Interactions of α-, β-, and θ-Defensins with Influenza A Virus and Surfactant Protein D

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Interactions of α-, β-, and θ-Defensins with Influenza A Virus and Surfactant Protein D

Mona Doss,* Mitchell R. White,* Tesfaldet Tcele,* Donald Gantz,† Erika C. Crouch,‡ Grace Jung,§ Piotr Ruchala,§ Alan J. Waring,§ Robert I. Lehrer,§ and Kevan L. Hartshorn1*

We have reported that the α-defensins human neutrophil peptides (HNP)-1 and HNP-2 neutralize and aggregate influenza A virus (IAV) and promote uptake of IAV by neutrophils. These α-defensins were also shown to bind to surfactant protein (SP)-D and reduce its antiviral activity. In this study, we examined retrocyclin (RC)1 and RC2, humanized versions of the antiviral θ-defensins found in the leukocytes of certain nonhuman primates. RC1 was just as effective as HNP-1–3 in neutralizing IAV, and RC2 and RC101 (an analog of RC1) were more effective. In contrast, human β-defensins (HBDs) showed less neutralizing activity. Human defensins 5 and 6 (mainly produced by intestinal Paneth cells) had viral neutralizing activity similar to HNP-1–3. Like HNP-1–3, RCs induced viral aggregation and promoted the uptake of IAV by neutrophils. We used surface plasmon resonance to evaluate binding of defensins to SP-D. HBDs, HD6, and HNP-4 bound minimally to SP-D. HNP-1–3 and RCs bound SP-D with high affinity; however, unlike HNP-1 and HNP-2, RCs did not inhibit SP-D antiviral activity. HBDs also did not inhibit antiviral activity of SP-D. Given their strong neutralizing activity and compatibility with SP-D, RCs may provide attractive prototypes for designing therapeutics that can prevent or treat respiratory infections caused by IAV.


In the United States alone, influenza virus infections cause ~36,000 annual deaths (1). In nonpandemic years, this virus is seasonal and primarily affects children and the elderly. However, the 1918 pandemic caused by the H1N1 subtype of avian origin caused millions of fatalities, prominently including many healthy young adults (2). As there is no proof-reading mechanism for RNA enzymes, numerous point mutations occur resulting in antigenic drift. Reassortment, the exchange of whole gene segments between human and animal strains, can also occur, resulting in novel pandemic strains (3).

The initial reactions to influenza virus are directed by the innate immune system. Upon exposure to a new influenza A virus (IAV) strain, it takes 5–7 days before strain-specific CTLs arrive in the lung. During these initial days, the body relies on the innate immunity to contain the infection without excessive inflammatory response. The innate response to influenza virus relies on pattern-recognition molecules, like collectins and TLR3 and TLR7. It also involves soluble components such as complement, cytokines (i.e., TNF-α, IFNγ), and defensins as well as cellular components such as neutrophils, macrophages, dendritic cells, and NK cells (4).

This study evaluates the antiviral activities of defensin peptides representing all three subfamilies found in primates, concentrating on their interactions with phagocytes and collectins. We use IAV strains that have been extensively characterized in prior studies. Defensins are small cationic peptides that contribute to innate defense. Neutrophils contain large stores of α-defensin human neutrophil peptides (HNP)-1–3 in their primary (azurophilic) granules, and other α- and β-defensins are found in epithelial cells and phagocytes (5). Collectively, their activity encompasses Gram-positive and Gram-negative bacteria, fungi, and viruses.

HNP-1–3 are active against IAV, HIV, and herpes simplex virus among other viruses (6). HNPs can inhibit influenza viral replication with treatment before and after infection (7, 8). Human β-defensins (HBDs) are of considerable interest with respect to respiratory viral infection. They are produced by oral, nasal, and airway epithelial cells in response to various activating stimuli and participate in signaling that promotes development of adaptive immune responses (9). Both α- and β-defensins have a similar triple stranded β-fold but differ in disulfide connectivity and peptide length between disulfide bonds. θ-defensins, such as retrocyclins (RCs) or the RTD peptides found in rhesus macaque leukocytes, have a cyclic backbone and a unique, ladder-like pattern of disulfide bonds (5). Unlike α- and β-defensins, θ-defensin peptides are only expressed in nonhuman primates, such as the rhesus macaque and olive baboon (Papio anubis) (10, 11). Humans express θ-defensin mRNA but lack the corresponding peptides because the human θ-defensin (DEFT) gene contains a stop codon in the signal sequence that aborts translation. RCs are synthetic humanized θ-defensin peptides whose sequences are based on those found in human DEFT genes. They have been shown to block steps of herpes virus infection, neutralize anthrax toxin, reduce HIV envidiated entry and inhibit influenza virus (12–15).

Collectins are collagogenous lectins with broad spectrum antimicrobial activity that are present in mucosal secretions (including respiratory lining fluid) and blood. The basic collectin structure is trimeric. Each monomeric subunit contains an N-terminal domain that is involved in disulfide formation between the trimers, a
structurally important collagen domain, a neck domain that drives trimerization, and a carbohydrate recognition domain that mediates binding to pathogens (16). Surfactant proteins (SP)-A and SP-D are collectins that are found in pulmonary secretions and at other mucosal locations that contribute to innate defense against IAV (17–20). Serum collectins (i.e., mannose-binding lectin) and SP-D inhibit IAV by binding in a calcium-dependent manner to carbohydrates on the viral hemagglutinin and neuraminidase (21). Among the collectins, SP-D is most potent at inhibiting IAV. The natural form of SP-D found in human bronchoalveolar lavage (BAL) fluids also has antiviral and opsonizing effects. SP-D may also contribute to the innate response by clearing apoptotic cells, inducing viral and bacterial aggregation, and increasing phagocyte uptake of pathogens (22). SP-D reduces inflammation in the lung during IAV infection as well (18, 19). SP-A also inhibits IAV replication and IAV-induced inflammation, but its antiviral activity is less than that of SP-D (23, 24). SP-A and SP-D have different mechanisms of antiviral activity. SP-A inhibits IAV not through its lectin activity, but by expressing a sialic acid rich ligand on its carbohydrate recognition domain to which the virus binds. We have shown that HNP-1 and HNP-2 bind to the carbohydrate recognition domain of SP-D and cause SP-D to precipitate out of BAL fluid (8). These defensins also modify the antiviral activity of SP-D and BAL fluid in vitro, in most instances reducing activity of SP-D.

Neutrophils contribute to the initial innate response to IAV infection probably through ingestion of IAV or of IAV-infected apoptotic cells (25, 26). Functional impairment of neutrophils during or after IAV infection predisposes some IAV-infected patients to bacterial superinfection (27). We have found that collectins and HNP-1–3 promote neutrophil uptake of IAV in vitro (24, 28). SP-D also protects neutrophils from the deactivating effects of IAV in vitro (17) and in vivo (18).

The initial goal of this study was to compare representatives of all three known mammalian defensin subfamilies, α-, β-, and θ-defensins, in their actions toward IAV and their binding to SP-D; as well as to ascertain whether any defensins can work in concert with SP-D more effectively than HNP-1 and HNP-2. During the course of these studies, we also observed for the first time that RCs promote IAV uptake by phagocytes.

**Materials and Methods**

**Virus preparation**

IAV was grown in the chorioallantoic fluid of 10-day-old chicken eggs and purified on a discontinuous sucrose gradient as previously described (27). The virus was dialyzed against PBS to remove sucrose, aliquoted and stored at −80°C until needed. Philippines 82/H3N2 (Phil82) strain was

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**Figure 1.** Inhibition of influenza virus infectivity by defensins. Neutralization was measured by inhibition of fluorescent focus formation in the indicated epithelial cells. Results are mean ± SEM (n = 3 or more experiments) and are expressed as a percentage of control infectious foci in all experiments. A and B, show the neutralizing activity of three θ-defensins (RC1, RC2, and RC101) for the Phil82 strain of IAV. MDCK cells (A) and A549 cells (B) were used. In both experiments, each concentration of RC1, RC2, and RC101 significantly reduced viral infectivity (p < 0.05) compared with control. The effects of both RC2 and RC101 were significantly greater than the effects of RC1, as determined by ANOVA at 2.5 and 5.0 μg/ml. C, Inhibition of the Phil82/BS strain of IAV by the RCs again using A549 cells. All RCs again inhibited IAV significantly but there was no difference between RC1, RC2, and RC101 at the concentrations tested. D, Inhibition of the PR-8 strain in MDCK cells. All RCs caused significant inhibition of infectivity compared with control and RC2 and RC101 again had greater activity than RC1.

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<table>
<thead>
<tr>
<th>Defensin</th>
<th>Phil82 Strain</th>
<th>PR-8 Strain</th>
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</thead>
<tbody>
<tr>
<td>RC1</td>
<td>4.05</td>
<td>8.5</td>
</tr>
<tr>
<td>RC2</td>
<td>1.25</td>
<td>1.76</td>
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<tr>
<td>RC101</td>
<td>1.25</td>
<td>3.03</td>
</tr>
<tr>
<td>HNP-1</td>
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<td>1</td>
</tr>
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<td>HNP-2</td>
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<tr>
<td>HNP-3</td>
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<tr>
<td>HD6</td>
<td>2.4</td>
<td>ND</td>
</tr>
<tr>
<td>HBD-1</td>
<td>33</td>
<td>ND</td>
</tr>
<tr>
<td>HBD-2</td>
<td>14</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Results are ~50% neutralizing activity for the indicated IAV strains and are derived from experiments shown in Figs. 1 and 2 (for RC, HNP-3, HD5, and HBD), or Hartshorn et al. (8) (for HNP-1 and HNP-2).

ND, Not determined.
provided by Dr. E. Margot Anders (University of Melbourne, Victoria, Australia). The A/PR/8/34/H1N1 (PR-8) strain was a gift of 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 (27). Postthawing the viral stocks contained \(5 \times 10^8\) PFU/ml.

**Defensin and SP-D preparations**

Recombinant human SP-D was produced in stably transfected CHO-K1 cells as previously described (29). For these studies the dodecameric fraction of recombinant human SP-D was used unless otherwise specified. The collectin preparations used in this report were tested for endotoxin using a quantitative endotoxin assay (Limulus Amebocyte Lysate; BioWhittaker). The final concentrations of endotoxin in protein samples containing the highest concentrations of collectins were \(20 \text{–} 100\) pg/ml (or \(6 \text{–} 12\) endotoxin U/ml using an internal assay standard). HNPs were purchased from Bachem Bioscience. HNP-3 was purchased from Bachem Bioscience. HBD-1 and HBD-2 were purchased from Peptide Institute. HD5 was purchased from Bachem Bioscience. RC1, RC2, and RC101 were chemically synthesized by Lehrer and colleagues (12, 15, 30). Surface plasmon resonance binding studies were performed with synthetic human \(\alpha\)-defensin and HBD peptides that were a gift of W. Lu (University of Maryland, Baltimore, MD). The preparation has been described elsewhere (31–33).

**Measurement of binding of collectins to defensins**

Binding of collectins to defensins was assessed by ELISA. Plates were coated with \(10\) g/ml defensins in coating buffer (15 mM Na\(_2\)CO\(_3\), 5 mM NaHCO\(_3\) (pH 9.6)) overnight at 4°C vs PBS containing 2.5% BSA (fraction V, fatty acid free, and low endotoxin, A8806) (Sigma-Aldrich) background control. Following washing three times with PBS with 2 mM calcium and magnesium, the plates were blocked with PBS containing 2.5% BSA for 3 h. These defensin-coated plates were then incubated with SP-D and then washed with PBS with 2 mM calcium and magnesium. Binding anti-SP-D mAb was detected with HRP-labeled goat anti-mouse Abs using tetramethylbenzidine as a substrate (Bio-Rad). The reaction was stopped using 1 N sulfuric acid. The OD was measured on an ELISA plate reader at 450 nm wavelength. Each individual data point was performed in duplicate. Background nonspecific binding was assessed by coating plates with fatty acid free BSA but no defensin and performing the collectin binding assay as outlined earlier. Binding to BSA was subtracted from the collectin binding values.

**FIGURE 2.** Comparison of IAV neutralizing activity of various \(\alpha\)- and \(\beta\)-defensins. The experiments were performed as in Fig. 1 and represent mean ± SEM of \(n = 4\) or more experiments. A and B. Using A549 cells, the effects of HNP1 (A) in its natural conformation vs HNP1 that was reduced to remove disulfide bonds and the latter was significantly less neutralizing than HNP-1 are shown. *, \(p < 0.02\). The neutralizing activity of HD6 and HNP4 (B) are also shown. HNP-4 had significantly less neutralizing activity than HNP-1 or HD5. C. The neutralizing activity of two other \(\alpha\)-defensins (HNP-3 and HD5) and two \(\beta\)-defensins (HBD-1 and HBD-2) for IAV (Phil82 strain) in MDCK cells is shown. All of the defensins used in this experiment significantly reduced infectivity of IAV compared with control (\(p < 0.05\)) at all concentrations tested.

**FIGURE 3.** Aggregation of IAV particles by RCs. Viral aggregation was tested by measuring reduction of light transmission through stirred viral suspensions (A) or through EM (B). RC2 caused the largest reduction in light transmission at 350 nm compared with control (virus alone). All RCs caused significant virus aggregation (\(p < 5 \times 10^{-4}\)), compared with the control. Results are mean ± SEM of \(n = 8\) experiments. RC2 caused significantly more aggregation (\(p < 0.005\)) when compared with RC1 and RC101. Representative pictures from EM studies (\(n = 3\)) are shown (B). Images show control virus alone (Phil82 strain of IAV) or virus that has been preincubated with 20 \(\mu\)g/ml RC.
Binding of defensins to the collectin, SP-D, was measured on a Biacore3000 surface plasmon resonance instrument (30). In brief, SP-D was immobilized by amine coupling on CM5 biosensor chips (Biacore). The rate constants, $K_a$ and $K_d$, and the equilibrium binding constant ($K_d$) were calculated with the assumption of 1:1 monomeric ligand to analyte binding, using BioEvaluation software (version 4.1; Biacore).

Binding was also expressed as a molar ratio, representing the moles of analyte (defensin) bound per moles of immobilized monomeric ligand (SP-D) at a defined time point, typically at the end of the 3- or 5-min binding phase. The molar ratio was calculated from the formula $abc/d$, where $b$ is the mass of monomeric SP-D (42,117), $c$ = mass of the defensin, and $d$ = resonance units of SP-D attached to the biosensor (see Table II). This formula follows logically from the fact that the magnitude of the binding response, in resonance units, is proportional to the total mass of analyte bound to the biosensor surface (34, 35), and from its corollary, that the resonance units resulting from a single binding or immobilizing event is proportional to the mass of the molecule that binds the biosensor.

**Fluorescent focus assay of IAV infectivity**

Viral neutralization was measured using a fluorescent focus reduction assay. In brief, A549 (respiratory epithelial cell line) or Madin Darby Canine Kidney (MDCK) cell monolayers were prepared in 96-well plates and grown to confluency. These layers were then infected with diluted IAV. Viral incubation with defensins, SP-D or control buffer, followed by addition of FITC-labeled IAV (Phil82 strain) was prepared and uptake of virus by neutrophils or RAW 264.7 cells was measured as previously described (36). IAV was incubated with neutrophils or RAW 264.7 cells for 30 min at 37°C in the presence of control buffer or RCs. Extracellular fluorescence was quenched by addition of trypan blue (0.2 mg/ml) to the samples. After they were washed, the neutrophils or RAW 264.7 cells were stained with 1% sodium phosphotungstate (pH 7.3) (Sigma-Aldrich) for 10 s, and excess stain was blotted off. The grids were then air dried and stored in a grid box until examined with a Phillips 300 electron microscope.

**Electron microscopy**

RCs were incubated with Phil82 IAV at 37°C for 30 min, and a 4-μl sample was placed on each copper grid. After the unbound virus was blotted off, the grid was fixed with 4 μl of 2.5% glutaraldehyde for 5 min. Samples were stained with 1% sodium phosphotungstate (pH 7.3) (Sigma-Aldrich) for 10 s, and excess stain was blotted off. The grids were then air dried and stored in a grid box until examined with a Phillips 300 electron microscope.

**Human neutrophil preparation**

Neutrophils from healthy volunteers were isolated to >95% purity by dextran sedimentation, followed by Ficoll-Paque gradient separation for the removal of mononuclear cells, and then hypotonic lysis to eliminate any contaminating erythrocytes, as previously described (27). 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. Neutrophil collection was done with informed consent, as approved by the Institutional Review Board of Boston University School of Medicine.

**Measurement of IAV uptake by neutrophils and RAW cells**

FITC-labeled IAV (Phil82 strain) was prepared and uptake of virus by neutrophils or RAW 264.7 cells was measured as previously described (36). Briefly, IAV was incubated with neutrophils or RAW 264.7 cells for 30 min at 37°C in the presence of control buffer or RCs. Extracellular fluorescence was quenched by addition of trypan blue (0.2 mg/ml) to the samples. After they were washed, the neutrophils or RAW 264.7 cells were stained with 1% sodium phosphotungstate (pH 7.3) (Sigma-Aldrich) for 10 s, and excess stain was blotted off. The grids were then air dried and stored in a grid box until examined with a Phillips 300 electron microscope.
fixed with 1% paraformaldehyde, and neutrophil/RAW cell-associated fluorescence was measured using flow cytometry. The mean intracellular fluorescence (2000 cells/sample) was measured. Neutrophil uptake by adherent neutrophils was performed as previously described (24). In brief, neutrophils were allowed to adhere to glass slides for 1 h at 37°C followed by washing and addition of Alexa-Fluor-labeled Phil82 IAV. After 45 min of incubation with the virus at 37°C, the neutrophils were again washed, and trypan blue was added to quench extracellular fluorescence. The cells were then examined under fluorescent microscopy at a magnification of ×40.

**Statistics**

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

**Results**

**Antiviral activities of defensins**

RC1, RC2, and RC101 inhibited infectivity of the Phil82 strain of IAV in MDCK cells (Fig. 1A). RC2 and RC101 had significantly greater activity than RC1. Similar inhibition was found in A549 cells that are derived from alveolar epithelium (Fig. 1B). Because RCs have lectin activity we tested their ability to neutralize two viral strains that have reduced glycosylation on their envelope proteins. The Phil82/BS strain of IAV is derived from Phil82 but differs in that it lacks a high mannose oligosaccharide on the globular tip of its hemagglutinin (40). The RCs all caused significant inhibition of Phil82/BS which was similar, if not greater than, their activity against the parental Phil82 strain (Fig. 1C). There was no significant difference in the activity of the three RCs against Phil82/BS at the concentrations tested. The PR-8 is of the H1N1 subtype and lacks N-linked oligosaccharides on its envelope proteins. The RCs also showed a similar pattern of inhibition for the PR-8 strain, although for each RC activity was slightly less than against Phil82 (Fig. 1D).

Of interest, although the RCs had slightly reduced activity against the glycosylation deficient PR-8 strain this was not true for HNPs in our prior studies. Table I shows the relative 50% neutralizing activity of defensins tested in this study and in Hartshorn et al. (8).Fig. 2 shows results of neutralization assays using a panel of human defensins and the Phil82 strain of IAV. Fig. 2A shows the activity of HNP-1 in its native conformation vs its activity after disulfide bond reduction. There was significant diminishment in the activity of HNP-1 with reduction, but it is notable that the reduced preparation did retain considerable activity. Fig. 2B shows that HNP-4 had lesser activity than HD6 (an α-defensin found primarily in small intestinal Paneth cells). The activity of HNP4 was also less than that of HNP-1, HNP-3 or HD5. HD1 and 2 inhibited infectivity of the Phil82 strain of IAV (Fig. 2C); however, their activity was significantly less than that of the α-defensins HNP-3 and HD5. The neutralizing activities of HNP-3 and HD5 were comparable to the activity we previously reported for HNP-1 and HNP-2.

When tested against the Phil82 strain, RC2 and RC101 had greater activity than the other defensins tested (see Table I). Inhibition of infectivity in the infectious focus assay is generally predictive of similar results on the plaque assay, as we have previously reported for SP-D (41). We confirmed this using HNP-1 (5 μg/ml), which reduced plaque formation to zero (n = 2; data not shown).

**Table II. Binding of defensins to immobilized SP-D**

<table>
<thead>
<tr>
<th>Defensin</th>
<th>Molar Ratio</th>
<th>Ke (nM)</th>
<th>Ke (1/Ms × 10⁻⁴)</th>
<th>Ke (1/S × 10⁻⁸)</th>
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</thead>
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<td>HNP-1</td>
<td>2.65 ± 0.10</td>
<td>63.1 ± 17.4</td>
<td>5.9 ± 2.4</td>
<td>0.92 ± 0.08</td>
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<td>HNP-2</td>
<td>2.40 ± 0.13</td>
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<td>10.4 ± 3.8</td>
<td>0.80 ± 0.09</td>
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<tr>
<td>HNP-3</td>
<td>2.47 ± 0.16</td>
<td>55.7 ± 13.5</td>
<td>7.6 ± 1.9</td>
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<td>HNP-4</td>
<td>0.18 ± 0.05</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>HD5</td>
<td>1.24 ± 0.26</td>
<td>48.7 ± 4.8</td>
<td>15.0 ± 3.3</td>
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<td>HD6</td>
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<td>NT</td>
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<td>HBD-1</td>
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<td>HBD-2</td>
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<tr>
<td>HBD-3</td>
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<tr>
<td>RC1</td>
<td>3.25 ± 0.26</td>
<td>115.5 ± 20.7</td>
<td>4.6 ± 0.47</td>
<td>4.2 ± 0.13</td>
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<tr>
<td>RC2</td>
<td>4.61 ± 0.21</td>
<td>72.1 ± 8.0</td>
<td>2.8 ± 0.30</td>
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<td>RC101</td>
<td>6.94 ± 0.78</td>
<td>30.1 ± 2.9</td>
<td>24.0 ± 2.9</td>
<td>6.0 ± 0.19</td>
</tr>
</tbody>
</table>

*Data are mean ± SEM. Molar ratios were measured in n = 7 experiments, all using an analyte concentration of 1 μg/ml defensin. Four CM5 biosensors were used, and they presented immobilized SP-D at the following resonance units (RU) levels: 8298 (n = 2), 8690 (n = 2), 8572 (n = 2), and 3403 (n = 1). Defensin solutions were prepared in HBS-EP buffer. The binding phase lasted for 5 min with a flow rate of 50 μl/min.

1 Molar ratio (MR) was calculated from the formula using the following: MR = ab/cd, in which a represents binding at 5 min in RU, b = monomeric mass of the immobilized SP-D (42,117 Da), c = mass of the defensin peptide, and d = RU of SP-D attached to the biosensor.

*Calculated using Biacore 4.1 software, assuming 1:1 binding, from 16–18 separate binding runs with 50–300 ng/ml peptide and the first three biosensors described for these results.

NT, Not tested.
Defensin-mediated viral aggregation

We have reported that HNP-1 and HNP-2 cause aggregation of IAV (28). The RCs had similar effects, as assessed by light transmission (Fig. 3 A). The activity of RC2 exceeded that of RC1 and RC101, which had similar effects. RC-mediated viral aggregation was also shown by electron microscopy (Fig. 3 B).

Effect on neutrophil and macrophage IAV uptake

As befits components of the innate immune defense system, neutrophils bind and take up IAV in the absence of Abs and are stimulated by IAV to generate H2O2 (42, 43). We have reported that HNP-1 and HNP-2, HD5, and HBD-2 cause increased uptake of IAV by neutrophils (28). The RCs also markedly increased neutrophil uptake of IAV (Fig. 4 A). All of the RCs increased uptake of the virus by RAW 264.7 macrophages as well (Fig. 4 B). Uptake of IAV was also evaluated using adherent neutrophils and fluorescent microscopy (Fig. 4 C), confirming markedly increased neutrophil-associated fluorescence when Alexa Fluor-labeled IAV was preincubated with RCs.

Interactions of defensins with SP-D

We have previously reported that HNP-1 and HNP-2 bind to SP-D and interfere with its neutralizing activity. As assessed by ELISA, SP-D bound to HNP-3 as well but bound minimally to HD5, HBD-1 or HBD-2 or RC1 or RC2 (Fig. 5). Binding of SP-D to HNP-3 was less than binding to HNP-2. The ELISA had to be performed by coating the plate surface with defensins because our attempts to bind soluble defensins to SP-D-coated surfaces were complicated by high background binding. There was no difference in the amounts of RC1, RC2 or HNP-2 protein binding to the ELISA plates (as measured by protein assays performed after the initial binding step; data not shown).

We also tested the binding of solution-phase defensins to immobilized SP-D by surface plasmon resonance (SPR). The results of these studies are summarized in Table II and Figs. 6 and 7. SPR experiments measure binding in real time and with high precision. Table II shows that HNP-1–3 had almost identical affinity for SP-D, with a $K_D$ ranging from 40 to 65 nM. At a concentration of 1 µg/ml (~300 nM), ~2.5 molecules of HNP-1, HNP-2, or HNP-3 had bound each SP-D monomer. HD5 bound SP-D with similar affinity to HNP 1–3, but evidently had fewer binding sites on a SP-D molecule because its molar ratio was half that of the HNPs. Two other human α-defensins, HNP-4 and HD6, failed to bind SP-D appreciably. Overall, the ELISA and SPR results were discordant for HNP-1, HNP-2, and HNP-3. Both assays also agreed that HBDs bound minimally, if at all, to SP-D.

When it came to RCs, however, the ELISA and SPR results diverged. Whereas the ELISA results indicated minimal binding of SP-D by RC1 and none by RC2, our SPR assays showed extensive

![FIGURE 6. Measuring the binding of defensins to SP-D by surface plasmon resonance in resonance units (RU). SP-D (8292 RU) was immobilized on a CM5 biosensor by amine coupling. Defensins were prepared at 1 µg/ml in HBS-EP buffer and flowed through the biosensor chamber at 50 µl/min for 5 min. Plasmon resonance measurements were taken every second. Note that the ordinates of the three data graphs all have different scales.](http://www.jimmunol.org/)

![FIGURE 7. Binding of low concentrations of HNP-2, RC2, and RC101 to SP-D. We used BiaEvaluation 4.1 software and nested binding isotherms (curves) such as those shown to calculate the association ($K_a$) and dissociation ($K_d$) rate constants and the equilibrium binding constant ($K_D$) shown in Table I. The CM5 biosensor used in the illustrated experiments presented 8298 resonance units (RU) of SP-D. The reactant masses were: 42,117 for monomeric SP-D, 3371 for HNP-2, 2017.6 for RC2, and 1890.6 for RC101. A formula described in Materials and Methods can be used to convert binding, in resonance units, to molar ratios, i.e., the number of defensin molecules bound per molecule of SP-D. Results show a 1:1 molar ratio would correspond to 664 RU (A), to 397.5 RU in (B), and to 372.4 RU (C).](http://www.jimmunol.org/)

![FIGURE 8. Binding of SP-D to immobilized RC101 and HNP-1. HNP-1 (1099 RU) and RC101 (1034 RU) were immobilized on CM5 biosensor chips by standard amine coupling. During the association phase, analyte solution containing 10 µg/ml SP-D (237 nM in HBS-EP buffer flowed over the biosensor at a rate of 50 µl/min for 3 min. During the dissociation phase, the flow cell was perfused with HBS-EP buffer alone. Association ($K_a$) and dissociation ($K_d$) rate constants and the calculated equilibrium dissociation constants ($K_D$) are shown.](http://www.jimmunol.org/)
**A HBDs and SP-D**

*HBD1+ SP-D in Viral Neutralization*

<table>
<thead>
<tr>
<th>Concentration (ug/ml)</th>
<th>% Fluorescent Foci</th>
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</thead>
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<tr>
<td>control</td>
<td>120</td>
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<tr>
<td>0.006 SP-D</td>
<td>100</td>
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<tr>
<td>0.012 SP-D</td>
<td>80</td>
</tr>
<tr>
<td>0.012 SP-D + 0.012 HBD</td>
<td>60</td>
</tr>
<tr>
<td>10 HBD</td>
<td>40</td>
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<tr>
<td>10 HBD + 0.006 SP-D</td>
<td>30</td>
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<tr>
<td>10 HBD + 0.012 SP-D</td>
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</table>

**B Retrocyclins and SP-D**

*RCs in combination with SP-D*

<table>
<thead>
<tr>
<th>Concentration (ug/ml)</th>
<th>% Fluorescent Foci</th>
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</thead>
<tbody>
<tr>
<td>control</td>
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</tr>
<tr>
<td>0.012SP-D</td>
<td>100</td>
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<tr>
<td>2.5 RC1</td>
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<td>0.012SP-D + 2.5RC1</td>
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<tr>
<td>2.5 RC2</td>
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<tr>
<td>5 RC2</td>
<td>0</td>
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</table>

**FIGURE 9.** Antiviral activities of defensins in combination with SP-D. **A,** The outcome of adding β-defensins (HBD-1 and HBD-2) in combination with SP-D and their IAV neutralizing ability. When HBD-1 and HBD-2 were added along with SP-D, the combination significantly decreased ($p < 0.00005$) the number of virally infected cells than β-defensins alone. Results are mean ± SEM of $n = 11$ experiments. **B,** RCs in combination with SP-D. Compared with the control (control = virus alone), the addition of 0.012 μg/ml SP-D alone significantly reduced the number of infected cells ($p < 0.05$), as did the addition of 2.5 and 5 μg/ml each of RC alone. When tested in combination, 2.5 μg/ml RC plus 0.012 μg/ml SP-D was significantly lower than the corresponding concentrations of SP-D alone, RC1 alone, and RC101 alone ($p < 0.05$), though not significant when compared with RC2. The combination of 5 μg/ml RC plus 0.012 μg/ml SP-D significantly decreased the number of infected foci than SP-D alone, though not significant with this higher concentration of RC alone. We have shown that the addition of RC plus SP-D can produce a cooperative effect.
binding by both. In the SPR assays, RC2 had much the same affinity for SP-D as did HNP 1–3, and at 1 µg/ml, its molar binding ratio was almost 2-fold higher than those of HNP 1–3 (Table I). RC101 had the highest affinity for SP-D ($K_d$ 30.1 ± 2.9 nM), and at 1 µg/ml RC101, almost seven RC101 molecules bound per SP-D monomer, the highest molar ratio in the study. Note, however, that RC101 had a more rapid on and off rate in binding as well (Figs. 6 and 7). The similarities and differences between HNP-2, RC2, and RC101 are also evident in studies performed at much lower defensin concentrations, from 50 to 300 nM (Fig. 7).

Because the ELISA studies with RCs had been performed with SP-D in solution and immobilized RCs, and the converse conditions were applied in the SPR assays, we also performed experiments in which RC101 (1034 response units) or HNP-1 (1099 response units) was immobilized on a CM5 sensor and 10 µg/ml SP-D (237 nM) was the analyte. The binding curves shown in Fig. 8 indicate that SP-D again binds both defensins with high affinity, although in this case SP-D bound with lower affinity to RC101 than to HNP-1.

We previously reported that binding of HNPs to SP-D interferes with neutralizing activity of SP-D (8). In contrast, HBD and RC did not interfere with neutralizing activity of SP-D (Fig. 9) and some concentrations had additive activity when combined with SP-D.

**Discussion**

We demonstrate that RC1 and RC2 and HNPs 1–3 have greater IAV-neutralizing activity than the β-defensins, HBD-1 and HBD-2. Among the tested RCs, RC2 and RC101 were more potent than RC1 at inhibiting infectivity of IAV for MDCK or A549 cells when using the Phil82 strain which has a wild-type hemagglutinin. The structures of RCs are shown diagrammatically in Fig. 10. RC2 differs from RC1 in having an additional arginine, whereas RC101 differs from RC1 by the replacement of one arginine with a lysine (30). Both RC2 and RC101 were previously shown to have increased activity against HIV, compared with RC1 (14, 44, 45), and we now demonstrate similar findings with regard to IAV. The increased activity of RC2 compared with RC1 is unlikely to result merely from its greater positive charge (+5 as opposed to +4 from RC1), because RC101 has exactly the same charge as RC1.

The RCs function as minilectins. The Phil82 H3N2 strain contains a wild-type hemagglutinin and neuraminidase from a recent human strain and these envelope proteins have numerous N-linked glycosylation sites to which SP-D and other lectins can bind. We compared the antiviral activity of RCs against Phil82 with activity against two viral strains with reduced glycosylation on their envelope proteins, Phil82/BS and PR-8. Both of these strains were neutralized by the RCs, although there was some reduction in activity against PR-8. This stands in contrast to prior results with HNP-1 and 2 (see Table I). It is possible, therefore, that the lectin activity of RCs contributes to some extent to their IAV neutralizing activity in this assay format. In contrast, the loss of the single high mannose glycan from the hemagglutinin of Phil82/BS did not reduce binding.

RCs, like HNP-1 and HNP-2, induce viral aggregation. RC2 again had greater viral aggregating activity than RC1; however, RC101 did not. Although increased viral aggregating activity may contribute to the increased antiviral activity of RC2, this is apparently not the case for RC101. In the case of RC2 the increased ability to self-aggregate could result in more effective cross-linking of cell membrane receptors through self-association on the cell surface. The increased aggregating activity of RC2 could also reflect a greater ability to form trimers or to self-associate once bound to viral particles (46). The finding of significant differences in antiviral activity between RCs or other defensins based on single amino acid changes is surprising, but not unprecedented (14, 44, 45). In addition, HNP-1–3 (which differ from each other by one amino acid) differ substantially in activity against Candida (47), and HNP-3 differed from HNPs 1 and 2 in not promoting neutrophil uptake of IAV (28). HNP-3 had slightly less neutralizing activity for IAV than HNPs 1 and 2 (Table I). HNP-4 differs substantially from HNP-1–3 in its protein sequence and had somewhat lower (but still significant) neutralizing activity than HNP-1–3, HD5, or the RCs. The HBDs had lower neutralizing activity than any of the α-defensins or RCs, but HBD-2 had greater activity than HBD-1. The neutralizing activity of HBDs may still be significant when present at high levels on the respiratory epithelial surface.

Another novel finding of our study is that RCs, like HNP-1 and HNP-2, increased neutrophil and macrophage uptake of IAV. It is unclear at this point how the defensins promote viral uptake by phagocytes, although it is likely that viral aggregation is in part responsible. Further studies will need to be done to determine the mechanisms through which defensins promote viral or bacterial uptake by phagocytes and to determine whether they also increase phagocytosis by human monocytes or alveolar macrophages. In any case, the ability of RCs to act as opsonins that promote viral uptake into nonpermissive cells could become important if they can be developed into peptide therapeutics. The ability of defensins to opsonize bacteria and fungi for ingestion by alveolar macrophages has been the subject of only one other published study, which involved two α-defensins that are produced by rabbit neutrophils and alveolar macrophages. Both defensins, called either NP1 and NP2 or MCP1 and MCP2, enhanced the uptake of Klebsiella pneumoniae, Staphylococcus aureus, Bordetella bronchiseptica, and Candida albicans by rabbit alveolar macrophages (48).

One concern about the potential use of HNPs as respiratory tract therapeutics arises from their ability to bind to and inhibit the activity of SP-D. This property may in part account for the depletion of SP-D from BAL fluid in disease states or conditions characterized by chronic neutrophil inflammation. Such states include chronic smoking, chronic obstructive lung disease, and cystic fibrosis (49). We have shown that activated neutrophils result in marked depletion of SP-D from BAL fluid in vitro (8) and that this effect is only partially accounted for by the known ability of neutrophil proteases to degrade SP-D (50). This action of HNPs may account for inflammation and deterioration of lung function.
that has been observed by direct instillation of HNPs into the lungs of mice (51).

We now show that the ability of defensins to bind to SP-D and alter its functional activity appears to be confined to HNP-1–3. It is of interest that HBDs and other some α-defensins (e.g., HNP-4 and HD6) did not bind significantly to SP-D. The lack of binding of HNP-4 may relate again to its sequence divergence from HNP-1–3. The results obtained in the current and prior studies using ELISA were broadly confirmed by SPR, at least for α- and β-defensins; however, ELISA studies failed to detect the binding of RC1 and RC2 to SP-D, whereas this activity was clearly evident in our SPR studies. For technical reasons the ELISA had to be performed with defensins bound to the plate. It is possible that binding of RCs to plastic uses the same, presumably hydrophobic, components of the molecule that would otherwise participate in its binding to SP-D. In addition, having the defensins bound to the plate prevents cooperative binding or self-association among the defensins that could amplify binding to SP-D. SPR assays not only provide an independent way to confirm (or not confirm) ELISA results, they yield useful estimates of binding affinity, association and dissociation binding constants, and molar binding ratios as described in this study.

Our finding that HBDs and RCs had additive activities when combined with SP-D (or at worst did not alter activity of SP-D), are in agreement with the reduced ability of HBDs to bind SP-D, as compared with HNPs. In the early phase of infection of the respiratory tract with IAV it is very likely that HBDs and SP-D would interact because both can be produced locally by the epithelium. Our findings suggest that the reversible binding of RCs to SP-D does not impair the intrinsic antiviral properties of SP-D and may, in fact, enhance them. It is unclear why RCs bound to SP-D as compared with HNPs but did not inhibit antiviral activity of SP-D. In the early phase of infection of the lung in vivo, mouse, human cervicovaginal secretions, and BAL fluids prevent cooperative binding or self-association among the defensins; however, ELISA studies failed to detect the binding of RC1 and RC2 to SP-D, whereas this activity was clearly evident in our current and prior studies using ELISA.


Disclosures

The authors have no financial conflict of interest.

References


