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Human Neutrophil Defensins Increase Neutrophil Uptake of Influenza A Virus and Bacteria and Modify Virus-Induced Respiratory Burst Responses

Tefaldet Tele,* Mitchell R. White,* Don Gantz,† Erika C. Crouch,‡ and Kevan L. Hartshorn†

Human neutrophil peptides (HNPs) are released from granules of neutrophils in response to various activating stimuli and they participate in the killing of bacteria and the stimulation of various inflammatory responses. HNPs also inhibit infectivity of enveloped viruses, including influenza A virus (IAV). In this study, we demonstrate that HNPs increase the uptake of IAV and bacteria by neutrophils. The dimeric HNPs also induced aggregation of IAV and bacterial particles, which may, in part, explain their ability to increase uptake. HNPs did not increase neutrophil respiratory burst responses to IAV. We have recently demonstrated direct interactions of HNPs with surfactant protein D (SP-D), another important effector of innate immunity and antimicrobial host defense. Although HNPs did not alter SP-D-dependent uptake of IAV, they counteracted the ability of SP-D to increase IAV-induced neutrophil H$_2$O$_2$ generation. Our studies reveal previously unappreciated functional effects of HNPs, expand our understanding of the antiviral properties of HNPs, and suggest important interactions between collectins and HNPs in the host response to viruses and bacteria.


Abbreviations used in this paper: IAV, influenza A virus; HNP, human neutrophil peptide; SP-D, surfactant protein D; HD5, human defensin 5; HBD, human β-defensin; RHSP-D, recombinant human SP-D; BALF, bronchoalveolar lavage fluid.

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Influenza A virus (IAV) infections are a major cause of morbidity and mortality, causing ∼40,000 deaths per year in the United States (1). Innate immune mechanisms provide important protection against IAV in the naive host. In this study, we evaluate three important innate defense mediators, neutrophils, human neutrophil peptides (HNP), and surfactant protein D (SP-D), with IAV. Neutrophils are recruited to the respiratory tract early in the course of IAV infection (2–4). Highly pathogenic IAV strains elicit more profound neutrophil influx (3, 5). Recent studies with the 1918 pandemic IAV strain demonstrate markedly increased neutrophil influx in the lungs of mice infected with this strain, as compared to the infection with other recent human strains. In this model, depletion of neutrophils before infection reduced survival. Hence, in this case, neutrophils clearly play a protective role. Other studies have shown both protective and adverse effects of neutrophil infiltration during IAV infection (4, 6).

Neutrophils bind to, and take up, IAV in the absence of Abs, and IAV stimulates various activation signals and H$_2$O$_2$ generation by neutrophils. In addition, various functional properties of neutrophils are impaired during IAV infection or after incubation with IAV in vitro. This dysfunction could contribute to bacterial superinfection, which is a major cause of morbidity and mortality during IAV epidemics (7, 8).

HNP may contribute to host defenses against IAV. Defensins have antimicrobial activity against a variety of organisms, such as bacteria, fungi, and enveloped viruses, including IAV and HIV, among others (9, 10). In humans, α-defensins are stored and released in large quantities, from neutrophil granules during infectious and inflammatory states (11, 12) and are referred to as HNP 1, 2, or 3. HNPs are basic peptides 29–30 aa in length. The peptides have a β-sheet-rich fold and six disulfide-linked cysteines, resulting in a spatial segregation of charged and hydrophobic residues. It has been proposed that this amphiphilic structure could permit defensins to insert into the membranes of target organisms. In addition to their direct antimicrobial activities, defensins have a variety of other activities relevant to innate or adaptive immunity. Defensins have chemotactic activity for monocytes, T cells, and dendritic cells; stimulate IL-8 production by epithelial cells; bind to lipid bilayers; and contribute to respiratory epithelial cell activation, injury, or growth under some circumstances (13–19).

SP-D plays particularly important roles in restricting IAV replication and limiting the severity of inflammatory responses during the first several days of infection (3, 20–25). SP-D directly inhibits the infectivity of IAV and also modulates the interaction of IAV with neutrophils. SP-D promotes the uptake of IAV by neutrophils, and can either increase or reduce respiratory burst responses of neutrophils upon exposure to IAV, depending on whether SP-D is first incubated with IAV or neutrophils (26). SP-D also protects neutrophils against IAV-induced neutrophil dysfunction (20, 21).

We have recently shown that HNPs bind to SP-D and significantly modify its interactions with IAV (27). In this study, we show that HNPs promote IAV and bacterial uptake by neutrophils and modify neutrophil respiratory burst responses to IAV.

Materials and Methods

Buffers

Dulbecco’s PBS containing 0.9 mM calcium and 0.493 mM magnesium and PBS without calcium and magnesium were purchased from Invitrogen Life Technologies. PBS with added calcium and magnesium (pH 7.2) was used, unless otherwise indicated.
Virus and bacterial preparations

IAV was grown in the chorioallantoic fluid of 10-day-old chicken eggs and purified on a discontinuous sucrose gradient as previously described (28). The virus was dialyzed against PBS to remove sucrose, aliquoted, and stored at \(-80^\circ\text{C}\) until needed. Philippines 82/H3N2 (Phil82) strain was provided by Dr. E. M. Anders (University of Melbourne, Melbourne, Australia). The A/PR/8/34/H1N1 (PR-8) strain was a gift from Dr. J. Abramson (Bowman Gray School of Medicine, Winston-Salem, NC). The hemagglutinin titer of each virus preparation was determined by titration of virus samples in PBS with thoroughly washed human type O, Rh- RBC as described previously (28). After thawing, the viral stocks contained \(5 \times 10^8\) PFU/ml. Labeled Escherichia coli and Staphylococcus aureus were purchased from Molecular Probes.

Defensin and SP-D preparations

HNP1 and HNP2 were purchased from Bachem. HNP3, human defensin 5 (HD5), human \(\beta\)-defensin (HBD) 1, and human \(\beta\)-defensin 2 (HBD2) were purchased from the Peptide Institute. Recombinant human SP-D (RhSP-D) was produced in stably transfected Chinese hamster ovary-K1 cells as previously described (22). For these studies, the dodecameric fraction of RhSP-D was used. The SP-D and HNP preparations used in this study were tested for the degree of contamination with endotoxin using a quantitative endotoxin assay (Limulus amebocyte lysate; BioWhittaker). The levels of endotoxin in the HNP preparations were 15 and 25 pg/\(\mu\)g of protein, respectively, for HNP1 and HNP2. The levels of endotoxin in SP-D preparations were \(-2\) pg/ml. Normal volunteer donor bronchoalveolar lavage fluid (BALF) was obtained under a protocol approved by the Boston University Medical Center Institutional Review Board.

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**FIGURE 1.** HNPs increase the neutrophil uptake of IAV. Neutrophil uptake of FITC-labeled IAV was measured by flow cytometry. A, IAV was preincubated with the indicated concentrations of HNP1 or HNP2, and aliquots of these samples were added to neutrophils. Both HNPs caused significant increases in the uptake of IAV by neutrophils \((p < 0.005\) for 20 and 40 \(\mu\)g/ml HNPs compared with control; \(n = 6\) experiments using separate blood donors). B, Neutrophils were preincubated with HNPs, followed by washing and addition of IAV. Using this method, HNPs still significantly increased the uptake of IAV \((p\) values as indicated); however, the increase in uptake was significantly less than when IAV was preincubated with the HNPs \((p < 0.05\) by ANOVA). C, The ability of \(\beta\)-defensins 1 and 2, HNP3, and HD5 were tested in a similar manner as in A \((i.e.,\) preincubation of defensins with the virus before adding to neutrophils). As shown, HD5 increased viral uptake, while BD1 and HNP3 did not, and BD2 caused a slightly increased uptake at the highest concentration tested \((p\) values are shown).

**FIGURE 2.** HNPs increase the uptake of IAV by adherent neutrophils. Neutrophils were allowed to adhere to plastic slides followed by washing and incubation with either IAV alone or IAV that had been preincubated with either HNP1 or HNP2. Left, Fluorescent microscopy; right, same fields in phase-contrast microscopy. Increased neutrophil-associated fluorescence was clearly evident in samples treated with HNPs. Results are representative of four similar experiments.
Measurement of aggregation of viral and bacterial particles

Aggregation of IAV particles was assessed following the addition of various concentrations of collectins by monitoring changes in light transmission on a SLM/Aminco 8000C (SLM Instrument) spectrofluorometer as described elsewhere (29). The aggregation of viral particles is demonstrated by a decline in light transmission (i.e., increased turbidity). Bacterial aggregation was measured by a similar method, as previously described (30). However, in the case of bacteria, aggregation is indicated by increased light transmission through the suspension as the bacteria sediment. In addition, viral and bacterial aggregation were assessed by fluorescence microscopy as described previously (30).

To demonstrate aggregation by electron microscopy, Phil82 virus was preincubated with HNPs 1 or 2 (or control buffer) under the identical conditions used in the neutrophil uptake experiments (i.e., same concentrations of virus and HNP for the same time). These samples were then allowed to adhere to glow-discharged (31), carbon-coated copper grids for 30 min at room temperature, fixed with 2.5% glutaraldehyde for 5 min, and counterstained with 1% sodium phosphotungstate (pH 7.4) (32). Micrographs were recorded on Kodak SO163 film in a CM12 transmission electron microscope (Philips). Images were developed in D19, digitized on a CreoScitex EverSmart Supreme Scanner, and assembled in Adobe Photoshop.

**FIGURE 3.** HNPs promote the neutrophil-mediated clearance of IAV from culture supernatant. IAV was incubated with HNPs or neutrophils alone or the combination of HNPs and neutrophils, followed by low-speed centrifugation and determination of viral titers in cell-free supernatant by hemagglutinin assay. Results are mean ± SEM of five experiments. HNP1 alone significantly reduced viral titers in the supernatant (p < 0.001 vs control); however, HNP1 and neutrophils together caused a significantly greater reduction in viral titers (p < 0.01 for HNP1 alone vs HNP1 + neutrophils).

**FIGURE 4.** HNPs cause aggregation of IAV particles. Aggregation of viral particles (Phil82 or PR-8 strain) was tested using a sensitive assay of light transmission through stirred viral suspensions (upper, n = 5 for Phil82; n = 2 for PR-8) and by examination under fluorescent microscopy (lower, rhodamine-labeled Phil82 IAV; representative of four experiments).

**FIGURE 5.** Viral aggregation by HNPs as demonstrated by electron microscopy. Representative electron micrographs from three separate experiments are shown. Exposures were taken at ×8,000 and ×60,000 of control virus (Phil82 strain) or virus that was pretreated with 40 μg/ml HNP1 or HNP2 in the identical manner as in Fig. 1.
Neutrophil preparation

Neutrophils from healthy volunteers were isolated to >95% purity by using dextran precipitation followed by Ficoll-Paque gradient separation for the removal of mononuclear cells and then hypotonic lysis to eliminate any contaminating erythrocytes, as previously described (28). Cell viability was determined to be >98% by trypan blue staining. The isolated neutrophils were resuspended at the appropriate concentrations in control buffer (PBS) and used within 2 h.

Measurement of IAV and bacterial uptake by neutrophils

During flow cytometry on neutrophils in suspension, FITC-labeled IAV (Phil82 strain) was prepared as described previously (29). Uptake of the virus by neutrophils was measured as previously described (23). In brief, IAV was preincubated with control buffer or various concentrations of collectins for 30 min at 37°C to test the effect of collectin on IAV binding, followed by incubation of aliquots of these samples with neutrophils for 30 min at 37°C. Trypan blue (0.2 mg/ml) was added to these samples to quench extracellular fluorescence. After washing, the neutrophils were fixed with paraformaldehyde, and neutrophil-associated fluorescence was measured using flow cytometry. The mean neutrophil fluorescence (>1000 cells counted per sample) was measured.

Measurement of neutrophil \( \text{H}_2\text{O}_2 \) production

\( \text{H}_2\text{O}_2 \) production was measured by assessing the reduction in scopoletin fluorescence as previously described (33).

Statistics

Statistical comparisons were made using a Student’s paired, two-tailed \( t \) test or ANOVA with post hoc test (Tukey’s).

Results

HNP1 and HNP2 and HD5 increase neutrophil uptake of IAV

SP-D increases the neutrophil uptake of IAV (20) and bacteria (30). Initially, we planned to determine whether this activity of SP-D is altered by HNP. Unexpectedly, we found that HNP1 and HNP2 alone strongly increase the neutrophil uptake of IAV (Fig. 1A). HNP1 and HNP2 caused very similar increases in the uptake of IAV, with peak effects at 20 – 40 \( \mu \text{g/ml} \). We tested the effect of preincubating neutrophils with HNP1 and HNP2 on the subsequent uptake of IAV (Fig. 1B). Neutrophils were preincubated with 50 \( \mu \text{g/ml} \) HNP1 or HNP2 for 30 min at 37°C, followed by the washing and addition of FITC-labeled IAV. HNP1 and HNP2 again significantly increased viral uptake (Fig. 1, lower panel); however, the degree of increase in uptake was less pronounced in these experiments than when the virus was preincubated with HNPs. We also found that the preincubation of IAV with HNPs increases uptake of IAV by plastic-adherent neutrophils (Fig. 2).

We have previously reported that the incubation of IAV with human BALF obtained from normal volunteers modestly, but significantly, increases the neutrophil uptake of the virus (26). To determine whether HNPs can further increase the viral uptake caused by BALF, we added HNP2 to the fluid and measured the neutrophil uptake of IAV. BALF alone increased the uptake of fluorescent IAV to 120 \( \pm \) 5 (\( p < 0.03 \) compared with control; \( n = 4 \)). If IAV was incubated with bronchoalveolar lavage and HNP2 simultaneously, uptake was increased to a much greater extent (i.e., 300 \( \pm \) 37% of control; \( p < 0.029 \) vs BALF alone). Hence, HNPs retain their ability to increase neutrophil uptake of IAV in the presence of BALF.

To determine whether other defensins have a similar ability to increase the neutrophil uptake of IAV, we preincubated Phil82...
IAV with HNP3, HD5, HBD1, or HBD2 and tested uptake by flow cytometry (Fig. 1 C). HD5 is an α-defensin produced by Paneth cells in the gastrointestinal mucosa and by vaginal epithelial cells. HNP3, like HNP1 and HNP2, is produced by neutrophils and other myeloid cells. HBD1 and HBD2 are produced by various epithelial cells. HD5 strongly increased IAV uptake by neutrophils, but HNP3 and HBD1 did not. HB2 slightly (but statistically significantly) increased viral uptake at 40 μg/ml.

HNPs promote clearance of IAV by neutrophils

Live, unlabeled IAV was incubated in vitro with HNPs or neutrophils alone or with combinations of HNPs with neutrophils. Following incubation, the samples were subjected to low-speed centrifugation to determine residual virus present in cell-free supernatants. Neutrophils alone did not cause a significant decrease in IAV in the supernatant in this assay as measured by hemagglutination titers (Fig. 3). HNPs alone caused a decrease in virus in supernatant; however, the decrease in virus was significantly greater when HNPs and neutrophils were combined. This supports the notion that HNPs promote neutrophil-mediated clearance of IAV. It is of interest as well that HNPs alone caused a drop in virus in the supernatant, presumably due to viral aggregation leading to precipitation of the virus at low-speed centrifugation.

HNPs cause aggregation of IAV

Our previous studies suggest that the ability of SP-D to increase neutrophil binding and uptake of IAV correlates with viral aggregation by SP-D. HNPs caused aggregation of two different strains of IAV (Phil82 and PR-8) as assessed by measuring light transmission through stirred suspensions of IAV (Fig. 4, upper panel) or by fluorescent microscopy using a rhodamine-labeled Phil82 strain of IAV (Fig. 4, lower panel). Fig. 5 shows the appearance of viral aggregates induced by HNPs on electron microscopy.

HNPs do not increase neutrophil H2O2 responses to IAV and inhibit the respiratory burst-enhancing activity of SP-D

HNPs have previously been reported to inhibit neutrophil respiratory burst responses (34). IAV alone triggers modest H2O2 production by neutrophils, an effect that is significantly increased by preincubation of the virus with certain concentrations of RhSP-D (Ref. 20 and Fig. 6). Preincubation of IAV with HNPs did not increase IAV-induced H2O2 generation. However, when IAV was preincubated with both RhSP-D and HNP1 or HNP2, the enhancing effect of RhSP-D was reduced (Fig. 6). HNP1 or HNP2 did not significantly alter H2O2 production induced by fMLP in parallel experiments (n = 4; data not shown).

HNPs increase the neutrophil uptake of bacteria

To determine whether the HNPs have a more general effect on neutrophil phagocytosis, we tested the effect of preincubation S. aureus or E. coli with HNPs on uptake of these bacteria. As shown in Fig. 7, HNP1 and HNP2 increased uptake of both of these bacteria by neutrophils as assessed by flow cytometry. Fig. 8 shows representative fluorescent micrographs of adherent neutrophils in which HNP1 and HNP2 increased the neutrophil uptake of E. coli.
and *S. aureus*. Adherent neutrophils appeared to phagocytose very few bacteria in the absence of HNPs, and HNPs caused clear-cut increases in cell-associated bacteria as shown. RhSP-D increased the neutrophil uptake of *E. coli* as previously reported (30); however, SP-D did not as clearly increase the uptake of *S. aureus*. Fig. 9 demonstrates that HNPs induced the aggregation of *E. coli* and *S. aureus*.

**Discussion**

We report that HNP1 and HNP2 increase the neutrophil uptake of IAV, *E. coli*, and *S. aureus*. HNPs showed similar activity in this regard. HNPs increased the viral and bacterial uptake by neutrophils in suspension as well as by adherent neutrophils. The effect of HNPs on viral uptake was most pronounced when the virus was preincubated with HNPs. Preincubation of IAV with HNPs in the presence of BALF greatly increased the ability of the fluid to promote viral uptake.

Preincubation of neutrophils with HNPs caused a much more modest (although still statistically significant) increase in viral uptake. β-Defensins appear to have lower activity than α-defensins, because HBD1 had no activity, and HBD2 had modest activity compared with HNP1 and HNP2 or HD5. Fleischman et al. (35) reported that MCP-1 and MCP-2 increase the uptake of bacteria and *Candida* by rabbit alveolar macrophages, perhaps representing a similar phenomenon to what we have reported in this study. Further studies involving additional cationic peptides and phagocytic cell types will, therefore, be of interest.

It is notable that HNP3 did not increase viral uptake, given its close relationship to HNP1 and HNP2. HNP1 and HNP3 differ from HNP2 only in having, respectively, an additional alanine or aspartic acid at the N terminus. However, there is evidence indicating that the N-terminal aspartic acid impacts the function of the molecule considerably. HNP3 exists as a dimer with an amphiphilic structure, and the N-terminal aspartic acid is located on the charged surface of the molecule. HNP3 has greatly reduced antibacterial and anti-candidal activity compared to HNP1 and HNP2 (36). Our results suggest that N-terminal amino acids on HNPs can modulate prophagocytic activity as well.

The mechanisms through which HNPs promote phagocytosis require more study; however, we demonstrate one possible mechanism: HNP-induced aggregation of IAV and bacteria. A similar effect appears to contribute to enhanced viral uptake caused by SP-D (21, 30, 37, 38). The need for the preincubation of IAV with HNPs to obtain optimal enhancement of uptake is consistent with a role for viral aggregation; however, a contribution of direct effects of HNPs on neutrophils is also possible.

The finding that HNPs can induce viral and bacterial aggregation is also of interest, because it implies that HNPs can cross-link viral or bacterial particles to each other. It is likely that the dimerization of higher levels of oligomerization (39) of the HNPs are involved in this activity (and, by extension, in increased neutrophil uptake). It is unlikely that the lectin activity of HNPs (40) contributes to their ability to aggregate IAV, because aggregation of the PR-8 strain was greater than aggregation of the Phil82 strain. The PR-8 strain is a mouse-adapted human strain that has greatly diminished glycosylation on its envelope proteins compared with the Phil82 strain (41, 42).

The ability of HNPs to promote neutrophil-mediated clearance of IAV from culture supernatants was confirmed by an independent technique involving live, unlabeled virus. It is likely that HNPs participate in host defense against IAV during the early phase of infection that is characterized by an influx of neutrophils into the infected respiratory tract (2, 5). It is intriguing to speculate that neutrophils themselves can promote phagocytosis through the release of HNPs during viral or bacterial infection.

We report that HNPs reduced neutralophil H$_2$O$_2$ production in response to SP-D-treated IAV. Under the conditions of our assay, this effect appeared to be specific to IAV-induced responses, since responses to fMLP were not reduced. The finding that HNPs increase IAV uptake without increasing virus-induced respiratory burst responses suggests that HNPs may promote viral uptake without exacerbating oxidant injury, which contributes to the pathogenesis of severe IAV infection in animal models (43).

SP-D has complex effects on neutrophil oxidant responses to IAV. In the absence of SP-D, IAV triggers an atypical activation response characterized by H$_2$O$_2$ release in the absence of a superoxide generation (33, 44, 45). IAV also depresses superoxide responses of neutrophils to a variety of other stimuli (46). However, preincubation of IAV with SP-D increases H$_2$O$_2$ production (20). In contrast, if neutrophils are first preincubated with SP-D, a reduced H$_2$O$_2$ response of the cells to IAV is observed (26). Of interest, it has been reported that HNPs suppress the oxidative metabolism of neutrophils and reduce their ability to kill *S. aureus* (34). We have found that HNPs bind to SP-D, which could account in part for the ability of HNPs to counteract the SP-D-induced increase in respiratory burst responses to IAV (27). We have found that several other components of respiratory fluids including surfactant protein A, mucins, and scavenger receptor-rich gp340 counteract the ability of SP-D to increase IAV-induced H$_2$O$_2$ responses and have shown that BALF per se does not increase H$_2$O$_2$ responses to IAV (26).

Our findings reveal a previously unappreciated feature of the functional activity of HNPs and suggest that HNPs modulate host defense in IAV or bacterial infection through direct antiviral and antibacterial effects and through inducing viral and bacterial aggregation with altered interactions of the pathogens with neutrophils. Future studies will address how HNPs modulate viral or bacterial trafficking within neutrophils or macrophages.

**Disclosures**

The authors have no financial conflict of interest.

**References**


HUMAN NEUTROPHIL DEFENSINS PROMOTE PHAGOCYTOSIS


