

# GENERATION OF THE NEUTROPHIL-ACTIVATING PEPTIDE NAP-2 FROM PLATELET BASIC PROTEIN OR CONNECTIVE TISSUE-ACTIVATING PEPTIDE III THROUGH MONOCYTE PROTEASES

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We have recently shown that cultures of human blood mononuclear cells stimulated with *Escherichia coli* LPS or PHA produce a 70 amino acid neutrophil-activating peptide, termed NAP-2,<sup>1</sup> which appears to be a cleavage product of the platelet  $\alpha$ -granule component platelet basic protein (PBP) and its derivative, connective tissue-activating peptide III (CTAP-III) (1). NAP-2 has similar size and marked sequence homology to other recently described inflammatory peptides, NAF/NAP-1 (2) and the murine protein MIP-2 (3). Unlike its presumed precursors, PBP and CTAP-III, NAP-2 has powerful neutrophil-stimulating effects and a profile of biological activity similar to that of NAF/NAP-1 (4).

We have now studied the conditions for the generation of NAP-2. This article shows that platelets are the source of the precursor proteins and that proteases secreted from monocytes process these proteins into neutrophil-activating peptides.

## Materials and Methods

**Media and Solutions.** Two culture media were used, MEM and MEM-PPL. MEM consisted of Eagle's MEM (Seromed GmbH, Munich, FRG) supplemented with 25  $\mu$ g/ml neomycin and buffered at pH 7.4 with 25 mM NaHCO<sub>3</sub> 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). Recombinant NAF/NAP-1 was obtained from the Sandoz-Forschungsinstitut GmbH, Vienna, Austria (5).

**Cell Preparations.** Monocytes (90% pure) and lymphocytes (95% pure) were obtained from buffy coats of donor blood by centrifugation on Ficoll-Paque followed by centrifugal elutriation (6). Neutrophils were isolated and used exactly as described previously (6) and platelets were prepared from platelet-rich plasma (7).

**Cell Cultures.** Monocytes ( $1.2 \times 10^6$  cells/cm<sup>2</sup>) and lymphocytes ( $2.3 \times 10^6$  cells/cm<sup>2</sup>) were cultured in 175-cm<sup>2</sup> flasks with 100 ml MEM-PPL for 22 h at 37°C in a 5% CO<sub>2</sub> atmosphere in the presence of the release supernatant from  $2 \times 10^{10}$  thrombin-stimulated platelets (4). Three different stimuli, 1  $\mu$ g/ml LPS (LPS from *E. coli* 055:B5; Difco Laboratories, Basel, Switzerland), 5  $\mu$ g/ml PHA (Difco) were used.

**Purification of Peptides.** Monocyte and lymphocyte culture supernatants (95 ml) were passed

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through 5-ml phosphocellulose columns (Whatman P11) equilibrated in 20 mM potassium phosphate, pH 7.2, 20 mM NaCl, 1 mM EDTA, and 5% glycerol at room temperature. The columns were washed with 10 ml equilibration buffer and 10 ml of the same buffer supplemented with 0.2 M NaCl. Elution was performed with three 3-ml portions of equilibration buffer supplemented with 1.2 M NaCl. Fractions with neutrophil-stimulating activity (kept at 4°C) were acidified with trifluoroacetic acid (TFA) and directly loaded onto an analytical HPLC reversed-phase cyanopropyl column (4.6 × 250 mm, 5 μm, wide-pore; Baker Research Products, Phillipsburg, NJ). The column was eluted at 0.5 ml/min with a gradient of acetonitrile in 0.1% TFA with an increment of 0.66%/min. 1-min fractions were collected and tested for neutrophil-stimulating activity.

**Processing of CTAP-III to NAP-2.** About 10<sup>9</sup> monocytes were cultured for 22 h in 200 ml MEM-PPL. The culture supernatant was cleared by centrifugation (20,000 g for 20 min) and concentrated 50-fold by means of CX-10 ultrafilters (Millipore, Bedford, MA). Aliquots of 20 μl of the concentrate were incubated with 5 μg of CTAP-III, purified as described (4), for up to 16 h at 37°C in a total volume of 1 ml PBS containing 1% PPL. PMSF (Fluka), leupeptin (Fluka AG, Buchs, Switzerland), or EDTA were added as potential inhibitors.

**Bioassay.** A microtiter plate fluorescence assay for elastase release was used to assess neutrophil activation (4).

**Amino Acid Sequence Analysis.** This was performed by automated phenyl isothiocyanate degradation with a gas phase sequencer (No. 477 A; Applied Biosystems, Inc., Foster City, CA). Samples of the purified peptides (500 pmol) were applied directly or after chemical modification. Reduction and alkylation was performed as described (1).

## Results

**Source of NAP-2.** The formation of NAP-2 and other neutrophil-stimulating peptides was studied with purified populations of monocytes and lymphocytes stimulated in different ways in the presence and absence of platelet release supernatant containing PBP and CTAP-III. Before biological testing, the conditioned medium was fractionated by cation-exchange chromatography followed by reversed-phase HPLC, resolving three peaks of activity with mean modal retention times of about 22, 26, and 34 min. Sequencing of the material recovered showed that peak 1 corresponded to NAP-2 (70 amino acids), peak 2 to a mixture of three NAP-2 variants with 73, 74, and 75 residues, and peak 3 to NAF/NAP-1 (Fig. 1).

Fig. 2 illustrates the role of monocytes in NAP-2 production. NAP-2 was produced whenever monocytes were cultured in the presence of platelet-release supernatant (Exps. C-E), but not when either the cells or the supernatant were omitted (Exps. A and B). When PHA or LPS were added as stimulus, NAF/NAP-1 was also produced and accounted for a major portion of the total neutrophil-stimulating activity. Stimulation of the monocytes and production of NAF/NAP-1 did not influence the yield of NAP-2, as shown by the comparison of Exp. C with Exps. D and E. Upon stimulation with LPS, however, a somewhat smaller intermediate peak containing

NAP-2 (70)	Ala-Glu-Leu-Arg-Cys-Met-Cys-Ile-Lys...
(75)	Asp-Ser-Asp-Leu-Tyr-Ala-Glu.....
(74)	Ser-Asp-Leu-Tyr-Ala-Glu.....
(73)	Asp-Leu-Tyr-Ala-Glu.....
NAP-1 (72)	Ser-Ala-Lys-Glu-Leu-Arg-Cys-Gln-Cys-Ile-Lys...

FIGURE 1. NAP-2 and its NH<sub>2</sub>-terminal variants with neutrophil-stimulating activity; comparison with NAF/NAP-1. The sequences are aligned according to the conserved cysteine residues and the total number of residues is indicated in brackets.

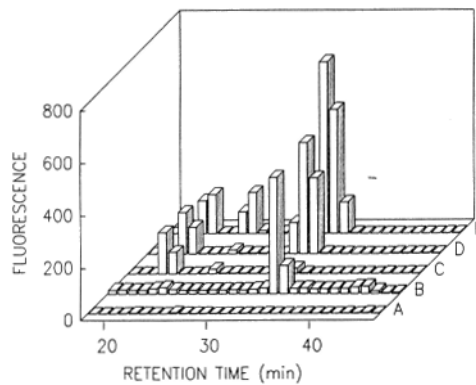


FIGURE 2. Neutrophil-activating peptides produced by cultured human blood monocytes. Culture supernatants were harvested after 22 h and chromatographed as described in Materials and Methods. Histograms representing the distribution of neutrophil-stimulating activity (fluorescence measurement of elastase release) versus retention time on reversed-phase HPLC are shown. The experimental conditions were: (A) platelet-release supernatant (PR) in the absence of cells; (B) LPS-stimulated monocytes in the absence of PR; (C) unstimulated monocytes in the presence of PR; (D) PHA-stimulated monocytes in the presence of PR; (E) LPS-stimulated monocytes in the presence of PR. The three peaks of activity seen in lane E are: NAP-2 (21.8 min), NAP-2 variants (26.2 min), and NAF/NAP-1 (34.6 min). The data are representative for two (A) and five (B-E) similar experiments.

the 73-75 residue variants of NAP-2 was obtained in addition. As estimated from the sequencing data, the additional peak was made up to 65, 20, and 15% by the 74, 75, and 73 residue form, respectively.

Similar experiments were performed with lymphocytes instead of monocytes. As shown in Fig. 3, Exps. C-E, neither NAP-2 nor NAF/NAP-1 were produced by stimulated or unstimulated lymphocytes in the presence of platelet release supernatant. Exps. A and B illustrate the stimulus-dependent production of NAF/NAP-1 by monocytes and the lack of production of NAP-2 when the platelet-release supernatant is omitted.

**Cell-free Generation of NAP-2.** The presumed role of monocyte proteases in the generation of NAP-2 was studied in cell-free assays using purified CTAP-III as the substrate. In preliminary experiments it was established that at least 80% of the total CTAP-III cleaving activity of 22-h cultures of purified monocytes was recovered in the medium (data not shown), indicating that the processing enzymes are secreted by the monocytes and are not appreciably stored intracellularly. The time course and inhibition of NAP-2 formation (detected as neutrophil-stimulating activity) were

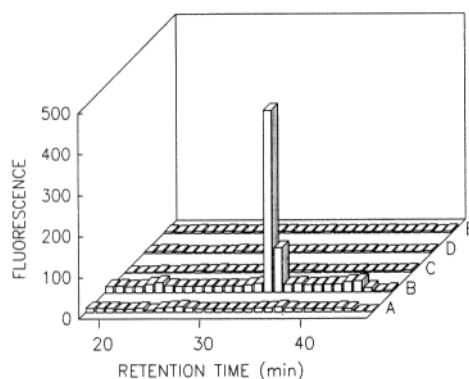


FIGURE 3. Lack of production of neutrophil-activating peptides by cultured human blood lymphocytes. Culture conditions and histograms as in Fig. 2. The experimental conditions were: Unstimulated (A) and LPS-stimulated (B) monocytes in the absence of platelet release supernatant; unstimulated (C), PHA-stimulated (D), and LPS-stimulated (E) lymphocytes in the presence of platelet-release supernatant. The data are representative for three similar experiments.

therefore studied using purified CTAP-III and concentrated monocyte conditioned medium (see Materials and Methods).

As shown in Fig. 4, a linear increase in NAP-2 formation was obtained in the presence of CTAP-III and the conditioned medium, while no change was observed when either one of the reactants were omitted. CTAP-III alone showed no activity, confirming our earlier observations (4), while a low, constant background was detected with medium alone, which could be attributed to NAF/NAP-1, on the basis of inactivation with an antiserum. Similar experiments were performed in the presence of inhibitors. NAP-2 formation was almost totally suppressed by PMSF but was not affected by EDTA, suggesting that the conversion was largely due to a serine protease. Leupeptin, a thiol-proteinase inhibitor, had a moderate but consistent effect, and no NAP-2 formation was obtained when PMSF and leupeptin were combined (not shown). While excluding a role for metallo-proteases, these experiments suggest that CTAP-III conversion to neutrophil-activating peptides is probably due to more than one enzyme.

**Neutrophil Stimulation.** The potency of the different peptides in inducing elastase release is compared in Fig. 5. In agreement with former observations (1, 4), NAP-2 was active in the same concentration range, but was about half as potent as NAF/NAP-1. An at least 10-fold higher threshold concentration was observed for the mixture of the 73-75 residue NAP-2 variants that were markedly less potent than NAP-2. The same ranking was obtained when the rise in cytosolic free calcium was measured (data not shown).

### Discussion

The present study demonstrates that the neutrophil-activating peptide NAP-2 is generated from the platelet  $\alpha$ -granule precursors, PBP and CTAP-III, through proteases released from monocytes. Lymphocytes have no role in NAP-2 production, and no NAP-2 is obtained when the monocytes are cultured in the absence of platelets

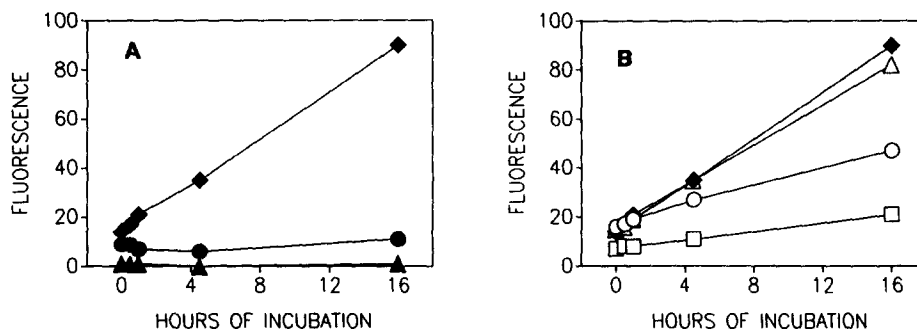


FIGURE 4. (A) Generation of neutrophil-stimulating activity (elastase release) from CTAP-III by monocyte-conditioned medium. CTAP-III alone (▲), conditioned medium alone (●), and CTAP-III plus conditioned medium (◆). (B) Inhibition of CTAP-III processing. CTAP-III and conditioned medium were incubated with the following additions: none (◆), 4 mM EDTA (Δ), 50  $\mu$ g/ml leupeptin (○), 2 mM PMSF (□). Each assay contained the medium equivalent of  $5 \times 10^5$  unstimulated monocytes cultured for 22 h, and 5  $\mu$ g of purified CTAP-III. Controls showed that the neutrophil-activation assay was not influenced by the inhibitors used. Similar data were obtained in four different experiments.

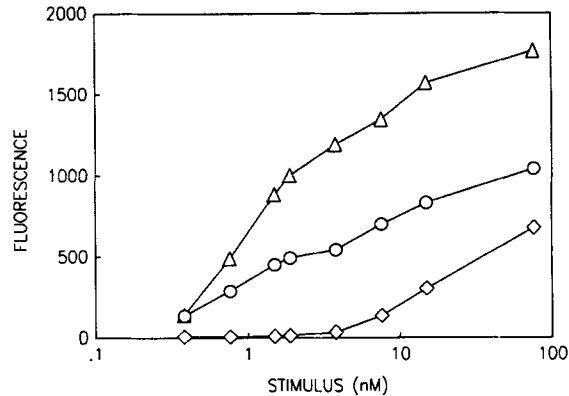


FIGURE 5. Neutrophil activation by recombinant NAF/NAP-1 ( $\Delta$ ), NAP-2 (O), and NAP-2 variants ( $\diamond$ ). Concentration-dependent induction of elastase release (fluorescence units) from cytochalasin B-treated human neutrophils. The data are representative for three similar experiments.

or platelet-release supernatant, even under stimulatory conditions leading to the generation of high amounts of NAF/NAP-1.

Two platelet-derived fractions with neutrophil-stimulating activity were identified in this study, one corresponding to NAP-2 and the other consisting of a mixture of three slightly larger variants. The latter were obtained with stimulated mononuclear phagocytes only, while the yield of NAP-2 was not influenced by stimulation. These observations suggest that different proteases or peptidases are involved in the formation of the two active fractions, and that the NAP-2 variants are not obligatory intermediates of NAP-2 formation. CTAP-III was used to study the generation of NAP-2 by monocyte-conditioned media since its concentration in platelet release supernatants is usually higher than that of PBP (8). From the effects of inhibitors, serine and thiol proteases appear to be involved. These enzymes are likely to be released constitutively by cultured monocytes (as stimulation did not enhance NAP-2 formation) and to act in concert since neither PMSF nor leupeptin alone were fully inhibitory.

The NAP-2 variants were considerably less potent than NAP-2. The fact that no peptide with less than 70 residues was identified, and that effectiveness decreased rapidly with  $\text{NH}_2$ -terminal elongation indicates that among the cleavage products of CTAP-III NAP-2 may be the one with highest neutrophil-stimulating activity.

In terms of its biological effects on neutrophils, NAP-2 is similar to NAF/NAP-1. Both peptides induce a transient rise in cytosolic free calcium, chemotaxis, and exocytosis in the lower micromolar concentration range (4), and thus qualify as pathophysiologically relevant inflammatory mediators. Neither peptide is generated constitutively. The production of NAF/NAP-1 (by mononuclear phagocytes and a wide variety of tissue cells [2]) is induced by inflammatory cytokines like TNF and IL-1, while NAP-2, as this study indicates, can only be generated when PBP and/or CTAP-III are liberated from platelets. Despite their functional similarity, the two peptides must, therefore, be expected to arise in dissimilar pathological situations and at different sites. Being platelet derived, NAP-2 will be produced mainly intravascularly, where platelet activation and aggregation occurs, e.g., in thrombi and atherosclerotic lesions (4), while NAF/NAP-1 will almost invariably form in the tissues (2).

### Summary

We studied the origin of the neutrophil-activating peptide NAP-2, a presumed 70 amino acid cleavage product of platelet basic protein (PBP) and connective

tissue-activating peptide III (CTAP-III). Purified human blood monocytes or lymphocytes were cultured with or without stimuli (LPS or PHA) in the presence or absence of platelet-release supernatant, and the formation of NAP-2 and other neutrophil-activating peptides was monitored. NAP-2 was generated whenever monocytes and platelet release supernatant were present. When a monocyte stimulus was added, NAF/NAP-1 was also formed, and in the presence of LPS a third, less potent neutrophil-stimulating fraction, consisting of NAP-2 variants with 73, 74, and 75 residues, also appeared. Monocytes alone did not yield NAP-2 and no neutrophil-activating peptide was generated by lymphocytes. The monocyte-conditioned medium was found to cleave purified CTAP-III into NAP-2 through proteinases that were highly sensitive to PMSF, moderately sensitive to leupeptin and insensitive to EDTA.

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