

## **Phagocytosing Neutrophils Produce and Release High Amounts of the Neutrophil-activating Peptide 1/Interleukin 8**

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### **Summary**

After phagocytosis of yeast opsonized with IgG, neutrophil leukocytes (polymorphonuclear leukocytes [PMN]) expressed high levels of neutrophil-activating peptide 1/interleukin 8 (NAP-1/IL-8) mRNA, which peaked after 3–5 h and were still elevated after 18 h. A similar but quantitatively less prominent effect was obtained with lipopolysaccharide (LPS). After phagocytosis, but not after exposure to LPS, the PMN progressively released considerable amounts of NAP-1/IL-8 into the culture medium (18.6–50 ng/ml in 18 h). The peptide released was biologically active, as indicated by the transient elevation of cytosolic-free calcium in PMN exposed to aliquots of the culture supernatants, and desensitization by prestimulation of the cells with recombinant NAP-1/IL-8. By producing NAP-1/IL-8 at sites where they phagocytose invading microorganisms, PMN could enhance the recruitment of new defense cells.

Two main responses of neutrophil leukocytes (PMN) are observed upon microbial invasion: migration from the blood stream to the site of infection and phagocytosis of the microorganisms. Several chemotactic agonists with different chemical structure, origin, and receptor specificity induce PMN migration. They include the anaphylatoxin C5a, *N*-formyl-methionyl peptides, leukotriene B<sub>4</sub> (LTB<sub>4</sub>), platelet-activating factor (PAF), and the recently identified chemotactic cytokine, neutrophil-activating peptide 1/interleukin 8 (NAP-1/IL-8) (1, 2). NAP-1/IL-8 differs from the classical chemotactic agonists in two important aspects: it is produced by mononuclear phagocytes and a wide variety of tissues cells, and it is highly selective for PMN (2, 3). These features indicate that NAP-1/IL-8 is a major mediator of PMN infiltration in diseased tissues (2).

Phagocytosis is the ultimate stimulus for PMN leading to abundant release of granule contents (4), massive production of superoxide and other toxic oxygen metabolites (5), and formation of LTB<sub>4</sub> (6) and PAF (7). Since PMN, despite their high degree of specialization, retain the ability to synthesize proteins (8, 9, 9a), we have investigated whether they produce NAPs. We report here that phagocytosing PMN express high levels of NAP-1/IL-8 mRNA and release considerable amounts of the mature peptide, suggesting that they may, in this way, promote the influx of new defense cells.

### **Materials and Methods**

**Cell Purification and Culture.** PMN were isolated by two consecutive Ficoll-Hypaque density gradient centrifugations to minimize contamination with monocytes (10). Monocytes were purified from the PBMC by adherence to plastic for 1 h (10). As assessed by Wright staining and unspecific esterase cytochemistry, PMN preparations contained <1% monocytes, and the purity of the monocyte preparations was on average 90%. PMN (10<sup>7</sup>/ml) and monocytes (1–2 × 10<sup>6</sup>/ml) were cultured in polystyrene flasks (37°C, 5% CO<sub>2</sub>) for up to 18 h in RPMI 1640 containing antibiotics and 10% FCS. The cells were stimulated at time zero with heat-killed yeast opsonized with IgG (11) (Y-IgG; particle/cell ratio of 2:1), or *Escherichia coli* LPS (1 μg/ml). Cell viability after 18 h in culture was >98% for both PMN and monocytes as assessed by trypan blue exclusion. All solutions were prepared with endotoxin-free water for clinical use (10).

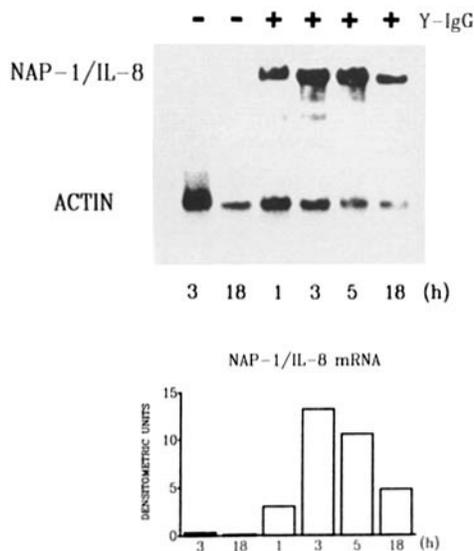
**RNA Isolation and Northern Blot Analysis.** Total RNA was purified from samples of 8 × 10<sup>7</sup> PMN or 5 × 10<sup>6</sup> monocytes by the guanidinium isothiocyanate/cesium chloride method (12), size separated by agarose gel electrophoresis, blotted onto a nylon membrane (Schleicher & Schuell, Inc., Keene, NH), and hybridized with a radiolabeled 0.7-kb EcoRI fragment prepared from a cDNA for human NAP-1/IL-8 kindly provided by Dr. I. Lindley (13). RNA loading of the gels was assessed with phosphoglycerate kinase (PGK) or β-actin cDNA. Several exposures of each autoradiograph were quantified by laser densitometry (ultrascan XS; LKB Instruments, Inc., Gaithersburg, MD) to ensure that the measurements were within a linear range of signal intensity.

**NAP-1/IL-8 Assay.** NAP-1/IL-8 was determined using a double-ligand ELISA method in cell-free supernatants of PMN or monocyte cultures stored at  $-70^{\circ}\text{C}$ . Samples and recombinant NAP-1/IL-8 standards (0.02–10 ng/ml) (13) were incubated for 2 h at  $37^{\circ}\text{C}$  in microtiter plates coated with a mouse anti-NAP-1/IL-8 mAb. After washing, a goat anti-NAP-1/IL-8 mAb conjugated to alkaline phosphatase was added, and finally, the activity was determined with *p*-nitrophenylphosphate.

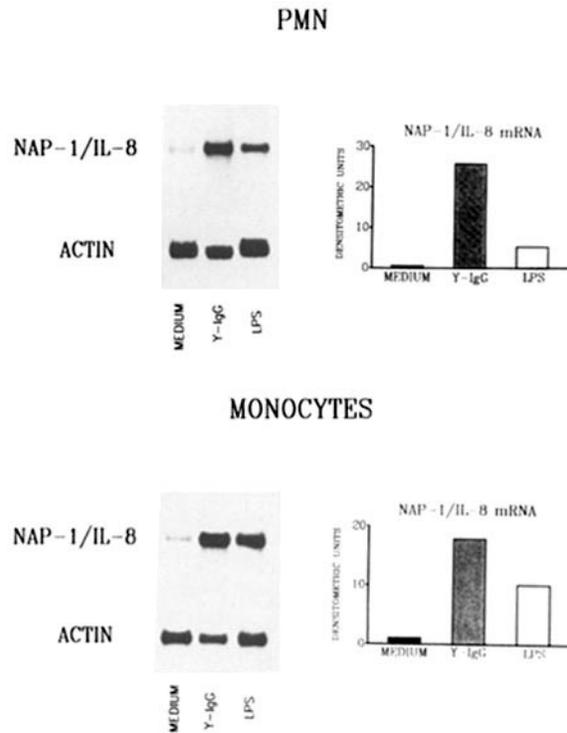
**Cytosolic-free  $\text{Ca}^{2+}$  Measurements.** Neutrophils were loaded with Fura-2 for 20 min at  $37^{\circ}\text{C}$  (0.2 nmol Fura-2-AM/ $10^6$  cells), washed, and resuspended in a buffer containing 136 mM NaCl, 4.8 mM KCl, 1 mM  $\text{CaCl}_2$ , 5 mM glucose, and 20 mM Hepes, adjusted to pH 7.4.  $[\text{Ca}^{2+}]_i$  changes were induced by addition of 10–50- $\mu\text{l}$  samples of the culture supernatants or standard solutions of recombinant NAP-1/IL-8 or fMet-Leu-Phe, and monitored as described (14).

## Results

PMN were exposed to Y-IgG or LPS, and the production of NAP-1/IL-8 was studied after different periods of time. When PMN were cultured in the presence of Y-IgG, phagocytosis proceeded rapidly, and after 45 min, the phagocytic index was  $117 \pm 13$  (number of particles per 100 PMN; mean  $\pm$  SD of three experiments performed with PMN from different donors). The expression of NAP-1/IL-8 mRNA in such cells is shown by the Northern blot analysis presented in Fig. 1. Phagocytosis of Y-IgG induced a significant, time-dependent increase in the level of the specific mRNA, which was already evident 1 h after addition of the particles. The levels reached a maximum after 3–5 h and were still elevated after 18 h. A similar time course of NAP-1/IL-8 mRNA en-



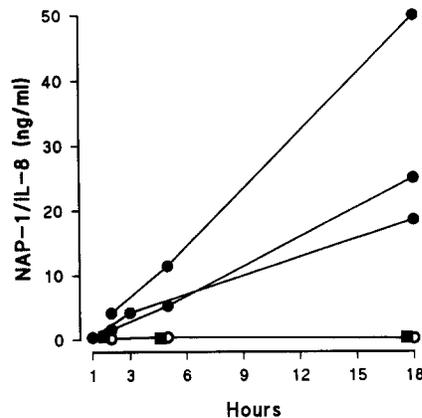
**Figure 1.** NAP-1/IL-8 mRNA expression in human PMN after phagocytosis of opsonized yeast (Y-IgG). PMN were cultured for up to 18 h in the presence (+) or absence (-) of Y-IgG, and mRNA was prepared at different times. (Top) Northern blots; 5  $\mu\text{g}$  of total RNA was loaded on each lane. (Bottom) Densitometry of the autoradiographs (filled columns, controls; open columns, Y-IgG). The values were normalized on the basis of hybridization to actin mRNA. Data are representative for three experiments performed under the same conditions with different neutrophil preparations.



**Figure 2.** NAP-1/IL-8 mRNA expression in human PMN and monocytes in control medium and after phagocytosis of opsonized yeast (Y-IgG) or exposure to LPS. mRNA was prepared after 6 h of culture, and Northern blot analysis was performed under identical conditions for PMN and monocytes. 6  $\mu\text{g}$  of total RNA was loaded on each lane. Densitometric evaluation (right) is as in Fig. 1. PMN and monocytes were prepared from the same donor. Similar results were obtained in two additional experiments.

hancement was observed when the cells were exposed to 1  $\mu\text{g}/\text{ml}$  LPS (data not shown). In most experiments, control PMN expressed small amounts of NAP-1/IL-8 mRNA.

Since monocytes are the main producers of NAP-1/IL-8



**Figure 3.** NAP-1/IL-8 release by human PMN cultured for up to 18 h in control medium (O) or in the presence of Y-IgG (●) or LPS (■). Data from three different PMN preparations exposed to Y-IgG are shown together with data from one preparation exposed to LPS or medium alone.

(2, 3), mRNA expression was compared in PMN and monocytes purified from the same donor and stimulated with Y-IgG or LPS under identical conditions. The results of a representative experiment are shown in Fig. 2. In both types of phagocytes, NAP-1/IL-8 mRNA transcripts were already detected under control conditions and the expression was greatly enhanced in response to either stimulus. As revealed by the densitometric evaluation corrected on the basis of actin mRNA expression, PMN and monocytes responded to similar extents to Y-IgG. The effect of LPS, by contrast, was markedly lower in PMN than in monocytes. Expression in PMN challenged with yeast particles was not altered by polymyxin B (5 µg/ml), indicating that the enhancement was due to phagocytosis and not to contamination with LPS (data not shown).

In subsequent experiments, we determined whether NAP-1/IL-8 mRNA was translated and the peptide secreted. As shown in Fig. 3, PMN stimulated with Y-IgG progressively released considerable amounts of immunoreactive NAP-1/IL-8 into the culture medium, reaching concentrations of 18.6–50 ng/ml in 18 h ( $34.3 \pm 14.9$ , mean  $\pm$  SD,  $n = 4$ ). By contrast, NAP-1/IL-8 release by PMN exposed to LPS for the same period was  $0.6 \pm 0.4$  ng/ml ( $n = 3$ ), and thus exceeded only slightly the values observed in control cultures (0.08–0.2 ng/ml). The release of NAP-1/IL-8 in monocyte cultures was considerably higher; on a per cell basis, monocytes stimulated with either LPS or Y-IgG produced 30–40-fold more NAP-1/IL-8 than Y-IgG-stimulated PMN. The marked difference in the production of NAP-1/IL-8 by PMN challenges with Y-IgG and LPS indicates that the NAP-1/IL-8 recovered in the cultures was not due to production by contaminating monocytes, which responded equally well to either stimulus.

The biological activity of NAP-1/IL-8 in the culture supernatants of PMN that had phagocytosed Y-IgG was assessed by measuring  $[Ca^{2+}]_i$  changes in PMN suspensions after loading with Fura-2. Supernatant samples induced a rapid and transient rise in  $[Ca^{2+}]_i$  that was qualitatively similar to that observed with recombinant NAP-1/IL-8 or fMet-Leu-Phe. In addition, the  $[Ca^{2+}]_i$  changes elicited by the supernatants were prevented or markedly attenuated by prestimulation (and desensitization) of the PMN with recombinant

NAP-1/IL-8. For desensitization, recombinant NAP-1/IL-8 was added to the cells 1 min before the supernatant sample to be tested.

## Discussion

This study shows that circulating, mature human neutrophils are capable of producing and releasing considerable amounts of NAP-1/IL-8. The induction of NAP-1/IL-8 mRNA was observed upon exposure of the cells to stimuli that they commonly encounter at sites of infection, endotoxin and opsonized particles, while the release of the NAP was largely restricted to PMN that had phagocytosed.

Since mononuclear phagocytes produce large amounts of NAP-1/IL-8 (2), it was important to exclude that monocyte contamination could account for the release of the peptide attributed to PMN. Multiple evidence argues against this possibility. (a) The purity of the PMN preparations used was at least 99%. (b) Northern blot analysis of equal amounts of total RNA extracted from PMN and monocyte preparations stimulated with Y-IgG yielded NAP-1/IL-8 mRNA bands of similar intensity (Fig. 2), indicating that expression occurred in both cells. (c) Total release of NAP-1/IL-8 in PMN cultures was 100–200-fold higher after Y-IgG phagocytosis than after exposure to LPS (Fig. 3), whereas in monocytes the release was similar with both stimuli. Additional evidence for the purity of the PMN population used stems from parallel studies of the expression of IL-6 mRNA, which was readily detectable in monocyte but not in PMN preparations (9a).

PMN have long been known to generate bioactive lipids, such as  $LTB_4$  and PAF, that act as chemotactic agonists, and may function as amplifiers of the defense response (6, 7).  $LTB_4$  and PAF are generated within seconds to minutes of stimulation, and are, therefore, early acting agents. The present demonstration of production and release of NAP-1/IL-8 after phagocytosis confers more strength to the notion that PMN have the ability to enhance antimicrobial defense by recruiting new cells. In contrast to the lipid agonists, which are short lived, NAP-1/IL-8 is known to persist in active form for long periods in the tissues (2), and its action is thus likely to be protracted.

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