LIPOPOLYSACCHARIDE-STIMULATED HUMAN MONOCYTES SECRETE, APART FROM NEUTROPHIL-ACTIVATING PEPTIDE 1/INTERLEUKIN 8, A SECOND NEUTROPHIL-ACTIVATING PROTEIN

NH_2-terminal Amino Acid Sequence Identity with Melanoma Growth Stimulatory Activity

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Monocytes are known to secrete upon LPS stimulation large amounts of a neutrophil-activating peptide originally termed MONAP (1), MDNCF (2), NAF (3), or GCP (4), and now called NAP-1/IL-8 (5). With respect to the primary sequence, this novel cytokine (consisting of 72 amino acids in its major form) shows structural homology to β-thromboglobulin-like host defense cytokines (2–4, 6) and is quite different from other cytokines, such as IL-1 or TNF.

In attempts to purify NAP-1/IL-8 from supernatants of LPS-stimulated monocytes by the use of HPLC techniques, we reproducibly observed the presence of additional neutrophil-stimulating activity eluting at a different retention time than NAP-1/IL-8. This indicates that monocytes secrete more than one neutrophil-activating peptide.

In this study, purification and characterization of a second monocyte-derived neutrophil-activating cytokine, which we tentatively termed NAP-3, will be described. Our data provide evidence that monocytes upon LPS stimulation are able to secrete a neutrophil-activating protein identical in its NH_2-terminal amino acid sequence with melanoma growth-stimulating activity (MGSA/gro)[1], which is known to belong to the same supergene family of β-thromboglobulin-like host defense cytokines as NAP-1/IL-8 does.

Materials and Methods

Neutrophil Isolation. Human polymorphonuclear leukocytes (PMNL) from normal human volunteers were isolated using a modification of the method of Henson (7), as described (1). PMNL preparations were identified to contain 96–98% neutrophils, usually <0.5% eosino-

This work was supported by Deutsche Forschungsgemeinschaft grant Schr 305, 1-2.

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[1] *Abbreviations used in this paper:* CI, chemotactic index; MGSA, melanoma growth-stimulating activity; PMNL, polymorphonuclear leukocytes.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/90/04/1091/10 $2.00 1091
Volume 171 April 1990 1091–1100

phils, and 0.5–2% mononuclear cells. Viability of the final PMNL preparation usually was >97% (trypan blue exclusion test).

Production of Monocyte-derived Neutrophil-activating Peptides. Human monocytes, isolated by counterflow elutriation (J2-21 M/E centrifuge; Beckman Instruments, Inc., Palo Alto, CA) of PBMC were incubated with LPS (*Salmonella minnesota*; Calbiochem-Behring Corp., Marburg, FRG) (1 μg/ml) in RPMI 1640 containing 1 mM glutamine and 20 mM Hepes (cell density, 5×10^6 cells/ml). After a 40–48-h incubation at 37°C, conditioned media were collected and frozen below −70°C until further use.

Purification of Neutrophil-activating Proteins. Supernatants of LPS-stimulating monocytes were acidified to pH 3, concentrated over Amicon YM-5 filters, and chromatographed on a G-75 gel column (2.5 × 70 cm; Pharmacia Fine Chemicals, Uppsala, Sweden) using 0.1 M ammonium formate, pH 5, as eluent. Fractions containing PMNL chemotaxis-stimulating activity (eluting in fractions corresponding to the area 40–5 kD) were concentrated and applied to a preparative wide-pore reversed-phase (RP)-8-HPLC column (300 × 7 μm C_8 Nucleosil, 250 × 12.6 mm; Macherey und Nagel, Düren, FRG) and separated by the use of increasing concentrations of acetonitrile, as described (8). Fractions were tested either for PMNL chemotactic activity, PMNL myeloperoxidase release, or both, and fractions containing PMNL-stimulating activity were further purified by wide-pore CN-propyl-HPLC (5 μm, 250 mm × 4.6 mm; J. T. Baker, Gross Gerau, FRG). Peptides were eluted with a gradient of increasing concentrations of *n*-propanol containing 0.1% (vol/vol) trifluoroacetic acid. Fractions containing PMNL-stimulating activity were finally purified by narrow-pore RP-18-HPLC (Nucleosil, 5-μm octadecyl silica column; Bischoff, Leonberg, FRG) using a gradient of acetonitrile in aqueous TFA (0.1% [vol/vol]).

SDS-PAGE. SDS-PAGE for peptides was performed as described by Schägger and von Jagow (9) using tricine and a discontinuous system optimized for detection of 1–20-kD polypeptides. For separation of polypeptides, a gel containing 16.5% T and 6% C in the presence of 8 M urea was used. Polypeptides were visualized by silver staining (Sigma Chemical Co., St. Louis, MO).

NH_2-terminal Amino Acid Sequence Analysis of Neutrophil-activating Peptide. Material made homogeneous by SDS-PAGE and RP-HPLC was subjected to gas phase sequencing using a sequencer (470A; Applied Biosystems, Inc., Foster City, CA) with on line HPLC analysis of the phenylthiohydantoin derivatives.

PMNL Functional Assays. Chemotactic activity for PMNL was assessed, as recently described in detail (1), using a modification of the endogenous component chemotaxis assay described by Creamer et al. (10). In some experiments, PMNL chemotaxis was determined by the use of a microscopic cell counting method, as described (11). Chemotactic activity was expressed as chemotactic index (CI): CI = No. of cell equivalents migrating upon stimulation/No. of cell equivalents migrating without stimulation. Release of lysosomal enzymes in cytochalasin B–pretreated PMNL was determined by estimating the amount of either β-glucuronidase or myeloperoxidase, as recently described (1).

Deactivation of chemotaxin-induced lysosomal enzyme release (β-glucuronidase) was performed, as recently described (1), by preincubating PMNL with chemotaxins followed by adding cytochalasin B and a second challenge with chemotaxins. Results are expressed as the percentage of inhibition of net enzyme release by buffer-preincubated cells.

Results and Discussion

Our results show that human monocytes upon stimulation with bacterial LPS secrete more than one neutrophil-activating peptide. Although there is no doubt that NAP-1/IL-8 is by far the most abundant PMNL-activating peptide, supernatants of LPS-activated monocytes reproducibly show several fractions containing less PMNL enzyme release, as well as chemotactic response–eliciting activity, which eluted at lower acetonitrile concentration than NAP-1/IL-8 did (Fig. 1).

One of the major neutrophil-activating proteins eluting at 30–35% acetonitrile from the RP-8-HPLC column could be purified by different RP-HPLC-techniques

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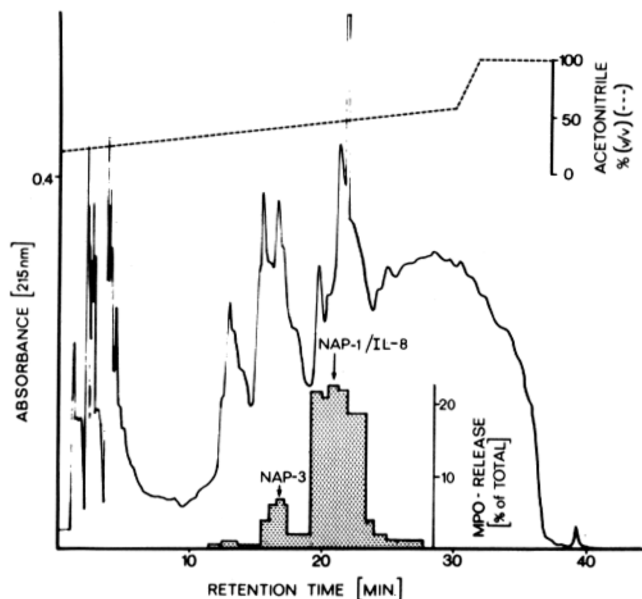

FIGURE 1. Preparative wide-pore RP-HPLC of neutrophil-activating peptides. Supernatants of LPS-stimulated human monocytes were partially purified by G-75 gel filtration. Biologically active fractions (5-40-kD area) were separated on a preparative wide-pore RP-8-HPLC-column by the use of an increasing concentration of acetonitrile. Elution of peptides was monitored at 215 nm. 10-μl aliquots of each fraction were tested either for secretion of myeloperoxidase (MPO) in cytochalasin B-pretreated PMNL (*shaded area*) or for chemotactic activity, as described in Materials and Methods. Note the appearance of additional PMNL enzyme-releasing activity eluting before NAP-1/IL-8 (the elution position of authentic NAP-1/IL-8 is indicated by the arrow).

(Figs. 2 and 3). This material appears to be homogeneous, giving a single band at M_r 5.3 kD (Fig. 4), which is of similar mobility as that seen for the 8.5-kD protein NAP-1/IL-8, migrating under these conditions with an unusual mobility like a 5.8-kD polypeptide (Fig. 4).

NH_2-terminal amino acid sequence analysis of purified material (Fig. 5) revealed

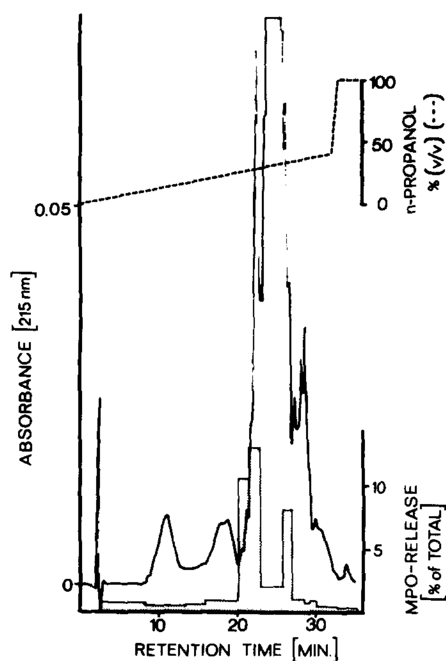

FIGURE 2. CN-propyl-RP-HPLC of the second monocyte-derived neutrophil-activating protein (NAP-3). PMNL-activating fractions off preparative RP-8-HPLC eluting at 30-35% acetonitrile (Fig. 1) were further purified by CN-propyl-RP-HPLC using a gradient of increasing concentrations of *n*-propanol. A major peak of PMNL-stimulating activity eluted at 21 min (NAP-3), whereas a second (minor) one eluted at higher *n*-propanol concentration (*shaded area*).

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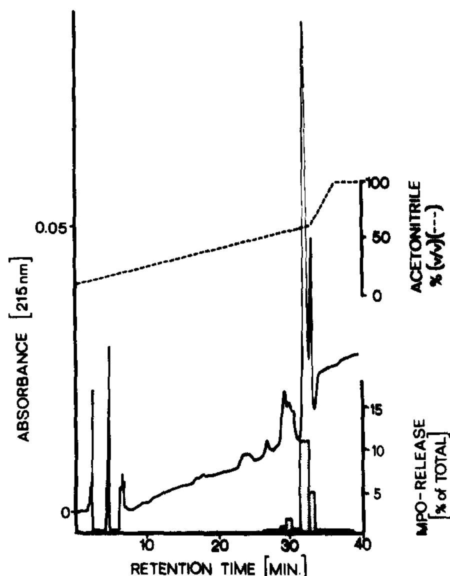

FIGURE 3. Final RP-18-HPLC purification of the second monocyte-derived neutrophil-activating protein. Fractions containing the major PMNL-stimulating activity off CN-propyl-HPLC eluting at 35% *n*-propanol (Fig. 2) were finally purified by narrow-pore RP-18-HPLC. Shaded area shows peaks containing PMNL enzyme release eliciting activity. Note the presence of a major and a minor peak absorbing at 215 nm.

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it to be identical with that of the 13-kD moiety of MGSA (12), known to be identical with the product of serum-induced growth-regulated gene, termed gro, which is differentially expressed by some human cell lines (13).

Whether the second monocyte-derived neutrophil-activating peptide we tentatively termed NAP-3 (because in a recent investigation, another structurally related neutrophil-activating peptide identical to a possibly platelet-derived fragment of β-thromboglobulin was detected in mononuclear cell supernatants, which was tentatively termed NAP-2 [14]) is, with respect to its COOH-terminal amino acid sequence and molecular weight, identical with MGSA is yet not clear. The M_r originally reported for a MGSA preparation showing identical NH_2-terminal amino acid sequence, as we found for NAP-3, has been estimated to be 13 kD (12). For NAP-3, we found a M_r of ~5.3 kD when a tricine-SDS-PAGE system together with CN-Br cleavage products of myoglobin as standards were used (Fig. 4). However, in a very recent report about the secretion of MGSA/gro by stimulated endothelial cells, a

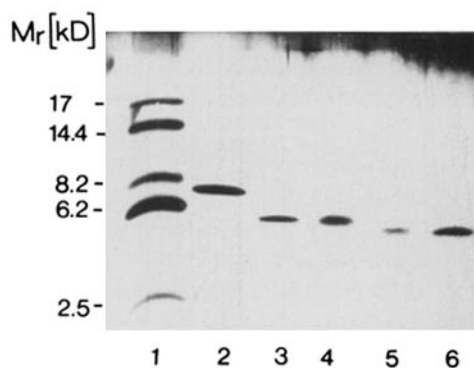

FIGURE 4. SDS-PAGE of the second monocyte-derived neutrophil-activating protein. Material obtained from the RP-18-HPLC-purification (Fig. 3) was analyzed in lanes *5* and *6* for its M_r by tricine-SDS-PAGE in the presence of 8 M urea using the method of Schägger and von Jagow (9). Lane *1* contains a mixture of CNBr cleavage products of myoglobin. In lanes *3* and *4*, authentic NAP-1/IL-8 is applied, whereas lane *2* contains the 77-residue form of NAP-1/IL-8 obtained from IL-1-stimulated fibroblasts (19). SDS-PAGE was performed under nonreducing conditions, except for the calibration mixture. Note the abnormal mobility of NAP-1/IL-8, which is known to be a 8.39-kD polypeptide migrating under these conditions as a 5.8-kD polypeptide.

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                  * ******  *     *** *    *
NAP-3          ASVATELRXQXLQTLQG IHPKNIQSVNVK?P
               SV
                L

MGSA           ASVATELRCQCLQTLQG IHPKNIQSVNVKSP...

gro            ASVATELRCQCLQTLQG IHPKNIQSVNVKSP...

NAP-1/IL-8       SAKELRCQCIKTYSKPFHPKFIKELRVIES...

NAP-2              AELRCMCIKTTSG-IHPKNIQSLEVIGK...
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FIGURE 5. NH_2-terminal amino acid sequence characterization of the second monocyte-derived neutrophil-activating peptide (NAP-3). NH_2-terminal amino acid sequencing of NAP-3 was performed without derivatization. Therefore, cysteins were detected as blanks (X). The single letter code for amino acids is used. Asterisks indicate homology with NAP-1/IL-8. (MGSA) Sequence as published for the 13-kD moiety of MGSA (12). (gro) Part of the sequence published for gro (13). (NAP-1/IL-8) NH_2-terminal sequence of NAP-1/IL-8 as recently shown (2–4, 6). (NAP-2) NH_2-terminal sequence of NAP-2 (14).

M_r of ~8 kD has been estimated for authentic MGSA made by Hs294 melanoma cells (15). The difference from the originally described M_r has been attributed to the use of a different gel system and molecular weight standards. When we used authentic NAP-1/IL-8, known to be a 8.386-kD polypeptide (6), as M_r standard, a M_r of ~8 kD could be estimated for NAP-3. This was supported by size exclusion HPLC of NAP-3, where we estimated a M_r of ~10 kD (data not shown).

The apparent molecular weight of NAP-3 is consistent with the value predicted from the amino acid sequence of gro/MGSA (12). Therefore, it appears likely that NAP-3 and MGSA/gro are identical cytokines. Further support for this suggestion was obtained from preliminary investigations, when we tested growth stimulatory activity of a NAP-3 preparation in fibroblasts. In a dose range between 0.5 and 10 ng/ml, NAP-3 showed growth stimulatory activity (unpublished results). This fits well with the mitogenic behavior reported for MGSA in fibroblasts (15).

Interestingly, sequence analysis of the monocyte-derived NAP-3 indicated the presence of an NH_2-terminal altered form of NAP-3 containing Ser (~50%) instead of Ala for the NH_2-terminal amino acid, as well as either Leu or Val instead of Ser (~30%) for the second amino acid. In the following cycles up to residue 31, no evidence for major changes was obtained. These results point towards the possible existence of different genetic forms of NAP-3, although further studies are needed to support this suggestion.

When NAP-3 was investigated for its PMNL stimulatory properties, it could be demonstrated that both chemotactic migration, as well as degranulation of lysosomal enzymes, are elicitable (Fig. 6). Interestingly, NAP-3 shows a similar ED_{50} in eliciting PMNL chemotaxis when compared with NAP-1/IL-8 (Fig. 6); however, the number of migrating cells at optimal stimulation conditions has been found to be only 60% of that seen for potent PMNL chemotaxins like C5a, LTB_4, or NAP-1/IL-8, which was supported by the use of experiments performed with a direct microscopical cell-counting chemotaxis method (data not shown). Moreover, the bell-shaped dose-response curve covers a more narrow area than NAP-1/IL-8, giving at concentrations >200 ng/ml no significant chemotactic responses (Fig. 6). A similar, more restricted dose-response profile in PMNL chemotaxis has been reported for platelet-derived growth factor (16).

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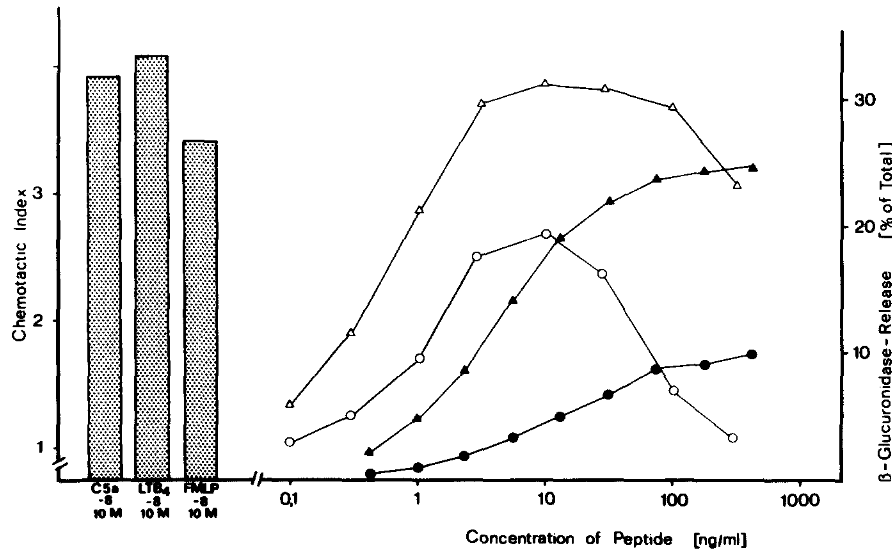

FIGURE 6. PMNL chemotactic and degranulation-eliciting activity of the second monocyte-derived neutrophil-activating protein. PMNL chemotactic activities of purified preparations of NAP-3 (○) as well as NAP-1/IL-8 (△) and PMNL degranulation (β-glucuronidase)-eliciting activities of NAP-3 (●), as well as NAP-1/IL-8 (▲) as a function of the peptide concentration used are shown. Shaded bars represent chemotactic indices of authentic C5a and LTB_4. Degranulation-eliciting activity with the use of 10^{-8} M FMLP or 10^{-8} M C5a has been found to be 48 and 35% of the total control, respectively. Data represent mean of three duplicate performed experiments.

Crossdesensitization experiments with NAP-3 and NAP-1/IL-8 show crossreactivities in chemotaxin receptor-dependent PMNL enzyme release by NAP-3 and NAP-1/IL-8, but not, however, with C5a or FMLP (Table I). This result may support the suggestion than NAP-3 binds to the PMNL-NAP-1/IL-8 receptor, which recently has been characterized (17). It appears, however, that NAP-3 is a weaker agonist than NAP-1/IL-8, because preincubation of PMNL with NAP-1/IL-8 completely abolished responses to a second challenge with NAP-3, whereas preincubation of cells with NAP-3 does not show the same amount of deactivation when NAP-1/IL-8 is used at a similar molar concentration for the second stimulation (Table I).

A PMNL-activating peptide with the same NH_2-terminal amino acid sequence as found for the 16-kD moiety of MGSA/gro (12) was recently isolated from lesional psoriatic scale material as one of its major peptide-like PMNL chemoattractants (18). Moreover, we detected in IL-1- or TNF-α-stimulated human dermal fibroblasts a neutrophil-activating peptide termed γ-FINAP (19), which is biochemically as well as biologically identical with the psoriasis-derived MGSA/gro. Both investigations support the observation of this study that a protein with similarity to MGSA/gro indeed is a neutrophil-activating factor. The biological significance of the PMNL function-activating property of NAP-3 remains to be speculative at the moment.

Recent studies have shown that MGSA, which originally has been isolated from melanomas as well as cultivated melanoma cells (20), apparently acts as a growth hormone for different cells (12).

Apart from this, autocrine growth-stimulating activity of MGSA/gro is well doc-

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TABLE I

Desensitization of Chemotaxin-elicitable PMN Enzyme Release by NAP-3

Preincubation	Stimulation with: NAP-1/IL-8 (10^{-8} M)	NAP-1/IL-8 (5×10^{-9} M)	NAP-1/IL-8 (2.5×10^{-9} M)	NAP-1/IL-8 (1.3×10^{-9} M)	NAP-1/IL-8 (5×10^{-10} M)	NAP-3 (80 ng/ml)	C5a (10^{-8} M)	FMLP (10^{-8} M)
NAP-1/IL-8 (10^{-8} M)	88	96	98	100	100	100	0	0
NAP-3 (160 ng/ml)	17	46	49	80	86	100	5	2
C5a (2×10^{-8} M)	4	ND	ND	ND	ND	0	54	5
BocMetLeuPhe (10^{-5} M)	7	ND	ND	ND	ND	2	0	89

Enzyme release (β-glucuronidase) was determined in chemotaxin-preincubated PMNL after subsequent stimulation with different chemotaxins as recently described (1). Results are expressed in percentage of inhibition of net enzyme release of the respective control (buffer-preincubated PMNL) after stimulation with the appropriate stimulus. The net enzyme release of buffer preincubated cells has been found to be 24, 22, 21, 19, and 13% of a total control for NAP-1/IL-8 at 10^{-8} M, 5×10^{-9}, 2.5×10^{-9}, 1.3×10^{-9}, and 5×10^{-10} M, respectively. For NAP-3 at 80 ng/ml, an enzyme release of 10% was found, whereas 10^{-8} M C5a and 10^{-8} M FMLP released 35 and 48% of a total control, respectively. Values represent the mean of three experiments.

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umented (12, 13, 15, 20). Therefore, it is possible that secretion of a MGSA/gro-related protein by LPS-stimulated monocytes could be considered as a mitogenic signal rather than a signal for PMNL activation upon inflammatory reactions. This is supported by sequence relatedness of this cytokine to both mitogens and neutrophil-activating proteins, because MGSA/gro is a member of the new supergene family of β-thromboglobulin-like host defense cytokines, as is NAP-1/IL-8 (2–5, 12). It is noteworthy that mouse macrophages secrete upon endotoxin stimulation a neutrophil attractant termed macrophage inflammatory protein 2, which shows strong sequence homology to human NAP-3 and MGSA/gro (21). However, it appears unlikely that macrophage inflammatory protein 2 despite its biological properties that are similar to NAP-3, is the murine equivalent of MGSA/gro, because another member of the β-thromboglobulin superfamily obtained from PDGF-stimulated murine macrophages, termed KC (22), shows higher sequence homology to human gro/MGSA.

Some of the members of the β-thromboglobulin superfamily–like connective tissue–activating peptide III are reported to be mitogenic (23), and NAP-1/IL-8 is known to be a powerful PMNL-activating cytokine (1–4).

It appears that MGSA/gro (or a COOH-terminal truncation product) represents the first member of this novel family of host defense cytokines, which expresses both growth-promoting and proinflammatory behavior at the nanogram level.

Summary

Purification of monocyte-derived NAP-1/IL-8 by preparative reversed-phase (RP)-HPLC led to the detection of a second peak with polymorphonuclear leukocyte (PMNL)-activating (degranulation, chemotaxis) properties. The monokine responsible for this biological activity, which we tentatively termed NAP-3, could be purified to homogeneity by three different RP-HPLC steps. Tricine-SDS-PAGE analysis gave a single line at M_r 5.3 kD (NAP-1/IL-8 = 5.8 kD). NH_2-terminal amino acid sequence analysis read as a major sequence (ASVATELRXCXLQT. .), which shows >40% homology to that of NAP-1/IL-8. The sequence is identical to that found for the 13-kD moiety of melanoma growth stimulating activity (MGSA) and the product of the oncogene gro.

Determination of neutrophil chemotactic activity of NAP-3 revealed a typical bell-shaped dose-response curve (ED_{50} = 2 ng/ml) with no significant neutrophil chemotactic activity at doses >200 ng/ml. Also, in cytochalasin B–pretreated PMNL, NAP-3 elicited release of myeloperoxidase and β-glucuronidase. Crossdesensitization studies in PMNL enzyme release revealed crossreactivities with the NAP-1/IL-8-R on PMNL. NAP-3 (MGSA/gro) appears to represent the first member of the novel supergene family of β-thromboglobulin-like host defense cytokines, which expresses both mitogenic as well as proinflammatory properties at the nanogram level.

We thank Mrs. Ch. Gerbrecht-Gliessmann and Mrs. E. Ohm for their excellent technical assistance, and Mrs. I. Brandt for editorial help.

Received for publication 13 September 1989 and in revised form 30 October 1989.

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