A NOVEL NEUTROPHIL-ACTIVATING FACTOR PRODUCED BY HUMAN MONONUCLEAR PHAGOCYTES

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In inflamed tissues, neutrophils are exposed to a variety of stimulatory molecules of different origin that condition their microbicidal and tissue-damaging activities. The best known stimuli are FMLP related to bacterial products (1), C5a formed upon complement activation (2), and two bioactive lipids, platelet-activating factor (PAF)[1] and leukotriene B_4 (LTB_4) (3–5). Products like PAF and LTB_4, which are released by stimulated phagocytes and have themselves stimulatory properties, may act as enhancers of antimicrobial defense and inflammation (6, 7). Of particular interest are products of activated mononuclear phagocytes, which are likely to influence neutrophil function at sites of chronic inflammation. We have cultured human blood mononuclear cells in the presence of LPS and different lectins and have tested the conditioned media for the presence of factors acting on the neutrophils. In this paper we describe a neutrophil-activating factor (NAF) produced by stimulated monocytes that induces exocytosis and the respiratory burst. The effects of NAF on human neutrophils are similar to those of FMLP and C5a, but appear mediated by a novel and selective surface rceptor.

The results presented here were obtained over the current year using a partially purified preparation of NAF. Only upon completion of the present study did we succeed in purifying NAF to homogeneity. The identification of 32 of 50 presumed residues by amino acid sequencing showed that NAF is a novel polypeptide (8). Pure NAF has an ~80-fold higher specific activity than the partially purified preparation. Both preparations, however, were qualitatively similar; they induced exocytosis and a rapid and transient respiratory burst response in human neutrophils, and showed complete cross-desensitization upon repeated application to the cells.

Materials and Methods

Materials. LPS (from *Escherichia coli* 055:B5) was obtained from Difco Laboratories Inc., Detroit, MI; BSA from Fluka AG, Buchs, Switzerland; Ficoll-Paque from Pharmacia

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[1] *Abbreviations used in this paper:* GRAM, granulocyte-activating mediators; LTB_4, leukotriene B_4; MOC, mononuclear cell–derived chemotaxin; NAF, neutrophil-activating factor; PAF, platelet-activating factor.

AG, Uppsala, Sweden; heparin, cytochrome *c* (type III from horse heart), trypsin (type III), and horseradish peroxidase from Sigma Chemical Co., St. Louis, MO; neomycin, cytochalasin B, and luminol, from Serva GmbH, Heidelberg, Federal Republic of Germany; sodium azide and polyethylene glycol 6000 from Merck AG, Darmstadt, FRG; chymotrypsin (Enzygel) and proteinase K from Boehringer GmbH, Mannheim, FRG; FMLP, 1-0-hexadecyl-2-O-acetyl-*sn*-glycero-3-phosphocholine (PAF) from Bachem AG, Bubendorf, Switzerland; and Quin-2/AM from Calbiochem AG, Luzern, Switzerland. The elastase substrate *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-valine-7-amido-4-methylcoumarin was provided by Dr. T. G. Payne, Preclinical Research, Sandoz Ltd., Basel, Switzerland. Human C5a was a gift of Dr. C. A. Dahinden, Department of Clinical Immunology, University of Bern, Bern, Switzerland.

Media and Solutions. Two culture media were used, MEM and MEM-PPL. MEM consisted of Eagle's MEM from Seromed GmbH, Munich, FRG, supplemented with 25 μg/ml neomycin and buffered to pH 7.4 with 25 mM $NaHCO_3$ and 20 mM Hepes. MEM-PPL contained, in addition, 1% pasteurized plasma protein solution (5% PPL SRK, Swiss Red Cross Laboratory, Bern, Switzerland) and 100 IU/ml penicillin/streptomycin (Gibco AG, Basel, Switzerland). Two PBS solutions (pH 7.4) were used: (*a*) PBS, a Ca^{2+}- and Mg^{2+}-free solution consisting of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, and (*b*) PBS-BSA containing, in addition to the above, 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, and 2.5 mg/ml BSA. Stock solutions were used for LPS (10 mg/ml in PBS), heparin (1,000 IU/ml in distilled water), FMLP (10 mM in DMSO), PAF (10 mM in PBS-BSA), and cytochalasin B (5 mg/ml in DMSO). The final DMSO concentration never exceeded 0.2%.

Cell Preparation. Citrated donor blood stored for up to 20 h at 4–10°C (Swiss Red Cross Laboratory) was used. Single buffy coats diluted fourfold with PBS supplemented with 13 IU/ml of heparin were layered on a Ficoll-Paque gradient for separation of neutrophils and mononuclear cells (9). The mononuclear cells were washed three times with MEM and resuspended at 5×10^6 cells/ml in MEM-PPL for culturing or in MEM for elutriation. Monocytes and lymphocytes were separated in a JE6 elutriation rotor operated by a J6-M refrigerated centrifuge (Beckman Instruments, Inc., Palo Alto, CA) as previously described (10). The elutriation medium consisted of PBS supplemented with 0.01% EDTA, pH 7.4, and 1% BSA. The flow rate was kept constant at 18 ml/min, the temperature was 10°C and the rotor speed was reduced stepwise from 3,130 to 2,100 rpm. Cells were collected in two 45-ml fractions each at five speeds, 2,930 and 2,790 rpm for lymphocytes and 2,430, 2,330, and 2,230 rpm for monocytes. They were washed once in MEM-PPL (250 *g* for 10 min), checked by differential counting, and the purest preparations (100% lymphocytes and 95% monocytes, respectively) were used for culture experiments. Neutrophils were isolated from the pellet following Ficoll-Paque centrifugation as described by Weening et al. (11) and were finally suspended (10^8 cells/ml) in isotonic NaCl containing 0.05 mM $CaCl_2$ and kept at 10°C until use. These preparations consisted of 95–98% granulocytes.

Cell Cultures. Mononuclear cells or purified lymphocytes and monocytes were cultured for different periods in MEM-PPL. The cells (5×10^6/ml unless stated otherwise) were seeded in 1 ml of medium into 24-well culture plates (Nunc-InterMed, Roskilde, Denmark). Adherent cells were obtained from the mononuclear cell fraction (5×10^6 cells per well) by incubation for 1 h at 37°C followed by removal of the medium and rinsing twice. Large scale cultures of mononuclear cells (1.2–1.5 l, containing 5×10^6 cells/ml MEM-PPL) were prepared in 2-liter glass culture bottles (Schott Glaswerke, Mainz, FRG) equipped with a stirring device.

Partial Purification of NAF. Mononuclear cells were cultured for 48 h in MEM-PPL supplemented with 100 ng/ml of LPS in stirred culture bottles. The culture supernatant was then collected, centrifuged at 20,000 rpm for 20 min at 4°C to remove particulate material, and loaded onto a 10-ml phosphocellulose column (Whatman P11, 1.4 × 6 cm) equilibrated with 20 mM potassium phosphate buffer, pH 7.2, containing 20 mM NaCl and 5% glycerol. The column was washed with 30 ml of the above buffer and was then eluted with 90 ml of a linear NaCl concentration gradient (0.02–1.5 M) in the same

buffer. Fractions of 2 ml were collected in 0.1% polyethylene glycol and tested for NAF and IL-1 activity. Absorbance at 280 nm was monitored continuously as a measure for protein (Uvicord SII; LKB Instruments, Bromma, Sweden). Fractions with the highest NAF activity were dialyzed overnight at 4°C against PBS using a membrane with a molecular weight cut-off of 1,000 (Spectrapor; Spectrum Medical Industries Ltd., Los Angeles, CA).

NAF Assay. A microtiter plate assay for elastase release from human neutrophils (12) was used with minor modifications. Briefly, 100-μl portions of a neutrophil suspension (10^7 cells/ml) treated for 10 min with 5 μg/ml of cytochalasin B were dispensed into the wells containing 125 nmol of the elastase substrate and the test sample in 150 μl PBS-BSA. For each plate, a background and a stimulation control were included in which the test sample was replaced with PBS-BSA or PAF in PBS-BSA (final concentration 0.1 and 1.0 μM, respectively). The assay was performed at room temperature using a Titertek Fluoroscan (Elflab, Helsinki, Finland).

IL-1 Assay. IL-1 activity was measured by the thymocyte proliferation assay according to Rosenwasser and Dinarello (13).

Resistance to Proteolysis and Denaturation. Samples of partially purified NAF were subjected to the following treatments: (*a*) incubation at 37°C with and without trypsin, chymotrypsin, or proteinase K (100 μg/ml), followed by the addition of an excess of BSA (2 mg/ml) to stop NAF proteolysis; (*b*) heating at 56, 80, or 95°C (as compared with 22°C for control); (*c*) exposure to pH 2 or 10 (at 22°C) followed by adjustment to pH 7.4 (as compared with pH 7.4 for control); (*d*) exposure to 2 M lithium chloride, 6 M guanidinium chloride, 1% 2-ME or 0.5% SDS for 3 h at 22°C, followed by dialysis for 24 h at 4°C against PBS. When additions were made, samples without NAF were handled in the same way and were tested for possible influence on the NAF assay.

Exocytosis. Neutrophils (5×10^6/ml) suspended in PBS-BSA were preincubated with or without cytochalasin B (5 μg/ml) at 37°C for 5 min. Exocytosis was then initiated by adding the appropriate stimulus and was stopped 15 min later by rapid cooling in ice followed by centrifugation. Vitamin B_{12}–binding protein, β-glucuronidase, and LDH were determined in the cell-free media and cell pellets, and marker release was expressed in percent of the total cellular content (14).

Superoxide Formation. Superoxide formation was measured at 37°C as the SOD sensitive reduction of ferricytochrome *c* (15). The assay mixture (1 ml) consisted of 3×10^6 cells/ml PBS-BSA containing 85 μM cytochrome *c*. Absorbance changes were recorded in a Hewlett-Packard Co. (Palo Alto, CA) 8451A diode array spectrophotometer equipped with a thermostated seven-place cuvette exchanger.

H_2O_2 Formation. H_2O_2 formation was determined by chemiluminescence as described by Wymann et al. (16). The assay mixture containing 0.1 mM sodium azide, 0.01 mM luminol, 9 U/ml horseradish peroxidase, and 10^6 neutrophils in 1 ml of PBS-BSA was preincubated for 10 min at 37°C and the reaction was started by stimulus addition.

Cytosolic-free Ca^{2+} Measurements. Neutrophils were loaded with quin-2 by incubating 0.2 nmol quin-2/AM per 10^6 cells for 15 min at 37°C. After centrifugation, the loaded cells were resuspended in a buffered saline medium containing 1 mM $CaCl_2$ (17). Changes in the cytosolic free calcium concentration ($[Ca^{2+}]_i$) were calculated from the fluorescence of the cell suspensions as described by von Tscharner et al. (17).

Results

NAF Assay. The stimulus-dependent release of elastase by cytochalasin B–treated human neutrophils was adopted as an assay for NAF activity. In Fig. 1 the dependence of elastase release on the amount of NAF-containing conditioned medium is shown. A linear relation was obtained when the amount of NAF was plotted on a logarithmic scale.

NAF Production. Mononuclear cell cultures (5×10^6 cells/ml) consisting of monocytes and lymphocytes in a ratio of ~ 1:5 produced NAF when stimulated

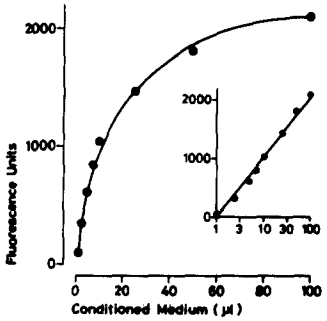

FIGURE 1. NAF induced elastase release by cytochalasin B–treated human neutrophils. Dependence on the amount of NAF-containing conditioned medium added. Conditions: 5×10^6 mononuclear cells were cultured for 24 h in 1 ml of medium containing 100 ng LPS. Inset, NAF plotted on a log scale.

with LPS (100 ng/ml), Con A (10 μg/ml), or PHA (5 μg/ml). NAF accumulated in the medium over 24 h and the production leveled off between 24 and 48 h. No NAF was formed in the absence of a stimulus. Being most potent, LPS was used in all subsequent experiments. Fig. 2 shows the time course of NAF production and dependence on the concentration of LPS that was already effective between 0.1 and 1 ng per 5×10^6 mononuclear cells. The source of NAF was studied using different mononuclear cell preparations. Fig. 3 *a* shows the LPS-dependent generation of NAF by the total mononuclear cell fraction and the

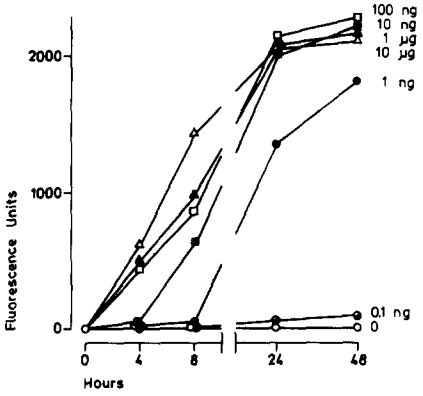

FIGURE 2. NAF production induced by LPS. Mononuclear cells were seeded in 24-well culture plates (5×10^6 cells in 1 ml) and were stimulated with various concentrations of LPS (0.1 ng to 10 μg/ml, as indicated). At given times, the media were collected, cleared by centrifugation, and the NAF activity was determined. Values obtained with cells from a single donor are shown (means of duplicate cultures). These results are representative for four similar experiments performed with cells from different donors.

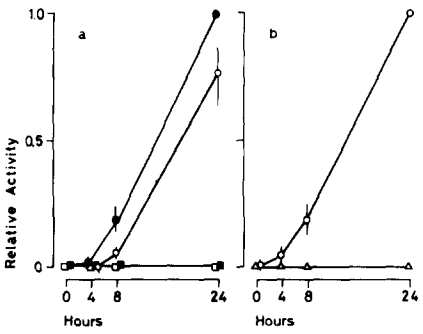

FIGURE 3. Cellular source of NAF. (*a*) The total mononuclear cell fraction (●, ■, 5×10^6 cells/ml) or cells derived from there by adherence (○, □) were cultured in the presence (●, ○) and absence (■, □) of LPS (100 ng/ml). (*b*) Monocytes (○, 10^6 cells/ml) and lymphocytes (△, 4×10^6 cells/ml) purified by elutriation were cultured in the presence of LPS (100 ng/ml). Media were collected at the indicated times and tested for NAF activity. Mean values from five (*a*) and six (*b*) separate experiments, each run in duplicate. Normalized data are shown, which were obtained by setting the 24-h value for the total mononuclear cell fraction (*a*) or the purified monocytes (*b*) equal to 1.0. This type of presentation was chosen to account for (*a*) the individual variation of NAF production by cells from different donors and (*b*) the variable responsiveness of different neutrophil preparations used in the NAF assay.

monocytes selected from there by adherence. These results confirm that NAF production is dependent on LPS and indicate that the monocytes are the source of NAF. The presence of lymphocytes does not appear to influence NAF production. Definite proof that NAF is a product of monocytes and not of lymphocytes is provided by the experiment shown in Fig. 3 *b* in which cell populations purified by elutriation were used. NAF production by mononuclear cell cultures was sensitive to cycloheximide. Complete inhibition was obtained with 10 μg/ml cycloheximide added together with LPS (100 ng/ml). Inhibition was only 76 and 30%, respectively, when cycloheximide was added 4 h and 8 h after LPS.

Partial Purification. The biological properties of NAF were investigated with a partially purified preparation obtained by phosphocellulose chromatography. Fig. 4 shows the distribution profiles of protein and two biological activities, thymocyte proliferation (IL-1) and neutrophil elastase release (NAF), obtained upon fractionation of the conditioned medium of LPS-stimulated monocytes on phosphocellulose. The bulk of the protein and most of the IL-1 activity were recovered in the flow-through volume. Elution with a linear NaCl gradient yielded the remaining IL-1 activity at low ionic strength followed by a peak of elastase-releasing activity (corresponding to NAF), which started to elute at 0.5 M and reached its maximum at 0.8 M NaCl. The peak was symmetric and was preceded by a small shoulder. The distribution of the UV-absorbing material eluted by the salt gradient did not coincide with the two biological activities determined. As shown in Fig. 4 and confirmed in 10 similar fractionations, IL-1 and NAF could be resolved completely. There was no elastase-releasing activity associated with IL-1 and no significant thymocyte proliferation-inducing activity associated with NAF. The final NAF preparation was obtained by pooling the fractions with at least 95% of the peak value, and dialyzing overnight against PBS using a 1,000 mol wt cut-off membrane. Such preparations contained on average 34 μg/ml protein and 24 × 10^4 fluorescence units of NAF activity per milliliter. The units are expressed as the fluorescence increase per 20 min measured in the 96-well fluorimeter (12) under the conditions described in Materials and Methods.

Resistance to Denaturation. Various treatments usually leading to the inactivation of proteins were applied to the NAF preparation. As shown in Table I, NAF turned out to be remarkably resistant. The activity of the preparation, however, was destroyed upon prolonged incubation with proteases, indicating that NAF is a polypeptide. It was therefore interesting to realize that NAF was not

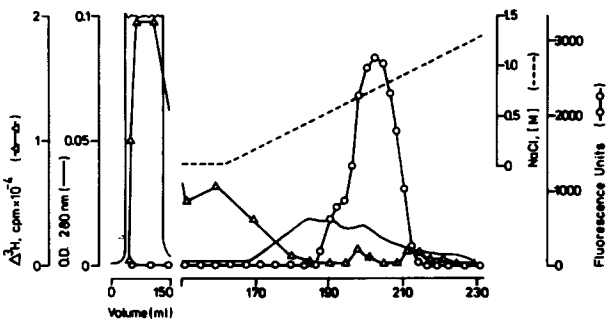

FIGURE 4. Partial purification of NAF by phosphocellulose chromatography. The distributions of protein (absorbance at 280 nm —), NAF (○), and IL-1 (△) are shown.

TABLE I
Physicochemical Properties of NAF

Treatment	Conditions	Relative activity
Temperature	22°C, 3 h	100
	56°C, 1 h	82
	80°C 15 min	80
	95°C, 5 min	66
pH 2.0	22°C, 3 h	76
pH 10.0	22°C, 3 h	90
SDS (0.5%)	22°C, 3 h	100
2-ME (1.0%)	22°C, 3 h	30
Lithium chloride (2 M)	22°C, 3 h	52
Guanidinium chloride (6 M)	22°C, 3 h	68
Trypsin (100 μg/ml)	37°C, 4 h	100
	37°C, 12 h	8
α-Chymotrypsin (100 μg/ml)	37°C, 4 h	80
	37°C, 12 h	1
Proteinase K (100 μg/ml)	37°C, 12 h	0

readily inactivated by heat, acid and alkaline pH, and exposure to SDS. Partial inactivation was obtained with 2-ME, lithium, and guanidinium chloride.

NAF-induced Exocytosis. The effects of NAF on the release of vitamin B_{12}–binding protein and β-glucuronidase, which are constituents of the specific and the azurophil granules, respectively, are summarized in Table II. NAF induced exocytosis of specific granules only, unless neutrophils pretreated with cytochalasin B were used, in which case release from both types of granules was obtained. Under the latter conditions, the release of β-glucuronidase was paralleled by that of elastase, the marker used to test for NAF activity (Fig. 1). In quantitative terms, the exocytosis-inducing properties of NAF were similar to

TABLE II
Stimulus-dependent Exocytosis by Human Neutrophils

Stimulus		CB	Percent release of			Number of experiments
			Vitamin B_{12}–binding protein	β-Glucuronidase	Lactate dehydrogenase	
None		−	6.0 ± 0.4	2.1 ± 0.4	6.2 ± 2.5	3
NAF	50 μl	−	12.9 ± 1.6	2.7 ± 1.3	5.8 ± 0.7	4
	100 μl	−	13.9 ± 2.1	3.0 ± 1.3	6.8 ± 2.2	4
FMLP	0.1 μM	−	14.0 ± 0.9	2.3 ± 0.8	5.7 ± 1.1	3
None		+	10.0 ± 1.1	2.4 ± 0.2	7.0 ± 3.2	3
NAF	50 μl	+	25.6 ± 3.4	7.9 ± 2.0	6.7 ± 1.6	6
NAF	100 μl	+	26.8 ± 4.9	8.5 ± 1.7	7.3 ± 2.1	4
FMLP	0.1 μM	+	35.6 ± 5.5	12.1 ± 4.4	5.3 ± 1.6	3

Mean values ± SD. CB, cytochalasin B, 5 μg/ml.

TABLE III
NAF-dependent Superoxide Production by Human Neutrophils

NAF	Cytochrome *c* reduction	Number of experiments
μl	*nmol/min/10^6 cells*	
10	1.45 ± 0.63	5
20	2.32 ± 0.56	8
30	2.51 ± 0.50	3
50	3.51 ± 0.69	8
100	3.69	2

Mean values ± SD

those of the chemotactic peptide FMLP. Neither stimulus increased the release of the cytosolic enzyme lactate dehydrogenase above control levels, indicating that cell damage was negligible.

NAF-induced Respiratory Burst. The results presented in Table III show that NAF elicits the respiratory burst in human neutrophils. A gradual increase in the rate of superoxide generation was obtained upon stimulation with increasing amounts of NAF. Recordings of the time course of H_2O_2-dependent chemiluminescence were used to compare the fine structure of the respiratory burst induced by NAF, FMLP, and C5a. As illustrated in Fig. 5, all three peptides elicited rapid and transient responses of similar shape and duration. The time between stimulus addition and onset of H_2O_2 production was ~ 2 s in all cases, a value previously determined upon stimulation with FMLP, C5a, PAF, and leukotriene B_4 (18).

It has recently been shown (6, 7, 19) that pretreatment with PAF enhances the respiratory burst response of human neutrophils to a subsequent challenge with FMLP or C5a. As shown in Fig. 6, the same phenomenon was observed with NAF. In PAF-pretreated cells, NAF-dependent superoxide production was markedly enhanced over controls.

Evidence for an NAF Receptor on Human Neutrophils. The remarkable similarity of the effects of NAF, FMLP, and C5a suggested that NAF, like the chemotactic peptides, must act through a surface receptor. This suggestion was substantiated by the demonstration of a NAF-dependent enhancement of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) and of a desensitization of the neutrophils upon repeated stimulation

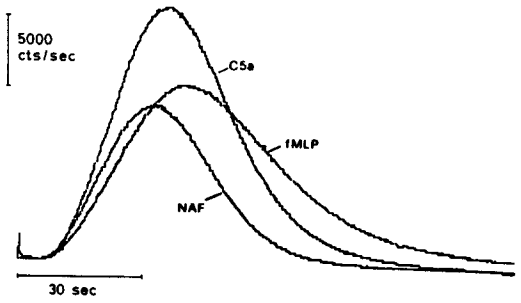

FIGURE 5. Respiratory burst response. H_2O_2-dependent chemiluminescence after stimulation with partially purified NAF and approximately equally active concentrations of C5a or FMLP.

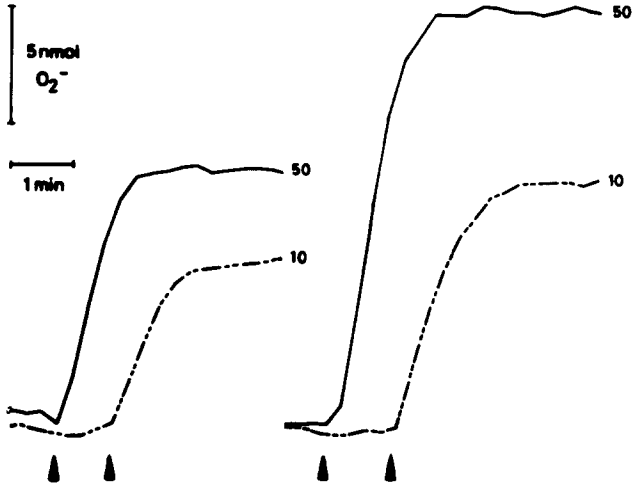

FIGURE 6. Enhancement of NAF-induced respiratory burst by PAF pretreatment. Neutrophils were preincubated for 2 min either in the absence *(left)* or presence *(right)* of 0.1 μM PAF and were then stimulated with 10 or 50 μl of partially purified NAF as indicated. As reported previously (7), PAF had no detectable O_2^--inducing activity under these conditions. Recordings of cytochrome *c* reduction are shown. Arrowheads indicate NAF addition.

with NAF. As shown in Fig. 7, NAF induced a rapid, transient increase in $[Ca^{2+}]_i$, characteristic of receptor agonists and similar to that induced by FMLP.

The effects of repeated stimulation with different combinations of NAF, FMLP, and C5a are shown in Fig. 8. When the neutrophils were first stimulated with NAF or C5a and then with FMLP, two independent respiratory burst responses were obtained (Fig. 8*a*). Superoxide production induced by FMLP was identical with or without prestimulation, showing that cells that had responded to either NAF or C5a remained fully sensitive to a structurally unrelated second stimulus. As shown in Fig. 8 *b*, analogous results were obtained when the neutrophils were first stimulated with NAF and then with C5a or vice versa. However, when the same stimulus was applied twice, no second response was obtained. Thus, complete densensitization to both NAF and C5a, but no cross-desensitization, could be demonstrated. Similarly, no cross-desensitization was observed in analogous experiments with combinations of NAF and PAF (see also Fig. 6) or NAF and LTB_4 (data not shown). Taken together, these results strongly suggest that NAF acts via a receptor, and provide evidence that the NAF receptor is unrelated to those of the already known agonists.

Effect of Serum. In serum or plasma, C5a is rapidly converted to its virtually inactive des-Arg derivative by a carboxypeptidase (20). In view of the functional similarities between NAF and C5a, their susceptibility to serum was compared. As shown in Fig. 9, a serum treatment that completely inactivated C5a did not affect NAF activity.

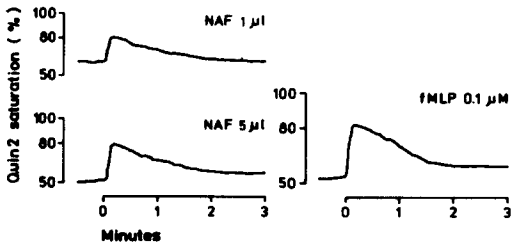

FIGURE 7. NAF-induced change in cytosolic free calcium. Quin-2 fluorescence curves of neutrophils stimulated with NAF (1 or 5 μl of the partially purified preparation) or FMLP (0.1 μM).

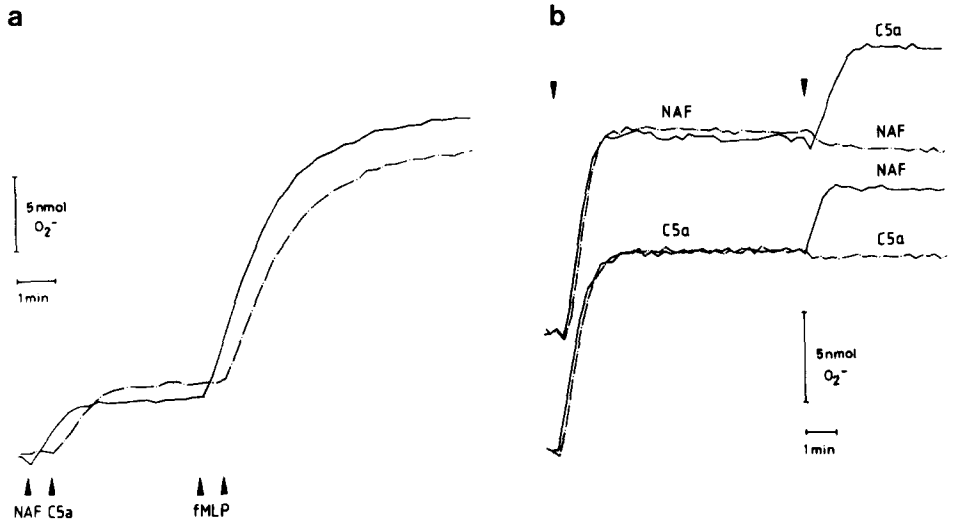

FIGURE 8. Superoxide production in response to sequential stimulation with partially purified NAF, C5a, and FMLP. (*a*) Neutrophils were first stimulated with NAF or C5a and then with FMLP. (*b*) Neutrophils were first stimulated with NAF or C5a and then either with the same or the alternate stimulus as indicated. Recordings of cytochrome *c* reduction are shown.

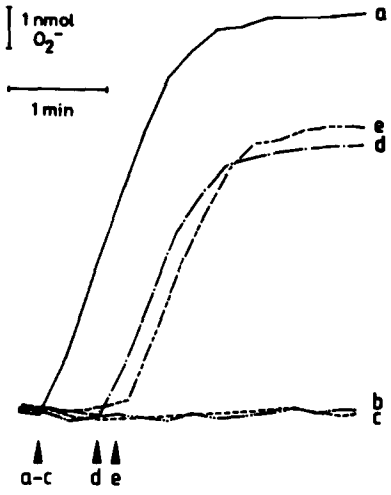

FIGURE 9. Effect of serum on NAF and C5a. Approximately equally active samples of partially purified NAF and C5a were incubated for 5 min at 37°C with or without fresh human serum, and were then added to the neutrophils to induce the respiratory burst *(arrowheads)*. (*a*) C5a in PBS-BSA; (*b*) C5a in serum; (*c*) serum alone; (*d*) NAF in PBS-BSA; (*e*) NAF in serum. Recordings of cytochrome *c* reduction are shown.

Discussion

This paper shows that cultured human blood monocytes stimulated with LPS, PHA, or Con A produce and release a neutrophil-activating factor, which we tentatively termed NAF. The time course of NAF production and the inhibition by cycloheximide indicate that *de novo* protein synthesis is required. Lymphocytes did not produce NAF, and there was no indication of their involvement as enhancers or inhibitors of production since the yield was proportional to the number of monocytes present and independent of lymphocyte additions.

A Novel Neutrophil Receptor Agonist. The goal of the present study was to characterize some of the biological effects of NAF on its target cell, the human neutrophil, and to compare it with well established neutrophil-activating agents. NAF elicited the two major neutrophil responses, the respiratory burst and granule release. Four lines of evidence indicate that NAF acts on a selective surface receptor and activates the neutrophils via a signal-transducing process similar to the one initiated by chemotactic peptides. (*a*) Like other receptor agonists, i.e. FMLP, C5a, PAF, and LTB_4, NAF induced a rapid, transient increase in $[Ca^{2+}]_i$; (*b*) real-time recordings of superoxide and H_2O_2 production showed that the respiratory burst elicited by NAF was similar in onset and time course to that induced by FMLP or C5a; (*c*) repeated stimulation with NAF showed full desensitization of the neutrophils, a phenomenon that was also shown to occur with both FMLP and C5a (21). No cross-desensitization, however, was observed upon sequential stimulation with NAF and FMLP or NAF and C5a; (*d*) the respiratory burst response to NAF was potentiated by pretreatment of the cells with PAF, as previously reported for the response to FMLP and C5a (6, 7, 19), suggesting a similarity in signal transduction.

NAF-like Factors. It was reported some years ago that alveolar macrophages release factors that are chemotactic for neutrophils (22–24), and that may thus condition the infiltration of pulmonary tissues by inflammatory cells (23). Other investigators showed subsequently that alveolar macrophage products enhanced the microbicidal activity of neutrophils, and suggested that such a process may be important in the defense of lung tissue against infection (25, 26). Purification by gel filtration and chromatofocusing led to the identification of a protease-sensitive factor termed NAF, with apparent molecular weight of 6,000 and an isoelectric point of 7.6 (26). The NAF described in the latter study was reported to be weakly chemotactic and to enhance oxygen-dependent killing of bacteria by neutrophils without, however, inducing the respiratory burst by itself. A similar mechanism of enhanced host defense was described by Ferrante and Mocatta (27), who showed that human neutrophils require conditioning with mononuclear phagocyte culture media to kill *Naegleria fowleri.* Granulocyte-activating mediators (GRAM) produced by LPS-stimulated human monocytes were described more recently by Kapp et al. (28) and Maly et al. (29). Two GRAM species with apparent mol wt of 60×10^3 and $< 10^4$ were reported to elicit a delayed and long-lasting chemiluminescence response in human neutrophils. GRAM was sensitive to heat and trypsin and its production was dependent on LPS stimulation and on *de novo* protein synthesis. Yet another activating factor, termed mononuclear cell–derived chemotaxin (MOC), apparently a peptide with a mol wt of 10^4, that differs from GRAM and C5a was reported by Kownatzki et al. (30). Most recently Yoshimura et al. (31) described a cationic chemotactic peptide of $\sim 10^4$ mol wt, that is released by LPS-stimulated monocytes, and that is functionally similar to MOC.

Some of the above reports as well as our present data clearly indicate that the neutrophil-activating peptides released by mononuclear phagocytes are distinct from the two well-known monokines, IL-1 and tumor necrosis factor (TNF). Several of the NAF-like molecules were shown to be considerably smaller than either monokine (26, 28, 30, 31), and to exert effects that could not be obtained

with recombinant and highly purified monokines (31). In addition, amino acid sequencing of purified NAF (8) showed no significant structural homology with IL-1α and β and TNF-α. In the light of the present evidence, we believe that early preparations of IL-1, which were shown to induce the respiratory burst and granule release (32, 33), might have been contaminated with NAF. The question about similarities among the NAF and the NAF-like factors discussed remains open due to the lack of sufficient structural information. Despite the biochemical and functional differences reported, it is conceivable that several of the NAF-like factors may turn out to be identical.

In view of its functional properties, NAF may be regarded as homologous to FMLP and C5a. NAF differs, however, in some important aspects from either chemotactic peptide and is likely to have a distinct role in host defense and inflammation. Like C5a, NAF is of host origin, but unlike C5a it is generated by inflammatory cells. Another distinction from C5a is its resistance to degradation by serum and exudate peptidases. NAF, therefore, is expected to persist and to accumulate in inflamed tissues (as it does in culture) and to have a prolonged influence on immigrating neutrophils. While C5a and N-formyl-methionyl peptides are considered to be early mediators of neutrophil activation upon infection, NAF presumably acts as a second-wave mediator since its generation depends on the influx of mononuclear phagocytes into the affected tissue. Mononuclear phagocytes are also major producers of PAF (34), and it is interesting to note that the effect of NAF on neutrophils is enhanced when this lipid agonist is present. These considerations suggest that NAF may have a particular role in chronic infection and inflammation. The rapid progress of the structural characterization (8) will soon lead to the development of analytical methods for the detection of NAF in inflamed tissues, which shall answer some of the questions proposed by our present work.

Summary

The biological properties of a neutrophil-activating factor (NAF), which was recently identified as a novel peptide of ~6,000 mol wt (8), are described. NAF is produced *de novo* by human blood monocytes upon stimulation with LPS, PHA, and Con A. It induces two main responses in human neutrophils, i.e., exocytosis (release from specific granules in normal, and from specific and azurophil granules in cytochalasin B–treated cells) and the respiratory burst (formation of superoxide and hydrogen peroxide). The action of NAF appears to be mediated by a surface receptor as shown by the following observations. (*a*) NAF induces a rapid and transient rise in cytosolic free Ca^{2+}; (*b*) interaction with NAF results in desensitization, since the cells do not respond to a second NAF challenge; and (*c*) the respiratory burst elicited by NAF is similar in onset, and time course to that induced by C5a or FMLP. The NAF receptor can be distinguished from the receptors of C5a, FMLP, platelet-activating factor, and leukotriene B_4 by the lack of cross-desensitization. Unlike C5a, the other host-derived neutrophil-activating peptide, NAF is not inactivated by serum and thus presumably accumulates in inflamed tissue.

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