Sephadex was packed in a 1.5 × 70 cm Pharmacia column with a 124 ml bed volume. Flow rate was 8 ml/hr and the fraction volume was 4 ml. The markers were RNase (13,700 mol wt), glucagon (3500 mol wt), vitamin B_{12} (1360 mol wt), and copper sulfate, which colored effluent at 100% bed volume. Fractions were diluted ¼ in Medium 199 before being assayed for inhibitory activity.

tracts by gel filtration on G-25 Sephadex columns equilibrated in Hanks' solution. All fractions were screened for NIF activity with neutrophils responding to an autologous serum stimulus. ANIF and ENIF showed one peak of chemotactic inhibitory activity at about 60% bed volume with an approximate molecular weight of 5000 (Fig. 5 A). PhNIF and the leukocyte extracts yielded two peaks of chemotactic inhibitory activity. The first was at the dextran blue col-

umn front just ahead of the RNase marker and the second was at 60% bed volume before the glucagon marker (Fig. 5 B). The 5000 mol wt species present in all four preparations was used further to distinguish NIF inhibitory activity from chemotactic activity.

Concentrations of pooled fractions from the 5000 mol wt region of G-25 Sephadex columns were adjusted to the same chemotactic inhibitory potency as starting supernatants. The ratio of column pool to starting supernatant chemotactic activity for ENIF, ANIF, and PhNIF preparations so standardized ranged from 0.39 to 0.24. Thus, during the gel filtration isolation of NIF activity, 61–76% of the chemotactic stimulating activity was removed. Heating at 56°C for 1 hr also eliminated 60–75% of the chemotactic activity from crude preparations without diminishing inhibitory potency. The residual chemotactic activity in column-purified NIF was completely destroyed by heating without affecting NIF inhibitory potency. The combination of isolation by gel filtration and subsequent heating clearly resolved NIF activity from the chemotactic activity of the starting material.

Cell Source of NIF.—In order to investigate the source of NIF in mixed leukocyte suspensions, 3.2×10^7 Ficoll-Hypaque-purified polymorphonuclear leukocytes in 2 ml of buffer were used for the generation of ANIF, ENIF, or PhNIF, or were extracted with 0.2 N H_2SO_4. A similar protocol was used with 2.6×10^7 mononuclear leukocytes. A purified suspension containing 32×10^7 platelets in 2 ml of Hanks' solution was employed in attempts to generate ENIF and ANIF activity. Significant chemotactic inhibitory activity was released from both polymorphonuclear and mononuclear leukocytes, but not from platelets (Fig. 6 A). The active preparations were then fractionated on G-25 Sephadex columns. In addition, the extracts which could not be studied in crude supernatants due to hyperosmolarity resulting from neutralization of the H_2SO_4 were also chromatogramed. These fractions were screened for chemotactic inhibition and the fraction showing peak NIF activity which was located at approximately 60% bed volume is depicted in Fig. 6 B. The presence of NIF in both extracts indicated that it is contained preformed in polymorphonuclear and mononuclear leukocytes. The soluble extracts and the PhNIF crude supernatants from both types of leukocyte also contained a chemotactic inhibitor of higher molecular weight.

Characteristics of Action of NIF.—To define further the action of NIF, test neutrophils were exposed for a given time period to either column-purified NIF preparations or column-purified supernatants from unstimulated leukocytes, and then washed twice to remove free NIF before loading the neutrophils into chemotactic chambers. 5–10 min was required for washing leukocytes and pipetting them into the chemotactic chambers. As shown in Fig. 7, the effect of each NIF preparation is maximal and irreversible within 5 min of preincubation with neutrophils. The time course of NIF action was the same when examined with the other chemotactic stimuli.

Both column-purified PhNIF (Fig. 8) and ANIF (not shown) concentrated to the original volume applied to the column showed a dose-response inhibition of chemotaxis when present on the cell side of the chemotactic chamber. In contrast, column-purified ENIF often showed an initial flat line relationship between chemotactic inhibition and dilution or even a slight prozone. Crude

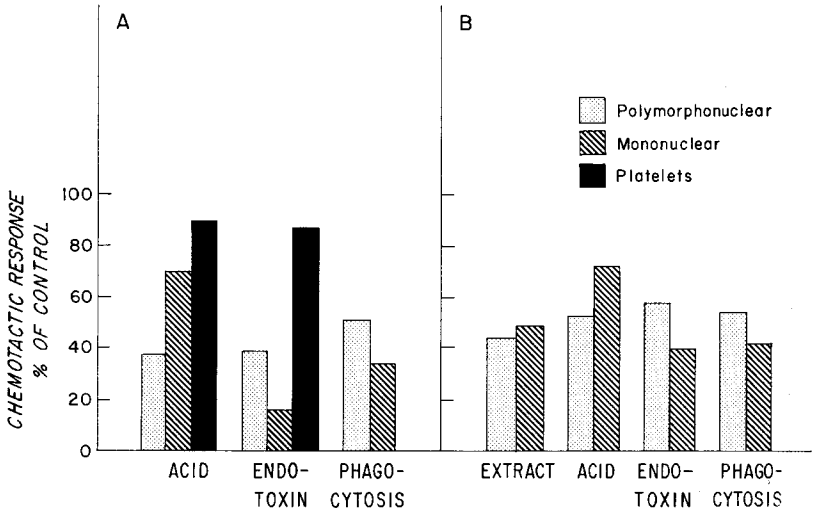

FIG. 6. Cell source of NIF. The details of NIF preparation were the same as in Fig. 5 and autologous serum was the chemotactic stimulus. (*A*) NIF activity in crude leukocyte supernatants. Results are expressed relative to supernatants from corresponding untreated leukocytes and were adjusted using a linear scale to 10^7 source leukocytes or 10^8 platelets. (*B*) NIF activity in G-25 Sephadex column fractions. NIF inhibitory activity was assayed at a ¼ dilution and adjusted to per cent chemotaxis per 10^7 source leukocytes as compared with the control supernatants.

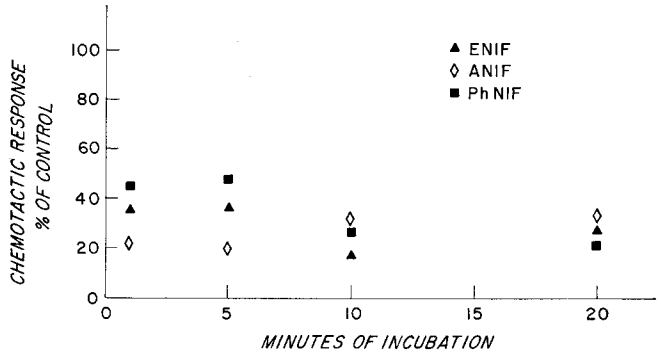

FIG. 7. Irreversibility of action of NIF. All NIF preparations and controls were pools of G-25 Sephadex column fractions of supernatants prepared as in Fig. 5. The chemotactic stimulus was autologous serum, and results are expressed as described in Fig. 1.

FIG. 8. Dose-response of G-25 Sephadex-purified PhNIF. PhNIF was generated from 1.5×10^7 leukocytes as in Fig. 5. Autologous serum was the chemotactic stimulus, and results are expressed as described in Fig. 1.

ENIF supernatants generally also showed a prozone at high concentrations while there was no prozone for crude PhNIF.

Mixtures of neutrophils and $\frac{1}{16}$ dilutions of column-purified ANIF, PhNIF, ENIF, or control leukocyte supernatants were preincubated for 1 hr, and then trypan blue dye was added. After a further incubation for 3 hr at 37°C, the neutrophils exposed to NIF or control supernatants exhibited the same viability of 85 ± 5% (mean ± 2 SD). NIF and control-treated neutrophils were also the same at 6 hr with a mean viability of 66 ± 8%.

Mononuclear leukocyte chemotaxis was carried out using the upper phase from Ficoll-Hypaque fractionation of human leukocytes. The total leukocyte count per high power field on the undersurface of 8-μ micropore filters was considered to represent the mononuclear leukocyte chemotactic response. ENIF was found to be much less effective in inhibiting the mononuclear leukocyte response than the neutrophil response to human C5a (Fig. 9). For example, 20% inhibition of mononuclear leukocyte chemotaxis required a $\frac{1}{16}$ dilution of ENIF while comparable inhibition of neutrophils occurred at a $\frac{1}{256}$ dilution. The data are the same with autologous serum as the chemotactic stimulus.

Enzymatic Digestion of NIF.—Both trypsin and chymotrypsin in insolubilized form were used to inactivate NIF by digestion at 37°C. Pools of NIF activity from G-25 Sephadex column fractions of crude supernatants were used throughout. The digestion buffer was 0.1 M ammonium acetate, pH 8.2, 0.01 M in $CaCl_2$. Fig. 10 demonstrates the destruction of all ENIF activity after 4–8 hr of chymotrypsin digestion with 50% destruction by 1–2 hr. The results with ANIF and PhNIF were similar. NIF activity was unaffected by incubation at 37°C without enzyme for up to 36 hr. Chymotrypsin treatment of identical

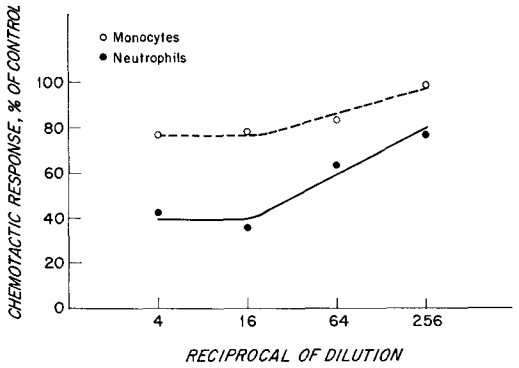

FIG. 9. ENIF inhibition of mononuclear leukocyte and neutrophil chemotaxis. C5a was the chemotactic stimulus. Cell counts in initial leukocyte test suspensions were 1.2×10^6 mononuclear leukocytes per ml and 2.2×10^6 neutrophils per ml, and results are expressed as described in Fig. 1.

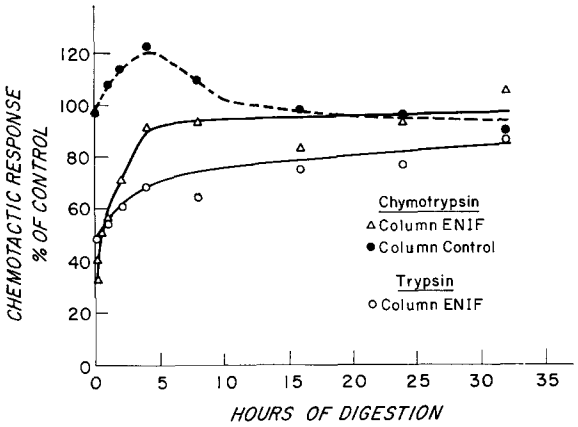

FIG. 10. Enzymatic digestion of NIF. Autologous serum was the chemotactic stimulus. Digestions utilized 5–10 μg insoluble trypsin or chymotrypsin per 1 ml of a ½ dilution of pooled column-purified ENIF or control. Results are expressed as in Fig. 1.

G-25 Sephadex column pools from control leukocyte supernatants generated neither significant neutrophil inhibitory nor stimulating activity (Fig. 10). Trypsin digestion similarly inactivated NIF but did so more slowly, so that 50% digestion was seen at 4–6 hr.

DISCUSSION

Three discrete procedures caused human polymorphonuclear and mononuclear leukocytes to release a preformed soluble low molecular weight inhibitor of the chemotactic response of human neutrophils (Figs. 1, 3, and 6). None of the release procedures resulted in decreased viability of the donor cells. Release

was related to a particular phase in the interaction of the leukocyte and the stimulating factor. Maximum PhNIF generation occurred during the period of maximum phagocytosis, whether achieved by dose-response of the initial available particles or by increasing the time allowed the leukocytes to ingest a fixed dose of starch (Figs. 1 and 2). Exposure of neutrophils to endotoxin alone did not result in maximal release of ENIF until there was a subsequent incubation in low-potassium medium (Fig. 3). Further evidence that the release of NIF from leukocytes can be a selective event was indicated by the single peak of activity obtained on G-25 Sephadex fractionation of ANIF and ENIF supernatants, whereas leukocyte extracts gave a minimum of two peaks of activity (Fig. 5). It is of interest that phagocytosis by either polymorphonuclear or mononuclear leukocytes also released at least two peaks of inhibitory activity. The mononuclear leukocyte population competent to liberate NIF (Fig. 6) has not been subfractionated, and contained 20–30% monocytes as well as lymphocytes. The failure of the platelet suspension to serve as a source of NIF (Fig. 6) indicates that the minor platelet contamination of the donor leukocytes could not be a factor in the generation of NIF from these leukocyte sources.

NIF inhibited neutrophil chemotaxis by a noncytotoxic action on the neutrophil itself. Blocking of the chemotactic stimulus is held unlikely since NIF effectively inhibited the neutrophil response to diverse unrelated stimuli (Figs. 1 and 3) and was more effective when preincubated with the neutrophils than with the stimuli (Fig. 4). The effect on the neutrophil, although irreversible after a brief interaction (Fig. 7), did not impair viability as assessed by dye exclusion. Further evidence for a direct effect on the target cell resides in the ability of NIF to suppress random migration of purified human neutrophils (17). The effect on the neutrophil is not rapid deactivation (18) by a chemotactic principle because purified heat-treated NIF had full inhibitory activity in the absence of any chemotactic activity (Fig. 5). NIF also showed a more rapid time course of action (Fig. 7) than neutrophil deactivation by any of the chemotactic factors studied to date (18, 19). Although NIF can be released from both neutrophils and mononuclear leukocytes, the human neutrophil was more sensitive to its action than the human monocytes (Fig. 9).

NIF is a low molecular weight, heat stable substance extractable from leukocytes under conditions which have been demonstrated to give high yields of lysosomal cationic peptides (20). The susceptibility of NIF to inactivation by chymotrypsin and trypsin (Fig. 10) suggested that NIF activity was dependent on a peptide present as at least part of its structure. The identical restricted chromatographic position of this heat stable molecule(s) from two different cell sources and under four discrete sets of conditions (Fig. 5) is consistent with NIF being a unique subspecies of the small molecules released from leukocytes.

NIF activity was generated by procedures which may share common mechanisms and which have all been previously shown to have profound effects on leukocytes. Both leukocyte exposure to endotoxin and active phagocytosis re-

sult in the release into the supernatant of numerous biologically active factors including endogenous pyrogen (21, 22), lysosomal enzymes (23, 24), and lysosomal cationic peptides and proteins (25, 26). NIF also shares with endogenous pyrogen and lysosomal enzymes the dependence on a low concentration of K^+ in the medium for full liberation after endotoxin exposure (27). At K^+ concentrations below 3 meq/liter, there is suppression of leukocyte cytoplasmic membrane ATPase activity which presumably facilitates release of these substances (28). In addition, incubation of leukocytes at a reduced pH results in significant depression of the cell membrane monovalent cation-activated ATPase activity (28), and significant disruption of leukocyte granules (29). Both endotoxin exposure and phagocytosis result in increased production of lactate by leukocytes, and it is possible that a reduced intracellular pH is a common factor in the various procedures used to release NIF (30, 31).

Inhibitors of human neutrophil chemotaxis have been observed in the sera of patients with alcoholism and cirrhosis (5) and glomerulonephritis (6). Both of these inhibitors apparently function by inactivating chemotactic factors although the action of the latter inhibitor was apparently reversible by heating. A chemotactic inhibitor which irreversibly blocks the activity of bacterial and complement-derived chemotactic factors has been isolated from the sera of some patients in high yield and is also present at low levels in normal sera (32); it is recoverable as both 3S and 7S molecular species. One child subject to frequent Gram-negative infections had in his serum an inhibitor of human neutrophil chemotaxis which acted directly on cells and did not affect the chemotactic stimuli (7). This inhibition could be reversed by washing the neutrophils, but physicochemical characteristics of the inhibitor are not available.

An inhibitor of rabbit neutrophil chemotaxis has been derived from rabbit leukocytes (33). It differs from NIF in being heat labile, having an approximate molecular weight of 28,000, occurring in the supernatants of resting but not phagocytizing leukocytes, and acting primarily by blocking the chemotactic stimulus. A heat stable human leukocyte-derived factor of approximately 4000 mol wt has been shown to inhibit the random migration of guinea pig macrophages in vitro by a direct but nonlethal action on the cells (34, 35). It is extractable from leukocytes and is also released during a prolonged incubation or a brief period of phagocytosis. This human leukocyte factor is reminiscent of NIF but the two preparations have not been tested in comparable assay systems. It should be noted, however, that NIF is not only more active in suppressing neutrophil than human monocyte chemotaxis (Fig. 9), but also exhibits the same target cell specificity in suppressing random migration.[3]

With regard to an in vivo biologic role, NIF may function at low concentrations to immobilize neutrophils in the early inflammatory focus without affecting their phagocytic and other functions. Later in the inflammatory response, higher

[3] Goetzl, E. J. Unpublished observations.

concentrations of NIF released from both polymorphonuclear and mononuclear leukocytes may suppress the migration of further neutrophils into the lesion, favoring the transition of polymorphonuclear to mononuclear cell populations in inflammatory foci. In addition, neutrophil lysates which are only weakly chemotactic for other neutrophils display marked chemotactic activity for mononuclear cells in vitro (36). Thus it is possible to suggest that there may be a leukocyte-directed transition from neutrophils to mononuclear cells in those inflammatory reactions in which there is early polymorphonuclear leukocyte predominance.

SUMMARY

A factor has been derived from human leukocytes which irreversibly inhibits the response of human neutrophils to diverse chemotactic stimuli without impairing their viability. It is released by both polymorphonuclear and mononuclear leukocytes during incubation in acidic medium, after endotoxin exposure and subsequent incubation in low potassium medium, and during phagocytosis of particles. It is extractable from both leukocyte types and therefore must be preformed. This chemotactic inhibitor is completely separable from contaminating chemotactic activity in the crude supernatants, has a mol wt of 5000, and is inactivated by digestion with trypsin or chymotrypsin. It has been termed a neutrophil-immobilizing factor because it inhibits neutrophils directly and independently of the chemotactic stimulus, and has relatively little effect on human monocyte chemotaxis.

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