

# Structure and Neutrophil-activating Properties of a Novel Inflammatory Peptide (ENA-78) with Homology to Interleukin 8

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## Summary

A new neutrophil-activating peptide, termed ENA-78, was identified in the conditioned media of stimulated human type II epithelial cell line A549. In response to stimulation with either interleukin 1 $\beta$  (IL-1 $\beta$ ) or tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), ENA-78 was produced and secreted concomitantly with IL-8, GRO $\alpha$ , and GRO $\gamma$ . ENA-78 consists of 78 amino acids (AGPAAA-VLRELRCVCLQTTQGVHPKMISNLQVFAIGPQCSKVEVVASLKNKGEICLDPEAPFLKK-VIQKILDGGNKEN) and has a molecular weight of 8,357. It has four cysteines positioned identically to those of IL-8 and analogues, and thus belongs to the CXC family of peptides. ENA-78 is related to neutrophil-activating peptide 2 (NAP-2) and GRO $\alpha$  (sequence identity, 53% and 52%, respectively) and IL-8 (22% identity). Like NAP-2 and GRO $\alpha$ , ENA-78 stimulates neutrophils, inducing chemotaxis, a rise in intracellular free calcium and exocytosis. Cross-desensitization experiments indicate that ENA-78 acts through the same type of receptors as IL-8, NAP-2, and GRO $\alpha$ .

Neutrophil activation and chemotaxis have recently acquired increased attention through the discovery of the potent neutrophil activating peptide 1/IL-8 (1–4). Subsequently, two structural homologues with similar biological activities on human neutrophils were detected, neutrophil-activating peptide 2 (NAP-2),<sup>1</sup> which is formed by proteolytic processing from platelet basic protein or connective-tissue activating peptide III released from platelet  $\alpha$  granules (5, 6), and GRO $\alpha$ , which was originally described as a mitogen for human melanoma cells (7) and was subsequently shown to activate neutrophils (8, 9). IL-8, NAP-2, and GRO $\alpha$  belong to a family of peptides with a molecular mass of 8–10 kD, containing four conserved cysteine residues, the first two spaced by one amino acid (CXC). Two other members of this family are platelet factor 4 (PF-4) and IFN- $\gamma$ -inducible peptide 10 ( $\gamma$ IP-10) (10, 11).

Whereas the formation of NAP-2 is probably limited to the vascular system, IL-8 and GRO $\alpha$  were shown to be secreted by a wide variety of cells (for review, see reference 12). This and the demonstration of their *in vivo* chemotactic activity

strongly support their involvement in a number of inflammatory processes through the triggering of neutrophil infiltration and activation.

Neutrophil infiltration into the alveolar space is prominent in a variety of acute and chronic pulmonary inflammatory disorders (13–15). Alveolar macrophages (16, 17), as well as lung epithelial cells (18) and fibroblasts (19), have been shown to produce IL-8 in response to stimulation. IL-8 is also released by endothelial cells, which, like macrophages, respond to LPS in addition to IL-1 and TNF- $\alpha$  (20). Lung fibroblasts were shown to produce GRO $\alpha$  in addition to IL-8 when stimulated with inflammatory cytokines (19). Since the pulmonary alveolus is in direct contact with the external environment, the alveolar macrophage might be the primary target of exogenous stimuli, such as viruses or bacterial products, which induce the expression of proinflammatory cytokines such as IL-1 and TNF- $\alpha$ .

Type II alveolar cells appear to have a prominent role in the generation of neutrophil attractants. Here we describe the isolation and complete primary structure of a novel neutrophil activating peptide (ENA-78) from the human type II epithelial cell line A549. ENA-78 has the same overall structure as the other members of the CXC family of peptides and matches IL-8, NAP-2, and GRO $\alpha$  in terms of biological activity.

<sup>1</sup> Abbreviations used in this paper: DEPC, diethylpyrocarbonate;  $\gamma$ IP-10, IFN- $\gamma$ -inducible peptide 10; MCP-1, monocyte chemotactic protein 1; NAP-2, neutrophil-activating peptide 2; PF-4, platelet factor 4; TFA, trifluoroacetic acid.

## Materials and Methods

**Reagents.** Human rIL-1 $\beta$  (30 U/ng), human rTNF- $\alpha$  (22 U/ng), and human rIL-8 (34) were kindly provided by Upjohn (Kalamazoo, MI), Genentech (San Francisco, CA) and Sandoz (Vienna, Austria), respectively. NAP-2 was purified as described previously (5). Synthetic oligonucleotides were prepared on a DNA Synthesizer (391; Applied Biosystems, Inc., Foster City, CA) and desalted on NAP 10 columns (Pharmacia Fine Chemicals, Piscataway, NJ).

**Cells.** A549 pulmonary epithelial cells (American Type Culture Collection, Rockville, MD) were grown to confluency in 225-cm<sup>2</sup> culture flasks (Costar, Cambridge, MA) with complete RPMI 1640, plus 10% FCS. Confluent monolayers were washed free of FCS with RPMI and stimulated for 24 h with 1  $\mu$ g/ml LPS, 20 ng/ml TNF- $\alpha$ , or 10 ng/ml IL-1 $\beta$  (300 ml RPMI per flask). Cell-free supernatants were then collected and stored at -75°C. Total cellular RNA was extracted from the monolayers as described below. Human neutrophils were isolated and used as described previously (21).

**Isolation of Poly(A) RNA.** Total cellular RNA from A549 cells was isolated using a modification of the methods of Chirgwin et al. (22) and Jonas et al. (23). Briefly, A549 monolayers were scraped into a solution of 25 mM Tris/HCl, pH 8.0, containing 4.2 M guanidine isothiocyanate, 0.5% sarcosyl, and 100 mM 2-ME. After homogenization, an equal volume of 100 mM Tris/HCl, pH 8.0, containing 10 mM EDTA and 1% SDS was added, and the mixture was extracted twice with chloroform-phenol and chloroform-isooamyl alcohol. The RNA was precipitated with isopropyl alcohol, washed with 80% ethanol in diethylpyrocarbonate-treated water (DEPC-water), and the pellet dissolved in DEPC-water. Poly(A) RNA was then isolated by two passages on an oligo(dT)-cellulose column.

**Preparation of cDNA.** First-strand synthesis was carried out in a total volume of 40  $\mu$ l in the presence of 0.5  $\mu$ g random hexamer primer or 4.2  $\mu$ g ENA-78 antisense primer (see Fig. 5 a), 2  $\mu$ g of poly(A) RNA, 40 U RNasin (Promega Biotec, Madison, WI), 1 mM each of dNTPs, 400 U of MMLV reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD), and 8  $\mu$ l of 5 $\times$  reverse transcriptase buffer (250 mM Tris/HCl, pH 8.3, 375 mM KCl, 15 mM MgCl<sub>2</sub>, 50 mM DTT). This reaction mixture was incubated for 2 h at 37°C.

PCR reactions were carried out on the first-strand cDNA in 50  $\mu$ l containing 4  $\mu$ l of 10 $\times$  PCR buffer (500 mM KCl, 100 mM Tris/HCl, pH 8.3, 15 mM MgCl<sub>2</sub>, and 0.01% gelatin), 10  $\mu$ l first-strand reaction, 3  $\mu$ g of ENA-78 antisense primer, 4.5  $\mu$ g of ENA-78 sense primer (see Fig. 5 a), and 1 U Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). The PCR was run for 35 cycles (40 s at 95°C, 90 s at 55°C, 90 s at 72°C) in a thermocycler (PHC-2; Techne Inc., Princeton, NJ). The final product was extracted three times with phenol/chloroform and four times with ether. The DNA was then precipitated with 0.3 M Na-acetate and 2.5 vol of ethanol.

**Cloning Procedures.** The PCR product was resuspended in sodium chloride, Tris/HCl, EDTA (STE), restricted with EcoRI and BamHI, and separated on a 5% NuSieve agarose gel in 40 mM Tris, 20 mM acetate, pH 8.0, 2 mM EDTA. DNA was visualized by staining with ethidium bromide, bands were cut out, electroeluted (Biotrap; Schleicher & Schuell, Inc., Keene, NH), and ligated into plasmid pTZ18R (Pharmacia, Duebendorf, Switzerland). The construct was then transfected into *Escherichia coli* HB101 by electroporation (Gene Pulser; Bio-Rad Laboratories, Richmond, CA) at 2.5 kV, 25  $\mu$ F, 400 Ohms. A number of positive clones were analyzed for their inserted cDNA by DNA sequencing with Sequenase (United States Biochemical Corp., Cleveland, OH).

**Isolation of Neutrophil-activating Peptides.** Serum-free culture su-

pernatants (450 ml) from the A549 pulmonary epithelial cells were diluted with one volume of loading buffer (20 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.5, 20 mM NaCl, 5% glycerol), and loaded onto a 12-ml phosphocellulose column (P11; Whatman Inc., Clifton, NJ) equilibrated with loading buffer. The column was washed with the same buffer containing 200 mM NaCl and then eluted with buffer containing 1 M NaCl (24 ml/min). Active fractions were pooled and further purified by repeated runs on a wide-pore analytical reversed-phase cyanopropyl column (4.6  $\times$  250 mm; Baker Research Products, Phillipsburg, NJ), which was eluted at 0.5 ml/min with a linear gradient of 0–50% acetonitrile in 0.1% trifluoroacetic acid (TFA) (0.83% increment per min). Active fractions were pooled, dried in a Speed-Vac centrifuge, resuspended in 0.1% TFA, and further purified on a C4 reversed-phase column (4.6  $\times$  250 mm; Baker Research Products), which was eluted at 0.5 ml/min with a linear gradient of 0–63% acetonitrile in 0.1% TFA (0.83% increment per min). Active fractions were rerun on a narrow-bore C4 column (2.1  $\times$  100 mm; 7  $\mu$ m, Brownlee/Applied Biosystems, Inc.) which was eluted at 0.3 ml/min with a linear gradient of 0–63% acetonitrile in 0.1% TFA (0.84% increment per min).

**Amino Acid Sequence Analysis.** This was performed with a gas phase sequencer (477A; Applied Biosystems, Inc.) (5). Cleavage at the Asp-Pro bond of purified, unmodified ENA-78 was performed in 75% (vol/vol) formic acid at 37°C for 60 h. The digestion mixture was then diluted four times with 0.1% TFA and loaded directly onto a narrow-bore reversed-phase C4 column as described above.

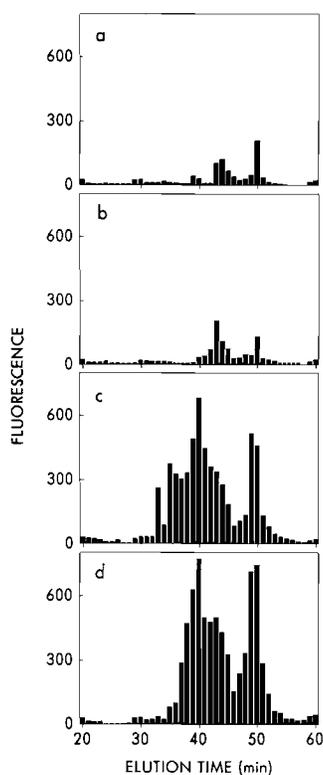
**Neutrophil Activation.** Established methods were used to assess elastase release (21) and cytosolic free calcium changes (24) in human neutrophils.

Neutrophil migration was determined in multiwell chemotaxis chambers (Neuro Probe, Cabin John, MD) (25). Dilutions of chemoattractants were loaded into the bottom wells in triplicates in prewarmed (37°C) MEM supplemented with 0.2% BSA, and the chambers (also prewarmed) were assembled with a 5- $\mu$ m pore diameter polyvinylpyrrolidone-free polycarbonate membrane (Nucleopore; Neuro Probe, Cabin John, MD). Freshly isolated human neutrophils were placed into the upper wells (25,000 cells per well in 50  $\mu$ l of MEM supplemented with 0.2% BSA) and chambers incubated at 37°C for 1 h in humidified air with 5% CO<sub>2</sub>. The polycarbonate filters were then removed and the cells on the upper surface were wiped off. After air drying, the filters were stained with DADE Diff-Quik (Merz & Dade AG, Düringen, Switzerland). Migrated cells were counted microscopically at 1,000 $\times$  magnification in five randomly chosen fields per well (0.03 mm<sup>2</sup>/field of 3.17 mm<sup>2</sup> total area per well). Each point in Fig. 6 represents the average of three independent experiments (15 fields per experiment).

## Results

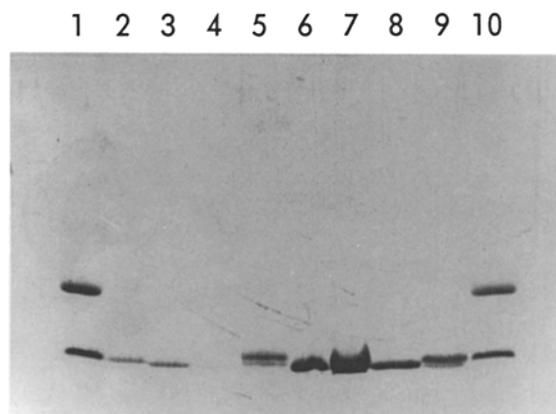
**ENA-78 and Other Neutrophil-activating Peptides.** As shown in Fig. 1, stimulation of pulmonary epithelial cells by TNF- $\alpha$  or IL-1 $\beta$  led to production and release of substantial amounts of neutrophil-activating peptides. On HPLC, the activity eluted into a broad peak with a retention time extending between 35 and 45 min, and a sharper peak eluting after 49 min. Only minor activity was recovered in the supernatant of unstimulated or LPS-stimulated cells.

For purification and sequence analysis, liter quantities of conditioned medium from IL-1 $\beta$ - and TNF- $\alpha$ -treated cultures were fractionated. HPLC on a CN-propyl column yielded three well-resolved peaks of activity (P1–P3) with mean reten-



**Figure 1.** Reversed-phase HPLC of neutrophil-activating peptides released by A549 cells. The cells were cultured for 24 h in medium without addition (a) or in the presence of 1 µg/ml LPS (b), 10 ng/ml IL-1β (c), or 20 ng/ml TNF-α (d). The culture supernatants were collected and fractionated by cation-exchange chromatography on phosphocellulose, followed by reversed-phase HPLC on a CN-propyl column. The graphs show neutrophil-stimulating activity (elastase release from cytochalasin B-treated human neutrophils) versus elution time on HPLC.

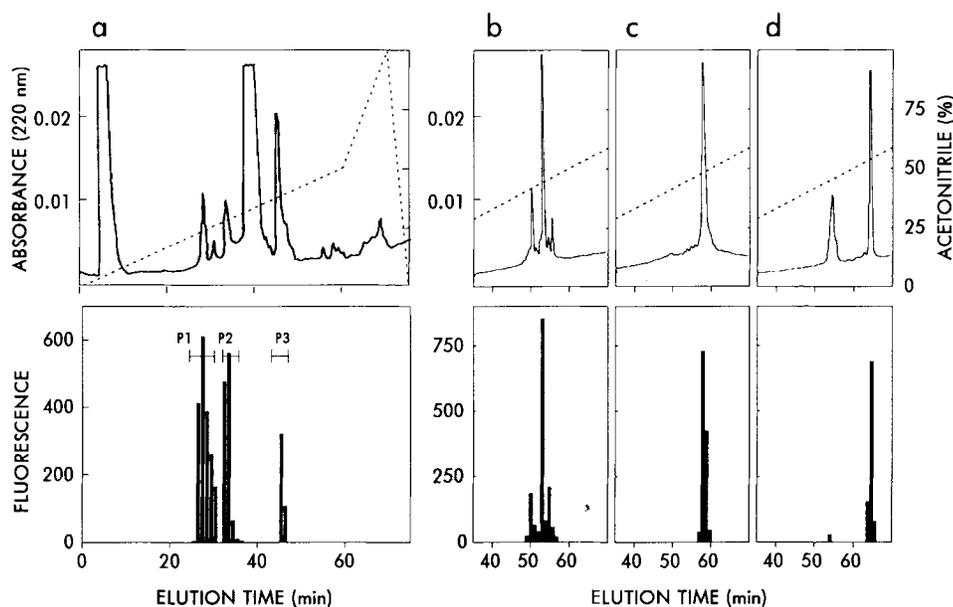
tion times of 29, 35.8, and 48.1 min (Fig. 2 a). The peak fractions were pooled, dried, and rechromatographed individually on a reversed-phase C4 column. Fraction P1 separated into three activity peaks with retention times 50.2, 53.3, and 55.5 min (Fig. 2 b). Urea-SDS-PAGE yielded single bands with apparent molecular weights corresponding to that of IL-8 (Fig. 3). All three preparations were subjected to Edman degradation. No defined sequence could be determined in the material eluting at 50.2 min due to the presence of more than one peptide species, while the materials eluting at 53.3



**Figure 3.** Urea-SDS-PAGE of purified neutrophil-activating peptides. Molecular mass markers aprotinin (6.5 kD) and lysozyme (14.4 kD) (lanes 1 and 10), active peptide from pool P1 (49.3-min peak) (lane 2), GROα (lane 3), GROγ (lane 4), IL-8 (77 aa) (lane 5), three preparations of ENA-78 (lanes 6-8), and recombinant IL-8 (lane 9).

and 55.5 min could be identified as GROα and GROγ, respectively, by sequencing the first 13 NH<sub>2</sub>-terminal residues (Fig. 4). Fraction P2 yielded one single peak with biological activity that could be identified as the 77-amino acid form of IL-8 (Fig. 4). The 72-amino acid form of IL-8, the major product of monocytes, was not detected in the lung epithelial cell supernatants. Fraction P3 contained a novel peptide with considerable sequence homology to NAP-2 and GROα, but clearly less homology to IL-8.

**Structure of ENA-78.** Three separate preparations of P3 were analyzed. On Urea-SDS-polyacrylamide gels, they all yielded a single band with slightly higher motility than IL-8 (Fig. 3). Sequencing led to the identification of 34 NH<sub>2</sub>-terminal and, after acid hydrolysis of the Asp57-Pro58 bond, 21 COOH-terminal residues (Fig. 4). The remaining 22 residues were identified indirectly using PCR methodology.



**Figure 2.** (a) Separation of neutrophil-stimulating activities from supernatants of IL-1β-stimulated A549 cells by reversed-phase HPLC on a CN-propyl column. Three pools of activity (P1-P3) were collected and purified separately by reversed-phase HPLC on an analytical C4 column: (b) pool P1, (c) pool P2, (d) pool P3.

ENA-78, NH<sub>2</sub>-terminus: AGPAAAVLRELCVCLQTTQGVHFKMISNLQVFA  
 ENA-78, COOH-terminus: DPEAPFLKKVIKLLDGGNKEN  
 GRO $\alpha$ : ASVATELRCQCLQ  
 GRO $\gamma$ : ASVVTELRCCQLQ  
 IL-8 (77): AVLPRSAKELRCQCIKTYSK

**Figure 4.** Amino acid sequences of neutrophil-activating peptides isolated from A549 cells. Underlined sequences were used for reversed translation and construction of sense and antisense primers for the first-strand synthesis and subsequent PCR.

The cDNA encoding amino acids 7–78 was amplified using sense and antisense primers and analyzed. PCR with antisense-primed cDNA yielded two prominent bands, the lower of which had the predicted size of ~235 bp (Fig. 5 *b*). PCR with random hexamer-primed cDNA yielded one single band of similar size (Fig. 5 *b*). The latter fragment was isolated from the gel and cloned into plasmid pTZ18R. Five clones were sequenced; three contained the correct sequence between the primers, and two were also identical in the primer region (Fig. 5 *a*).

These results show that ENA-78 consists of 78 amino acids. It has a molecular mass of 8,357 daltons and a calculated isoelectric point of 8.73. The position of the four cysteine residues is identical as in the sequence of IL-8, strongly suggesting that ENA-78 belongs to the CXC family of inflammatory peptides. The NH<sub>2</sub>-terminal sequence up to the first cysteine consists of 12 residues and approximately corresponds in length to that of the 77-residue form of IL-8 (see Fig. 9). No truncated variants of either peptide were detected. The sequence of ENA-78 contains two potential phosphorylation sites, one for casein kinase II at residue 40 (pqcSkve) and one for protein kinase C at residue 47 (vvaSlkn). No apparent sites for N-linked glycosylation are present. Sequence identities be-

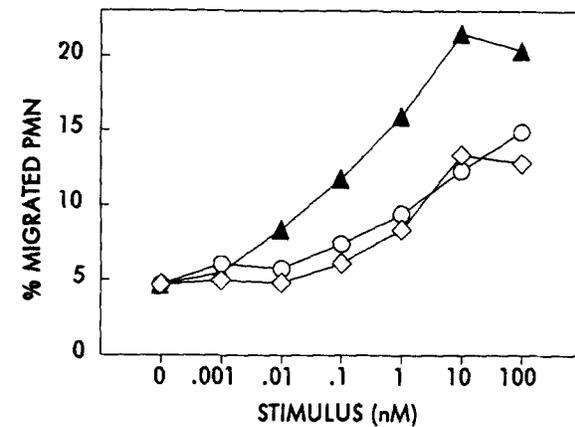
**a**

5' tcgaattcGTIYTISGIGAAGTISGITGYGTITG 3'  
 EcoRI  
 GTGTTGCGGGAAGTGCAGTGTGTTTACAGACCACGCAGGGAGTTCATCCC  
 ValLeuArgGluLeuArgCysValCysLeuGlnThrThrGlnGlyValHisPro

AAAATGATCAGTAATCTGCAAGTGTTCGCCATAGGCCACAGTGCTCCAAGGTGGAAGTG  
 LysMetIleSerAsnLeuGlnValPheAlaIleGlyProGlnCysSerLysValGluVal

GTAGCCTCCCTGAAGAACGGGAAGGAAATTTGTCTTGATCCAGAAGCCCTTTTCTAAAG  
 ValAlaSerLeuLysAsnGlyLysGluIleCysLeuAspProGluAlaProPheLeuLys

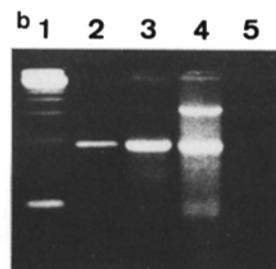
3' TADRAICTRCCICCTTRTYCTYTTI cctaggat 5'  
 BamHI  
 AAAGTCATCCAGAAAATCCTCGACGGCGGCAACAAAGAAAAC  
 LysValIleGlnLysIleLeuAspGlyGlyAsnLysGluAsn



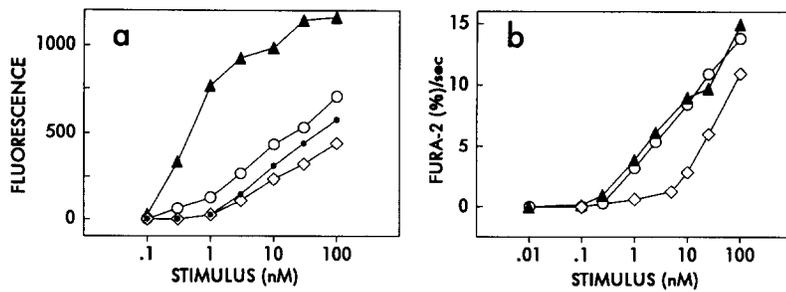
**Figure 6.** Human neutrophil chemotaxis induced by ELISA-78 ( $\diamond$ ), IL-8 ( $\blacktriangle$ ), and NAP-2 ( $\circ$ ). The response was calculated as the percent neutrophils of input number migrated to the lower side of the polycarbonate membrane. The results shown here are the mean of three independent experiments.

tween ENA-78 and NAP-2, GRO $\alpha$ , PF-4, IL-8, and  $\gamma$ IP-10 are 53, 52, 44, 22, and 15%, respectively.

**Biological Activities.** The effects of ENA-78 on neutrophils were studied in comparison with NAP-2 and IL-8. As shown in Fig. 6, ENA-78 induced a concentration-dependent migration response in vitro between 0.1 and 100 nM. NAP-2 was active in the same concentration range and showed a similar efficacy. The percentages of migrating cells at the maximum-effective concentration of 100 nM were 22, 15, and 13% for IL-8, NAP-2, and ENA-78, respectively (mean of three experiments). ENA-78 also induced the release of elastase from cytochalasin B-pretreated cells at concentrations ranging between 1 and 100 nM, as was the case for NAP-2 and IL-8.



**Figure 5.** (a) Partial amino acid sequence of ENA-78 as determined by oligonucleotide-primed synthesis of cDNA isolated from A549 cells. Mixtures of degenerated sense primer, encoding amino acids 7–17, and antisense primer, encoding amino acids 70–78, were used for the PCR. Nucleotide sequences of three individual cDNAs cloned in pTZ18R from the 226-bp band (see *b*). (I) Inosine; (R) A and G; (Y) C and T; (D) G, A, and T (S) A and C. (b) Gel electrophoretic analysis of the PCR products. Products of PCR amplifications were separated on a 5% NuSieve agarose gel and stained with ethidium bromide. Molecular size markers (lane 1), control PCR reaction, 234 bp (lane 2), PCR product obtained from A549 cDNA by random hexamer priming (lane 3), PCR product obtained from A549 cDNA by priming with antisense primer (lane 4), reaction mix without template (lane 5).



**Figure 7.** Effects of ENA-78 on human neutrophils. (a) Release of elastase from cytochalasin B-treated cells stimulated with two preparations of ENA-78 ( $\diamond$  and  $\bullet$ ), IL-8 ( $\blacktriangle$ ), and NAP-2 ( $\circ$ ). (b) Rate of cytosolic free calcium rise in cells stimulated with ENA-78 ( $\diamond$ ), IL-8 ( $\blacktriangle$ ), and NAP-2 ( $\circ$ ).

At 100 nM, IL-8 was consistently two- to threefold more effective than NAP-2 and ENA-78 (Fig. 7 a).

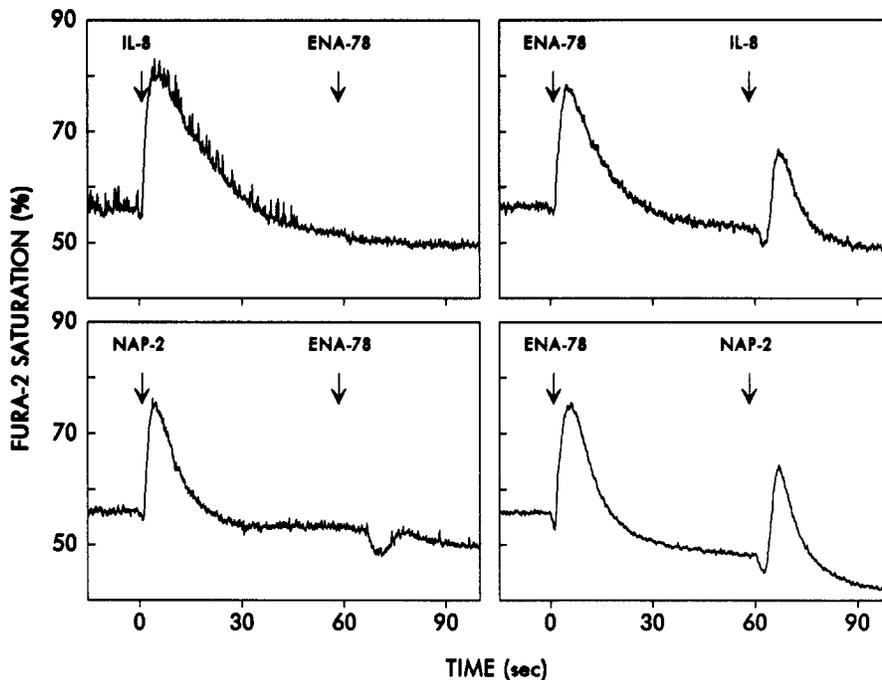
The somewhat lower potency of ENA-78 with respect to NAP-2 and IL-8 was also reflected by the measurements of stimulus-dependent changes in cytosolic free calcium. As illustrated in Fig. 7 b, the curve relating the rate of the calcium rise with the agonist concentration was virtually identical for IL-8 and NAP-2, and displaced to the right for ENA-78. Half-maximal rates were reached at ENA-78 concentrations that were  $\sim 10$  times higher than those required for the reference peptides.

$[Ca^{2+}]_i$  changes in response to sequential stimulations with IL-8, NAP-2, and ENA-78 were studied to explore the possibility of functional interactions among these related agonists. Desensitization of the neutrophils was always observed whenever the same agonist was applied twice at the same concentration, as reported previously for IL-8 (21, 26). The results obtained upon sequential stimulation with combinations of the three agonists are shown in Fig. 8. A first stimulation with 100 nM ENA-78 decreased only slightly the  $[Ca^{2+}]_i$  rise induced by the subsequent stimulation with

100 nM IL-8 or NAP-2. By contrast, prestimulation with 100 nM IL-8 or NAP-2 abolished the response to the subsequent challenge with 100 nM ENA-78. These results suggest that ENA-78 activates neutrophils via the same or a closely related receptor system as IL-8 and analogues.

### Discussion

A novel neutrophil-activating peptide was identified in the conditioned medium of the human type II epithelial cell line A549. The peptide was named ENA-78 to indicate its cellular origin, its neutrophil-activating properties, and its total number of amino acids. ENA-78 has considerable sequence identity with NAP-2 and  $GRO\alpha$ , and somewhat less with IL-8. It shares with these peptides and other analogues four conserved cysteine residues, therefore, it qualifies as a new member of the CXC family of inflammatory peptides. ENA-78 is a potent neutrophil activator with similar biological properties as demonstrated for IL-8, NAP-2, and  $GRO\alpha$ . It induces cytosolic free calcium changes, chemotaxis, and exocytosis at threshold concentrations ranging between 0.1 and



**Figure 8.** Cytosolic free calcium changes in response to sequential stimulation of human neutrophils with combinations of ENA-78, IL-8, and NAP-2 at 100 nM.

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ENA-78      A G P A A A V L R E L R C V C L Q T T Q - G V H P K M I S N L Q
IL-8        S A K E L R C Q C I K T Y S K P F H P K F I K E L R
NAP-2       A E L R C M C I K T T S - G I H P K N I Q S L E
GRO $\alpha$       A S V A T E L R C Q C L Q T L Q - G I H P K N I Q S V N
GRO $\beta$        . P L . . . . . L . . . . . K
GRO $\gamma$       . . . V . . . . . L . . . . .
PF-4        E A E E D G D L Q C L C V K T T S - Q V R P R H I T S L E
 $\gamma$ IP-10    V P L S R T V R C T C I S I N Q - P V N P R S L E K L E

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ENA-78      V F A I G P Q C S K V E V V A S L K N - G K E I C L D P E A P F L K K V I Q K I L D G G N K E N
IL-8        V I E S G P H C A N T E I I V K L S D - G R E L C L D P K E N W V Q R V V E K F L K R A E N S
NAP-2       V I G K G T H C N Q V E V I A T L K D - G R K I C L D P D A P R I K K I V Q K K L A G D E S A D
GRO $\alpha$       V K S P G P H C A Q T E V I A T L K N - G R K A C L N P A S P I V K K I I E K M L N S D K S N
GRO $\beta$        . . . . . Q . . . . . M . . . . . K N G . . .
GRO $\gamma$       . R . . . . . K . . . . . M . Q . . . . . I . . K G S T .
PF-4        V I K A G P H C P T A Q L I A T L K N - G R K I C L D L Q A P L Y K K I I K K L L E S
 $\gamma$ IP-10    I I P A S Q F C P R V E I I A T M K K K G E K R C L N P E S K A I K N L L K A V S K E M S K R S P

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**Figure 9.** Amino acid sequences of ENA-78 and related peptides aligned according to their four cysteine residues (*bold*). For GRO $\beta$  and GRO $\gamma$ , only amino acids that differ from GRO $\alpha$  are shown.

3 nM. Crossdesensitization studies indicate that ENA-78, IL-8, and NAP-2 act on neutrophils via the same or a related receptors.

During the last few years, a superfamily of 8–10-kD inflammatory cytokines with four characteristic cysteine residues has been recognized. On the basis of the arrangement of the first two cysteines, which can be adjacent (CC) or separated by one amino acid (CXC), and the chromosomal location of their genes, two subfamilies can be distinguished. The CC subfamily includes, among other peptides, the monocyte chemotactic protein 1 (MCP-1), RANTES, and I-309, which were shown to be chemotactic for monocytes (27). In addition, RANTES was reported to attract certain populations of lymphocytes (28). No member of the CC subfamily has been reported to stimulate neutrophils. Two murine homologues, however, MIP-1 $\alpha$  and MIP-1 $\beta$ , have been shown to induce neutrophil infiltration in vivo (29, 30). As already pointed out, the newly discovered peptide ENA-78 belongs to the CXC subfamily, which includes IL-8, NAP-2, and three forms of GRO (31) (Fig. 9). It was recently shown that GRO $\alpha$ , GRO $\beta$ , and GRO $\gamma$  are encoded by related, but distinct genes (32), and that GRO $\beta$  and GRO $\gamma$  are the human homologues of the murine inflammatory proteins MIP-2 $\beta$  and MIP-2 $\alpha$ , respectively (33).

The present study revealed an unusual versatility of A549 cells that produced, in addition to ENA-78, the 77-amino acid form of IL-8 as well as GRO $\alpha$ , GRO $\gamma$ , and a minor unidentified peptide, which on the basis of its HPLC elution profile and physicochemical properties appears to be closely related to GRO $\beta$  (MIP-2 $\alpha$ ). Unstimulated A549 cells constitutively released small amounts of ENA-78 and IL-8. The release of these factors was not increased by LPS stimulation. However, IL-1 $\beta$  and TNF- $\alpha$ , two proinflammatory cytokines that induce the production of IL-8 in a variety of cells (4), led to a massive release of ENA-78, the GRO peptides, and

IL-8 in A549 cells. Stimulus dependency is in agreement with recent observations by Standiford et al. (18) that the levels of IL-8 mRNA in A549 cells are low in the absence of stimulation or in the presence of LPS, and high upon exposure to IL-1 $\beta$  or TNF- $\alpha$ . IL-8 was recovered exclusively as the 77-amino acid form, which is obtained in minor amounts in monocyte cultures (34), and was the most abundant variant in cultures of endothelial cells stimulated with IL-1 $\beta$  (35). By analogy to the 77–72-amino acid truncation of IL-8, resulting in enhanced activity, it is possible that NH<sub>2</sub>-terminal processing of ENA-78 is also required for maximum activity.

Several acute and chronic lung disorders are characterized by the presence of high numbers of neutrophils. Enhanced chemotactic activity for neutrophils has been observed in the bronchoalveolar lavage fluids of patients with adult respiratory distress syndrome (36), idiopathic pulmonary fibrosis (15), and asbestosis (37). Elevated levels of elastase were observed as well, indicating the presence of activated neutrophils (38). Up to now, IL-8 was the only peptide to be considered as mediator of neutrophil recruitment in the lung (16–18). The present experiments demonstrate the potential involvement of several IL-8 analogues. Since A549 cells have retained distinguishing properties of type II alveolar epithelial cells, such as the characteristic multilamellar inclusion bodies and the capacity to produce surfactant (39), it must be assumed that the concomitant production of several members of the IL-8 family of peptides may also occur in vivo within the alveolar space. Epithelial cells, which also produce monocyte chemotactic peptide 1 (40), may thus function as second-stage producers of leukocyte attractants in response to proinflammatory cytokines released from alveolar macrophages. Such a multipotency possibly denotes an important role in directing neutrophils and monocytes to the alveolus during inflammatory states.

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