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Human α- and β-Defensins Block Multiple Steps in Herpes Simplex Virus Infection

Ehsan Hazrati,* Benjamin Galen,* Wuyuan Lu,‡ Wei Wang,§ Yan Ouyang,§ Marla J. Keller,† Robert I. Lehrer,§ and Betsy C. Herold‡**

This study examined the ability of nine human defensins (HD) to protect against herpes simplex virus infection. Noncytotoxic concentrations of all six α-defensins (HNPs 1–4, HDS, and HD6) and human β-defensin (hBD) 3 inhibited HSV infection. Two other β-defensins, hBD1 and 2, lacked this protective activity. Synchronized assays revealed that HNP-4, HDS, and hBD3 interacted primarily by preventing binding and entry, whereas HNP-1 and HD5 also inhibited postentry events. Even when added several hours after entry, substantial reduction in viral gene expression ensued. Human cervical epithelial cells incubated with HNP-1 or HD5 accumulated the peptides intracellularly. Surface plasmon resonance studies revealed that HNPs 1, 2, 3, and HD5 bound HSV glycoprotein B (gB) with high affinity, but showed minimal binding to heparan sulfate, the receptor for attachment. In contrast, HNP-4 and HD6 bound heparan sulfate, but not gB. HBD3 bound both gB and heparan sulfate, but hBD1 and hBD2 bound neither. Admixture of HD5 with hydroxyethylcellulose significantly protected mice from a viral challenge lethal to controls receiving an inactive peptide or hydroxyethylcellulose alone. These findings demonstrate that HDs act at multiple steps in the HSV life cycle and support the development of defensins or defensin-like peptides as microbicides. The Journal of Immunology, 2006, 177: 8658–8666.

Epithelial cells, glands, and leukocytes produce antimicrobial proteins, including lysozyme, lactoferrin, calprotectin, and secretory leukocyte protease inhibitor (SLPI). They also secrete antimicrobial peptides, such as cathelicidins and defensins. Protective factors present in the human female genital tract provide an inherent ability to resist sexually transmitted infections (STI) (1–3).

Defensins are small, cysteine-rich cationic peptides with antimicrobial, antiviral, and immunomodulatory properties (4, 5). The human genome encodes at least 35 different defensin peptides that belong to two structurally distinct subfamilies, α- and β-defensins (4, 6). Human α-defensin genes and several β-defensin genes are clustered on the short arm of chromosome 8. Four of the six human α-defensin peptides, human neutrophil peptides (HNP)s 1–4, are found primarily in neutrophils and other leukocytes. The other two, human α-defensin 5 and 6 (HD5 and HD6), are expressed by Paneth cells in the small intestine (7). HD5 is also produced by epithelial cells of the female genital tract (8). Although 30 or so β genes exist in the human genome (6), only a few have been studied to date. Certain human β-defensins (e.g., hBD1) are expressed constitutively (9), and others (e.g., hBD2 and 3) show increased expression in response to inflammation or infection (10–12). In addition to their direct antimicrobial actions, β-defensins enhance other innate and adaptive immune responses (13–15). Several reports have indicated that α-defensins HNP1–4 and hBD2 and 3 are active against HIV (16–23).

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mouse anti-VP16 (final concentration 1:500; sc7545; Santa Cruz Biotechnology) for 1 h followed by incubation with a secondary Ab and developed as described for binding gels.

**ICP27 and gB expressions**

CaSkI cells were infected with HSV-2(G) (MOI, 1 PFU/cell) in synchronized infectivity assays and defensins were added to the medium immediately posttreatment. Cells were harvested 3 or 8 h posttreatment and expression of ICP27 and gB were determined by Western blotting using mAbs for ICP27 and gB (P1119 and P1123; Virusys). An Ab to β-actin was used to control for total protein loading. In select experiments, defensins were added at different times posttreatment (0, 2, 4, 8, or 12 h). After 2 h, cells were harvested for Western blotting 12 h postinfection, whereas controls treated cells with peptide-free buffer were harvested 2, 4, 6, and 12 h posttreatment.

**Surface plasmon resonance**

Experiments were performed on a Biacore 3000 system (Biacore AB). Running buffer (pH 7.4) contained 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% polysorbate 20, and was delivered at 50 μl/min. The HSV glycoproteins (gB1 and gB2) were dissolved in 10 mM sodium acetate (pH 5.0) and immobilized on a CM5 sensor chip by the amine coupling method after activating the chip with 400 nM ethyl-N-(3-dimethylaminopropyl)-carbodiimide hydrochloride and 100 mM N-hydroxysuccinimide. The immobilization levels of gB1 and gB2 were 790 resonance units (RU) and 1227 RU, respectively. Residual reactive groups on the biosensor were blocked with 1.0 M ethanolamine-HCl (pH 8.5). The control flow-cell compartment lacked immobilized protein but was otherwise treated as described above. Signals were corrected for nonspecific binding by subtracting the background signal. To regenerate the chips, bound ligands were removed with 10 mM HCl. Binding data obtained with defensin concentrations of 0.1–0.5 μg/ml were used to determine the k_app, k_doff, and k_d. Binding isotherms were analyzed with BIAevaluation 3.1 software, using curves fitted on the assumption of one-to-one binding. Binding of defensins to glycosaminoglycans was measured in competition binding assays. The glycosaminoglycan experiments were performed with gB1 and gB2 biosensors (described above), which did not bind these glycosaminoglycans.

**Confocal microscopy**

CaSkI cells were grown on glass coverslips in 12-well plates, infected in synchronized infectivity assay with HSV-1 (K26GFP) (MOI 10 PFU/cell), and exposed to defensins or control buffer postentry. Four hours postentry, cells were washed three times with PBS, treated for 30 min with EZ-Link sulfoBiotinimidoiodobiotin reagent (1/500 dilution) to biotinylate cell surface proteins before fixation with 4% formaldehyde. Thereafter, cells were reacted with Alexa Fluor 488-conjugated streptavidin (1/500; Invitrogen Life Technologies) either with or without permeabilizing the cells for 5 min at room temperature. Nucleic acids were detected with Alexa Fluor 647-conjugated streptavidin (1/500; Invitrogen Life Technologies). Nuclei were stained with 4’,6-diamidino-2-phenylindole, dihydrochloride (Molecular Probes). Images were examined using a Leica TCS-SP (UV confocal microscope) fitted with ×100 objective. Images were scanned and analyzed using the LSM confocal software package.

Alternatively, to monitor the uptake of peptide by epithelial cells, cells were exposed to HNP-1, FITC-conjugated HDS (conjugated by the Bio logicals from the synthetic peptide) or control buffer for 2 h. Following an extensive wash with PBS, and fixation with 4% formaldehyde for 15 min, the HNP-1-treated cells were blocked with 10% goat serum and 1% BSA and then with 5 μg/ml BD Fc Bloc (no. 553141; BD Biosciences Pharmingen), which blocks Fc-mediated binding of Abs to mouse Fc receptor bearing cells. The HNP-1-treated cells were then incubated for 2 h with a biotinylated mAb directed at HNP-1 (1/500 dilution, HM2059; Hycult Biotechnology) either with or without permeabilizing the cells for 5 min with 1% Triton X-100 (Bio-Rad). Thereafter, cells were treated with Alexa Fluor 488-conjugated streptavidin (1/500 dilution) for 30 min. Nucleic acids were detected by staining both HNP-1- and HD5-treated cells with propidium iodide (4 μM) for 15 min before examining images using a Leica TCS-SP (UV confocal microscope) fitted with ×60 objective.

**Binding of defensins to viral DNA**

HSV-2(G) DNA was isolated from infected cell lysates by phenol-chloroform extraction. To visualize whether viral DNA bound to defensins, 0.5 μg of HindIII-digested HSV-2 DNA was incubated with or without 2 μg of HNP-2 or HDS for 5 min at room temperature. These samples were loaded onto a 0.6% agarose gel and electrophoresed in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA; pH 8.3). At the conclusion of electrophoresis, the
gels were stained with 4 μg/ml ethidium bromide. A HindIII digest of λ DNA (New England Biolabs) was used as a size standard.

Spectrofluorimetry was used to examine the ability of defensins to quench ethidium bromide-induced DNA fluorescence. The experiment was performed on a GeminiXS Spectramax microplate fluorometer (Molecular Devices) in black 96-well microplates using excitation and emission wavelengths of 518 nm and 610 nm, respectively. The final assay volumes were 100 μl/well, containing 1 μg/ml ethidium bromide, 0–500 ng of HindIII-digested HSV-2 DNA ± 8 μg of defensin/well. Samples were preincubated for 5 min at room temperature before recording their fluorescence.

Mouse model of genital herpes

Murine studies were conducted with the approval of the Mount Sinai School of Medicine Institutional Animal Care and Use Committee. Female BALB/c mice (weight, 18–21 g; age, 6–8 wk) were pretreated with medroxyprogesterone acetate subcutaneously (2 mg/mouse in a volume of 50 μl). Five days later, mice received an intravaginal instillation of 40 μl of HEC that had been diluted 1/1 with PBS. A peptide-free HEC/PBS mixture was used for the control group, and the treatment groups received HEC/PBS mixture containing HD5 or a linearized inactive version of HD5. The latter was chemically synthesized by substituting all six cysteines with β-aminobutyric acid. β-Aminobutyric acid is isosteric to Cys and has been routinely used for “clean” removal of disulfide bridges in peptides and small proteins (33). The final peptide concentration was 10 mg/ml, 1% w/v. Fifteen minutes later, all mice received an intravaginal challenge with HSV-2(G) (5 log10 PFU/mouse). The inoculated mice were evaluated for 16 days for signs of clinical disease, including genital ulcers, perineal erosion, hair loss, and hind-limb paralysis. Mice were sacrificed if severe ulceration or hind-limb paralysis developed.

Statistics

GraphPad Prism version 4 was used for statistical analysis. Differences between groups were compared by unpaired two-tailed Student’s t tests. Kaplan-Meier survival curves were assessed by log-rank test. All p values <0.05 were considered significant.

Results

Defensins inhibit HSV infection

This study examined the ability of all six human α-defensins and three hBD to protect human cervical epithelial cells from infection by HSV-2. All six α-defensins significantly inhibited HSV infection at concentrations between 25 and 50 μg/ml (Fig. 1a). Of the hBD tested, only hBD3 inhibited HSV infection in a dose-dependent manner (Fig. 1b). Neither HBD1 or HBD2 blocked HSV-2 infection although these synthetic peptides were active against HIV (19). Cell viability was quantified following 48 h exposure of CaSki cells to 50 and 100 μg/ml each defensin or nonoxynol-9 (0.005 and 0.01%) as a control. No cytotoxicity was observed with any of the defensins, whereas nonoxynol-9 was toxic at both concentrations (data not shown).

Temporal characterization of antiviral activity

HSV binding, which occurs at 4°C, can be experimentally differentiated from penetration/entry, which occurs after a shift to 37°C and from postpenetration events by treating cells with a low pH buffer, which inactivates any bound but nonpenetrant virus (31). Accordingly, the defensins were added during binding (for 5 h at 4°C), penetration (for 30 min at the time of temperature shift), or immediately postpenetration (after citrate treatment) for the remaining duration of the experiment (Fig. 2a). Heparin, a competitive inhibitor of HSV binding, and acyclovir, an inhibitor of viral DNA replication, served as positive controls. As expected, heparin significantly inhibited HSV plaque formation if present during the binding period, but had little effect if added at the temperature shift (penetration) or postentry. In contrast, acyclovir was only effective if present postentry. HNP-4, HD6 and hBD3 significantly inhibited binding and penetration, whereas only HNPs 1, 2, 3, and HD5 were also active if added after viral entry was completed (Fig. 2b). Similar results were observed with clinical isolates of HSV-2 (data not shown).

To more specifically evaluate the impact of defensins on the different steps in viral infection, we conducted synchronized experiments and compared the relative number of viral particles bound (binding); the nuclear transport of VP16, a surrogate marker for entry; and viral protein expression in the absence or presence of each defensin (Fig. 2a). In the binding studies, virus was added to peptide-treated cells and incubated for 5 h at 4°C. After removing unbound virus by washing, cell lysates were prepared and bound virus was examined in Western blots (38). To assess the effect of defensins on entry, peptides were added at the time of temperature shift, and the nuclear transport of VP16 was assessed in nuclear extracts prepared 3 h later (27). To evaluate postentry effects, defensins were added immediately after citrate treatment, and the expression of representative immediate early (ICP27) and late (gB) genes was compared in cell lysates that were prepared at 3 or 8 h (39).

Fig. 3 shows representative blots and data derived from scanning them. Consistent with the plaque assays (Fig. 2b), each of the defensins reduced viral binding (top panel) and entry (middle panel), whereas heparin only inhibited viral binding. Additionally, when HNPs 1, 2, and 3 or HD5 were first added after citrate treatment, they decreased viral gene expression, as shown by the reduction in ICP27 in the 3 h- and of gB in the 8 h-cell lysates (bottom panel). The extent of anti-viral activity observed for individual defensins during different steps in viral infection differed when comparing results obtained from synchronized plaque assays (Fig. 2) with those obtained in Western blot assays (Fig. 3). For example, hBD3 had greater effects on entry than binding in Western blot assays, whereas the effects on binding were greater than entry in the synchronized plaque assays. HDS consistently showed the greatest anti-viral activity at all steps in both assays. Despite the observed differences, the results obtained from either assay support the contention that defensins block multiple steps in HSV infection.
Effects on viral gene expression

The postentry effects of defensins on viral gene expression suggested that they might block viral DNA transcription and/or protein synthesis. To explore these possibilities, the kinetic studies were extended by introducing defensins at different times after citrate treatment. We focused on HNP-1 and HD5 for these studies, as they demonstrated the greatest postentry effects. Shortly after infection, HSV DNA enters the nucleus where it is transcribed. Thereafter, coordinated viral protein synthesis proceeds in the cytoplasm. Synthesis of ICP27 is detected 2–4 h postinfection and gB is detected by 8 h (Fig. 4a, right and left, respectively). Accordingly, we added HNP-1 or HD5 0, 2, 4, 6, or 8 h after citrate treatment, and prepared cell lysates 12 h postcitrate treatment. When defensins were added immediately or 2 h after infection, reduced ICP27 expression (relative to ICP27 detected 12 h postinfection in untreated cells) ensued (Fig. 4b, right). However, this effect was diminished if the defensins were added 4 h postinfection. Both defensins blocked gB expression if added up to 4 h postcitrate, but not if added 8 h postcitrate treatment (Fig. 4b, left). The number of viral plaques was significantly reduced if HNP-1 and HD5 were added up to 8 h postinfection (Fig. 4c), but not if added 12 h postinfection (Fig. 4c and d, respectively). No reduction in viral plaques was observed if HNP-4 or HD6 were added postinfection, which is consistent with results obtained in Figs. 2 and 3. The observation that viral plaques were reduced even when HNP-1 and HD5 were initially added 8 h postinfection, a time when minimal effects on gene expression were observed, suggests that other late stage events in viral replication may also be inhibited by defensins.

Surface plasmon resonance studies

Except for hBD1 and hBD2, which lacked activity against HSV-2, all of the other defensins tested reduced viral binding (Fig. 2). To obtain insights into the mechanism, we studied the binding of defensins to viral and cell surface components implicated in HSV-2 binding. Viral envelope glycoproteins B and C bind to heparan
sulfate, the major cell surface receptor for HSV attachment. Glycoprotein B plays the predominant role for HSV-2 (38), whereas both gC and gB contribute to the binding of HSV-1 to cells (31). Moreover, for both serotypes, gB is essential for viral penetration, although its precise role in this process has not been fully elucidated (38). Thus, we examined the ability of HDs to bind recombinant gB from HSV-1 and HSV-2. In studies conducted with 1 μg/ml each defensin (Fig. 5), HNP-4, HD6, hBD1, and hBD2 bound minimally to either gB1 or gB2. In contrast, HNPs 1, 2, and 3 bound both gB1 and gB2, with HNP-1 showing significantly increased binding if added up to 8 h postcitrate treatment as indicated by the asterisks ($p < 0.05$).

Heparan sulfate is the primary receptor for HSV binding. Because defensins might inhibit HSV binding by interacting with heparan sulfate or a structurally similar glycosaminoglycan, we performed a series of surface plasmon resonance experiments that also examined the binding of HDs to five glycosaminoglycans: heparan sulfate, heparin, chondroitin sulfate A and B, and chondroitin 6-sulfate. HNP-1 bound none of these molecules effectively, even when the glycosaminoglycans were present in 100-fold excess by weight (Fig. 6a). HNP-4 bound four of the glycosaminoglycans (heparin > heparan sulfate > chondroitin sulfate A > chondroitin 6-sulfate) with a 1:1 weight ratio, but did not bind chondroitin 6-sulfate (Fig. 6b). HBD3 showed extensive binding to heparin, even when the peptide and heparin were present at a 1:1 weight ratio (Fig. 6c). HBD3 also bound heparan sulfate, but to a considerably lesser extent. Other studies revealed that HD6 bound to heparan sulfate and to heparin (data not shown). Thus, defensins that inhibited HSV-2 binding bound heparan sulfate (HNP-4, HD6 and hBD3) or else they bound to gB-2 (HNPs 1, 2, 3, and HD5) or to both (hBD3), whereas defensins that failed to inhibit binding bound neither gB nor heparan sulfate (hBD1 and hBD2).

Confocal microscopy

We focused on the postentry effects seen most prominently with HNP-1–3 and HD5 (Figs. 2–4). Although a mixture of HNPs 1–3 can be taken up by human CD8+ T cells (20–22), it is not known if epithelial cells also manifest this behavior. First, we used confocal microscopy to examine CaSki cells that had been incubated for 2 h with HNP-1 (50 μg/ml) or control buffer (Fig. 7, a–c). The cells were fixed and then treated with a biotinylated mAb to gB1–3, HD5, or HBD3 ($p < 0.01$, unpaired Student’s $t$ tests).
HNP-1 with or without first permeabilizing the cells. Nonpermeabilized cells displayed surface-associated defensin, whereas permeabilized cells also revealed intracellular defensin. To determine whether HD5 also accumulates intracellularly, FITC-conjugated HD5 was prepared from the synthetic protein. In pilot studies we confirmed that the FITC-conjugated peptide retained anti-HSV activity. CaSki cells were incubated with buffer or 50 ng/ml FITC-labeled HD5 (Fig. 7, d and e). FITC-labeled HD5 was easily detected both in the cytoplasm and nucleus of the cells. Intracellular accumulation of defensins was also observed if HNP-1 or FITC-labeled HD5 were added 1–2 h postinfection (data not shown).

The observation that defensins accumulate intracellularly coupled with their ability to block viral gene expression and their known cationicity, suggests that some of the postentry anti-HSV activity may be mediated by interactions of defensins with viral DNA. We demonstrated that defensins bind to mammalian DNA by surface plasmon resonance assays (data not shown). Agarose gel electrophoresis revealed an interaction between HSV DNA and defensins; 0.5 ng of HindIII-digested HSV-2 DNA was incubated with or without 2 µg of HNP-2 or HD5 for 5 min before being electrophoresed and then stained with ethidium bromide. Fluorescence was evident only in lanes that contained DNA without defensin as shown for HNP-2 in Fig. 8a. Seeking to explain the disappearance of the viral DNA’s fluorescence, we performed a spectrophotometric study, which revealed that defensins quenched ethidium bromide’s ability to render the viral DNA fluorescent (Fig. 8b). The quenching of ethidium bromide-induced DNA fluorescence by HNP-2 and HD5 parallels findings obtained with AFP, a 51-residue, DNA-binding, β-sheet peptide derived from Aspergillus giganteus (40). Ethidium bromide is a planar molecule whose fluorescence is enhanced when it intercalates between adjacent DNA bases. We speculate that binding of HNP-2 and HD-5...
to DNA hinders the intercalation of ethidium bromide, thus quenching the fluorescence. In another set of experiments, we examined the effects of defensins on viral gene expression using an HSV-1 construct that expresses a GFP-VP26 fusion protein (30). Cells were infected synchronously (MOI ~ 10 PFU/cell) and then HNP-1 or HD5 or defensin-free buffer was added immediately after citrate treatment. Four hours after citrate treatment, viral GFP was readily detected in the nucleus of cells infected with HSV in the absence of defensins. In contrast, little or no GFP was detected in cells treated with either HNP-1 or HD5 immediately postcitrate treatment (Fig. 9). HD6, used as a negative control, failed to block viral GFP expression (data not shown). Because this experiment was performed with an HSV-1 construct, in addition to confirming that HNP-1 and HD5 block viral gene expression, it indicates that the effects of defensins on herpes virus gene expression are not viral serotype specific.

The occurrence of postentry inhibition of HSV-1 was further confirmed in a synchronized plaque assay where each of the α-defensins was added postcitrate treatment (Fig. 10). Cells treated with HNPs 1, 2, and 3 or HD5 showed a significant reduction in PFU relative to controls (p < 0.05) with HD5 showing the greatest effects, >95%. Little or no reduction in PFU was observed in cells treated postentry with either HNP-4 or HD6.

**Cumulative anti-HSV activity of defensins**

The observation that defensins have multiple effects on HSV infection supports their potential usefulness as vaginal microbicides.

To explore this notion, plaque assays were modified so that the defensins were present throughout the course of infection, as may be achievable if formulated for vaginal delivery. CaSki cells were exposed to each peptide and then challenged with serial 10-fold dilutions of HSV-2(G) (MOI 0.01–1000 PFU/cell). After 2 h, the inoculum was removed and the cells were overlaid with fresh medium containing the same concentration of peptide. Virus yields were determined after counting plaques 48 h later. Consistent with their postentry activity, HD5 and HNP-1 inhibited HSV infection over 10-million-fold (>7 logs) at a concentration of 100 μg/ml. In contrast, defensins with little postentry effect inhibited viral infection by only 2–3 logs (Fig. 11).

**In vivo protection from genital herpes virus infection**

Given these encouraging in vitro results, we used a murine genital herpes model to test the ability of defensins to protect against HSV-2 in vivo. Because it consistently had the greatest anti-HSV activity, we focused on HD5 in these studies. The active preparation contained HEC (50% v/v final) and either HD5 or linearized HD5 diluted in PBS (final peptide concentration 1% w/v). The placebo contained a 1:1 mixture of HEC with PBS. The linearized HD5 had no anti-HSV activity in vitro (data not shown). All HSV-2 challenged, placebo-pretreated mice developed genital herpes and succumbed to the infection by day 14 (10/10). In contrast, 9/13 (70%) of HSV-2 challenged mice pretreated with the HD5 preparation were protected from disease, whereas only 1/5 (20%) of those treated with the inactive linearized peptide were protected (p = 0.0005 and 0.04, log-rank test, HD5 vs HEC and vs linearized HD5, respectively) (Fig. 12).

**Discussion**

In the present study, all six human α-defensins and three hBDs were examined individually for their ability to block HSV binding, penetration, and postentry events. Surface plasmon resonance studies demonstrated that only four α-defensins (HNPs 1, 2, 3, and HD5) bound gB2 with high affinity, yet all six inhibited HSV binding. Because binding involves the interaction of gB2 with its receptor, heparan sulfate, we tested the ability of the defensins to bind this and other glycosaminoglycans. HNPs 1, 2, 3, and HD5 bound neither heparan sulfate nor other glycosaminoglycans. In contrast, HNP-4 and HD6 bound both heparan sulfate and heparin, but showed minimal binding to gB2. Thus, α-defensins may interfere with HSV binding either by binding gB2 or the heparin sulfate cell surface receptor. This duality could account for the strong protective activity of hBD3; the only HD that bound both heparan sulfate and gB2. Neither hBD1 nor hBD2 inhibited HSV.
binding or infection, although both synthetic peptides are fully functional in other assays. For example, hBD1 and hBD2 are chemotactic for monocytes, inhibit R5 and X4 HIV infection in a dose-dependent manner, and are bactericidal against Escherichia coli (19, 33, 41).

Our experiments also revealed that four α-defensins (HNP-1, HNP-2, HNP-3, and HD5) and the β-defensin, hBD3, inhibited viral penetration, when they were added after binding had occurred. In contrast, the α-defensins, HNP-4 and HD6, which failed to bind gB, lacked this ability. These findings suggest that the ability to block penetration maps, in part, to the affinity for gB. HSV entry is a complex process that requires the concerted activities of gB, glycoprotein D (gD) and hetero-oligomers of glycoproteins H and L (gH-gL). The effects of individual defensins on these other essential glycoproteins (gD, gH-gL) and on viral coreceptors remain to be studied. However, because all of the glycoproteins are glycosylated and HNPs 1, 2, 3, and HD5 are lectins that bind N-linked and O-linked glycans, it seems plausible that defensins may also bind these other essential glycoproteins.

Additionally, both gB and gH contain a pair of heptad repeats (HR1 and HR2) and it was recently reported that a peptide mimetic of HR2 can inhibit HSV-1 infection in a specific and dose-dependent manner (42, 43). Thus, a process akin to the 6-helix bundle formation implicated in the HIV-1 fusion/entry mechanism may also operate in herpesvirus entry (44). Given the recent report that α-defensins inhibit HIV-1 by preventing 6-helix bundle formation (45), it will be interesting to determine whether α-defensins affect entry by interacting with the HRs of HSV glycoproteins.

The ability of several α-defensins to inhibit HSV gene expression replication even when added after entry has occurred is of considerable interest. An ability of HNP-1 to accumulate within the cytoplasm of cells that do not synthesize it was noted in other studies, including those of human CD4+ T cells (20, 21) and smooth muscle cells (46). These studies expand on those earlier observations and demonstrate that both HD5 and HNP-1 accumulate intracellularly in epithelial cells. Learning how defensins enter and accumulate intracellularly and what regulates their uptake requires further study. Possibilities include receptor-mediated uptake, receptor-independent endocytosis, or transport across channels. In addition, because defensins such as HNP-1 bind to serum glycoproteins, some of which, such as transferrin, are endocytosed, defensin uptake may occur indirectly by “piggyback.” Notably, defensins also bind directly to viral DNA, which, coupled with their ability to accumulate intracellularly, may provide a mechanism to explain their ability to block viral gene expression. Precisely where defensins bind DNA, whether they bind viral DNA more effectively than DNA of mammalian origin, and whether they also directly interact with viral RNA requires further study.

The current studies also illustrate the specificity and diversity of defensin-mediated antiviral activity. We found that HDs inhibited HSV at different stages of the viral life cycle and did so by different mechanisms. Whereas all of the α-defensins and hBD3 inhibited HSV binding and entry, hBD1 and hBD2 had no anti-HSV activity and only HNPs 1, 2, 3, and HD5 blocked infection postentry. The mechanisms of defensin-mediated postentry anti-HSV activity may differ from those described for other viruses, such as HIV-1. It was suggested that HNP-1 may inhibit HIV-1 infection in CD4+ T cells postentry by interfering with PKC activity. This inference was based on observing that pretreating the T cells with a PKC activator, bryostatin 1, partially reversed the anti-HIV activity of HNP-1 (16). In contrast, we found that pretreating human epithelial cells with bryostatin 1 had no impact on the anti-HSV activity of HNP-1 (data not shown).

6-Defensins and hBD3 are believed to block influenza infection by a mechanism directly related to their ability to cross-link cell membrane glycoproteins into a fusion-resisting barricade (47). Defensins inhibit human papillomavirus (HPV) infection postentry by yet another mechanism preventing viral escape from endocytic vesicles (48). Notably, similar to our findings with HSV, HD5 and HNP1–3 showed the greatest inhibitory activity against HPV. HPV remained susceptible to defensins for hours after initial binding to cells. Kinetic and confocal microscopy studies suggest that the defensins prevent HPV escape from endocytic vesicles. Together these findings indicate that the mechanisms of anti-viral activity of defensins are complex and vary depending on specific peptide, cell type, and virus.

The extent to which defensins contribute to innate immunity against STI is not yet known. Although the concentration of defensins in the fluid phase of vaginal secretions is believed to be in the low microgram range (2), defensins such as HNPs 1, 2, 3, HD5, and hBD3 have a high affinity for cell surface glycoproteins that could lead to much higher local concentrations on or near cell surfaces. The ability of defensins to enter epithelial cells shown in this study could also position these peptides to contribute to postentry antiviral resistance.

Several studies in transgenic mice provide evidence that intestinal α-defensins contribute to antimicrobial defense. Oral challenge experiments have been performed in mice that either express transgenic human HD5 or are deficient in a processing enzyme that converts inactive intestinal prodefensins to active α-defensins (“cryptdins”). HD5-expressing mice were more resistant (49) and intestinal defensin-deficient mice were less resistant (50) to challenge with virulent Salmonella typhimurium. In humans, accumulating evidence has linked deficient production of HD5 and HD6 to Crohn’s disease (51).

Although the contribution of endogenous defensins to antiviral defenses in vivo has not been established, epidemiological studies have begun to support a role for defensins in host defense against HIV. A nested case-control study of 32 HIV-positive women who transmitted HIV to their infants and 52 randomly selected HIV-positive women who did not transmit HIV to their infants was conducted in Lusaka, Zambia. After adjustment for milk HIV RNA quantity, α-defensin concentration was significantly associated with a decreased risk of intrapartum and postnatal HIV transmission (odds ratio = 0.3, 95% confidence interval: 0.09–0.93) (52).

As the levels of defensins in vaginal secretions may be lower than those required to completely block viral infection in vitro, the findings suggest that exogenous defensins could potentially be developed as vaginal microbicides to reinforce their innate activity. The postentry effects for HIV, HSV, and HPV are especially exciting as they suggest that vaginally applied defensins might provide protection even if applied postexposure. The ability to inhibit multiple pathogens and to act at multiple steps in the viral life cycle is another potential advantage of defensins as candidate microbicides. As a “proof of concept,” we pretreated mice with HD5 that had been premixed with HEC gel, and then challenged with virus. Although the amount of defensin delivered under these suboptimal circumstances is unclear, the defensin provided significant protection. These findings are encouraging and support formulation of synthetic defensin or defensin-like peptides as vaginal microbicides. Advancing defensins as therapeutics will require de-
Disclosures

The authors have no financial conflict of interest.

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