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Direct Inactivation of Viruses by Human Granulocyte Defensins†

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Human neutrophils contain a family of microbicidal peptides known as defensins. One of these defensins, human neutrophil peptide (HNP)-1, was purified, and its ability to directly inactivate several viruses was extensively tested. Herpes simplex virus (HSV) types 1 and 2, cytomegalovirus, vesicular stomatitis virus, and influenza virus A/WSN were inactivated by incubation with HNP-1. Two nonenveloped viruses, echovirus type 11 and reovirus type 3, were resistant to inactivation. Purified homologous peptides HNP-2 and HNP-3 were found to have HSV-1-neutralizing activities approximately equal to that of HNP-1. Inactivation of HSV-1 by HNP-1 depended on the time, temperature, and pH of incubation. Antiviral activity was abrogated by low temperature or prior reduction and alkylation of the defensins. Addition of serum or serum albumin to the incubation mixtures inhibited neutralization of HSV-1 by HNP-1. We used density gradient sedimentation techniques to demonstrate that HNP-1 bound to HSV-1 in a temperature-dependent manner. We speculate that binding of defensin peptides to certain viruses may impair their ability to infect cells.

Granulocytes, especially polymorphonucleated neutrophils (PMN), are key effector cells involved in host defense. Long recognized as participants in the restriction of bacterial and fungal infections (11, 13, 26), these cells have also been shown to inhibit viral replication (1, 7, 8, 18–21). PMN predominate in herpetic lesions (9, 15, 28) and may prevent viral spread by a variety of mechanisms. Human PMN have been shown to carry out antibody-dependent cellular cytotoxicity against herpes simplex virus (HSV)-infected target cells (16, 21, 25). Bovine granulocytes release an interferonlike antiviral substance in response to incubation with certain viral antigens (18, 19). It is also likely that PMN participate in antiviral defenses by phagocytosis of extracellular opsonized viral particles (1).

Both oxidative and nonoxidative mechanisms are used by granulocytes to restrict microbial infections. H_2O_2, O_2^-, HOCl, and reactive chloramines are examples of microbicidal agents that are derived from PMN oxidative metabolism (2, 11, 26). The granules of PMN also contain diverse, oxygen-independent antimicrobial substances such as lysozyme, chymotrypsinlike cationic proteins, and the recently described defensins (4–6, 27). The human defensin family includes three small (molecular weight, ~3,500) peptides from human granulocytes named human neutrophil peptide (HNP)-1, HNP-2, and HNP-3, which demonstrate antibacterial, antifungal, and antiviral properties in vitro. The human neutrophil defensins are 29 or 30 amino acids long and identical in amino acid sequence except at their amino-terminal residue (24). These peptides share significant sequence homology with a family of microbicidal peptides purified from rabbit PMN and alveolar macrophages (23). Two of the purified rabbit defensins, MCP-1, and MCP-2, were shown to have direct neutralizing activity against a variety of animal viruses including HSV (12). We examined and now report the antiviral effects of the HNPs.

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MATERIALS AND METHODS

Peptides. HNPs were purified to homogeneity from human neutrophils as previously described (6). Purity was assessed by analytical high-performance liquid chromatography and visualization on acid urea-polyacrylamide gels stained with Coomassie brillant blue (17). Peptide concentrations were determined by amino acid analysis.

Reduction and alkylation of HNP 1-3. Lyophilized HNP 1-3 (a purified mixture of HNP-1, HNP-2, and HNP-3 in approximately 2:2:1 relative abundance) was dissolved to a concentration of 1 mg of peptide per ml in buffer containing 6 M guanidine hydrochloride, 0.1 M Tris hydrochloride (pH 8.5), and 2 mM EDTA. The solution was heated to 50°C for 30 min and then cooled to room temperature. Dithiothreitol was then added to a concentration of 1 mg/ml, and the mixture was incubated for 4 h at 50°C under N_2. The solution was brought to room temperature and iodoacetamide, in threefold molar excess with respect to dithiothreitol, was added. After a 20-min incubation in the dark, the sample was repurified and desalted by reversed-phase high-performance liquid chromatography as previously described (6).

Iodination of HNP-1. At room temperature, 1 mCi of sodium ^{125}I (Amersham Corp., Arlington Heights, Ill.; 13.9 mCi/μg) and two iodobeads (Pierce Chemical Co., Rockford, Ill.) were added to a solution of HNP-1 (2 mg/ml) in 50 mM sodium phosphate buffer (pH 7.0). An additional iodobead was added after 30 min, and incubation was continued for another 60 min at room temperature. Labeled HNP-1 was then separated from free iodide by reversed-phase high-pressure liquid chromatography. The iodinated HNP-1 had an activity of 8×10^7 cpm/mg.

Cells and viruses. The viruses listed in Table 1 were purchased from the American Type Culture Collection, Rockville, Md., except for influenza virus A/WSN, which was a gift from Debi Nayak (University of California Los Angeles), and vesicular stomatitis virus, which was provided by the Cetus Corp. (Berkeley, Calif.). All virus stocks were grown in Vero monkey kidney cell monolayers except for influenza virus, which was grown in Madin-Darby bovine

TABLE 1. Direct inactivation of viruses by HNP-1

Virus (strain)	Mean (±SD) $\log_{10}$ reduction in PFU/ml (no. of expts) by HNP-1 at:			
	25 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml
HSV-1 (MacIntyre)	2.03 ± 0.38 (12)	2.89 ± 0.17 (4)	3.04 ± 0.54 (3)	ND[a]
HSV-2 (MS)	0.77 ± 0.48 (3)	1.15 ± 0.14 (2)	2.04 ± 0.53 (3)	ND
Vesicular stomatitis virus (Indiana)	0.41 ± 0.25 (3)	0.74 ± 0.22 (3)	0.86 ± 0.26 (2)	ND
Influenza virus (A/WSN)	0.36 ± 0.07 (3)	0.50 ± 0.03 (4)	0.74 ± 0.04 (2)	ND
Cytomegalovirus (AD 169)	−0.02 ± 0.20 (3)	0.09 ± 0.08 (3)	0.29 ± 0.16 (3)	0.81 ± 0.28 (4)
Echovirus type 11 (Gregory)	−0.09 ± 0.08 (2)	0.04 (1)	−0.01 ± 0.01 (2)	ND
Reovirus type 3 (Dearing)	0.13 ± 0.04 (2)	0.13 ± 0.08 (2)	0.07 ± 0.06 (3)	0.19 (1)

[a] ND, Not determined.

kidney cells, and cytomegalovirus, which was grown in MRC-5 human embryo lung cells. Vero and MRC-5 cells were obtained from the American Type Culture Collection. Madin-Darby bovine kidney cells were a gift from D. Nayak. Virus-infected cells were cultured in Eagle minimal essential medium (MEM; Irvine Scientific, Santa Ana, Calif.) supplemented with 2% fetal calf serum (FCS; Irvine Scientific), penicillin G (100 U/ml), and streptomycin (100 μg/ml). Serum-free viral stocks were prepared by rinsing virally infected cells twice with phosphate-buffered saline (PBS) at pH 7.4 and then collecting the cells into PBS or 1 mM phosphate (pH 7.4). Infected cells collected into PBS were lysed by two cycles of freeze-thawing. Infected cells collected into 1 mM phosphate were kept on ice for 30 min to allow hypotonic lysis and were further disrupted by mild Dounce homogenization. All lysates were then cleared of cells and debris by centrifugation at 800 × *g* for 15 min at 4°C. All virus preparations were stored under liquid nitrogen until used.

Direct inactivation. Various concentrations of defensins (in PBS [pH 7.4] unless otherwise noted) were mixed with 5 to 40 μl of serum-free virus stock to a final volume of 200 μl. Control mixtures without defensins were similarly set up and incubated in parallel. Unless otherwise noted, incubations were carried out for 60 min at 37°C. After the incubation period, the mixtures were placed on ice and immediately serially diluted into MEM containing FCS (2%), penicillin (100 U/ml), streptomycin (100 μg/ml), and HEPES buffer (20 mM, pH 7.4). The serial dilutions were plated on appropriate tissue culture cell monolayers for determination of surviving PFU/ml.

HSV-1 yield reduction assays. Vero or MRC-5 cells were grown to confluency in 12-well tissue culture plates (Costar, Cambridge, Mass.) and thereafter maintained with 1.0 ml of viral growth medium (VGM) per well, which consisted of MEM containing FCS (2%), penicillin G (100 U/ml), streptomycin (100 μg/ml), and HEPES (20 mM, pH 7.4). Cells were treated with HNP-1 in VGM with or without FCS or mock treated with identical medium lacking HNP-1 for timed intervals as described in the Tables. After treatment, the cells were rinsed twice with PBS and then incubated in VGM. Viral inoculation was carried out by diluting HSV-1 (MacIntyre) into VGM and adding the suspension to the cell monolayers in a final volume of 250 μl at a multiplicity of infection of 1.0 PFU per cell. After 90 min at 37°C, the inoculum was removed and the cells were rinsed twice with PBS. VGM (1.0 ml) was added to each well, and the plates were incubated for 10.5 (Vero) or 22 (MRC-5) h after initiation of infection. Virus was harvested by scraping the cells into the medium and collecting the entire culture. The culture was freeze-thawed twice, and the HSV-1 titer was determined by plaque assay on Vero cell monlayers.

Preparation of labeled HSV-1. Confluent monolayers of Vero cells were infected with HSV-1 at a multiplicity of infection of 2.0. Infected cells were cultured in VGM until 6 h postinfection, when the medium was replaced with fresh VGM containing [^{3}H]thymidine ([^{3}H]TdR; 0.67 μg/ml, 18 μCi/ml; New England Nuclear Corp., Boston, Mass.). After an additional 24 h of culturing, the infected cells were rinsed twice with PBS, scraped from the flask, and collected into PBS. The cells were subjected to two cycles of freeze-thawing to release virus and were then pelleted at 5,865 × *g* for 15 min at 4°C. Nycodenz (Accurate Chemical and Scientific Corp., New York, N.Y.) density step gradients were prepared by layering 1-ml volumes of 40, 35, 30, 25, 20, 15, and 10% (wt/vol) Nycodenz in 0.13 M NaCl,–5 mM Tris hydrochloride–3 mM KCl–0.3 mM EDTA (pH 7.4) into 10-ml polyallomer centrifuge tubes. Supernatant virus (2.5 ml) was layered over the Nycodenz solutions, and the gradient was centrifuged at 37,000 × *g* for 3 h at 4°C in an SH-80 rotor (Ivan Sorvall, Inc., Norwalk, Conn.). The centrifuge tube containing the gradient was punctured at its bottom, and the gradient was collected dropwise. HSV-1-containing fractions were pooled and dialyzed against PBS containing penicillin (100 U/ml) and streptomycin (100 μg/ml). The dialyzed [^{3}H]TdR-labeled HSV-1 had a titer of 1.6×10^5 PFU/ml and was labeled to 1.5×10^4 cpm/ml.

RESULTS

Purity and relative antiviral activity of defensins. The purification and biochemical characterization of defensin peptides have been described in detail elsewhere (6). The inset to Fig. 1 demonstrates the purity of the defensins used in the experiments described herein. For comparison, an extract of whole neutrophils is shown. The extent of HSV-1 inactivation by various doses of the defensins is shown in Fig. 1. Each of the defensins and a mixture of the defensins (HNP 1-3) had potent activity against HSV-1. The three peptides had approximately equivalent HSV-1-neutralizing activity and showed no synergy with each other as evidenced by a lack of increase in activity of the HNP 1-3 mixture (Fig. 1).

Importance of native structure. To determine the importance of native structure on the antiviral properties of the defensins, HNP 1-3 was reduced with dithiothreitol and alkylated with iodoacetamide. Such treatment alters conformation by thiolytically cleaving the three disulfide bonds of HNPs and preventing their reformation without affecting the polypeptide net charge. Under conditions wherein native HNP 1-3 reduced HSV-1 infectivity by 99.7%, equal amounts of reduced and alkylated HNP 1-3 had no antiviral effect. These findings demonstrated that the cationic charge of HNP 1-3 was insufficient to confer antiviral properties on

these peptides and suggest that the molecular topology required for antiviral activity is conferred, at least in part, by intramolecular disulfides.

Spectrum of antiviral activity. One of the defensins, HNP-1, was chosen for more extensive characterization of its antiviral properties. Our findings are summarized in Table 1. HSV-1 and HSV-2 were both very susceptible to inactivation by HNP-1. In contrast, cytomegalovirus was inactivated only by relatively high doses of HNP-1 (100 and 200 μg/ml). The two enveloped RNA viruses tested, influenza virus and vesicular stomatitis virus, showed similar and intermediate susceptibility to HNP-1. HNP-1 was inactive against echovirus or reovirus, both nonenveloped viruses. The results shown in Table 1 represent means of independent experiments, each performed with duplicate or triplicate samples. Within-assay coefficients of variation (standard deviation divided by the mean) were always less than 20%.

Modulating factors. The ability of HNP-1 to inactivate HSV was greatly influenced by incubation temperature (Fig. 2). Although virtually no inactivation occurred at 0°C, inactivation was markedly increased as more physiologic temperatures were used. Note that much greater inactivation occurred at 42 then 37°C. The effect of temperature was also studied in temperature shift experiments wherein paired samples of HSV-1 were treated with 25 μg of HNP-1 per ml and incubated at 37°C. After 0, 10, or 30 min, one of the pair was shifted to incubation at 0°C for the remainder of the 1-h total incubation period while the other remained at 37°C. After 1 h, all samples were chilled and serially diluted, and the surviving PFU/ml were determined. We found that shifting the sample to 0°C greatly slowed the ongoing process of viral neutralization (Fig. 3). Figure 3 also demonstrates the time dependence of the inactivation process.

Inactivation of HSV-1 by HNP-1 was modestly influenced by incubation pH (Fig. 4). HNP-1 was approximately equally effective between pHs 7.0 and 8.5, but its activity fell off at pH 6.0. Studies at lower pHs were precluded by the acid

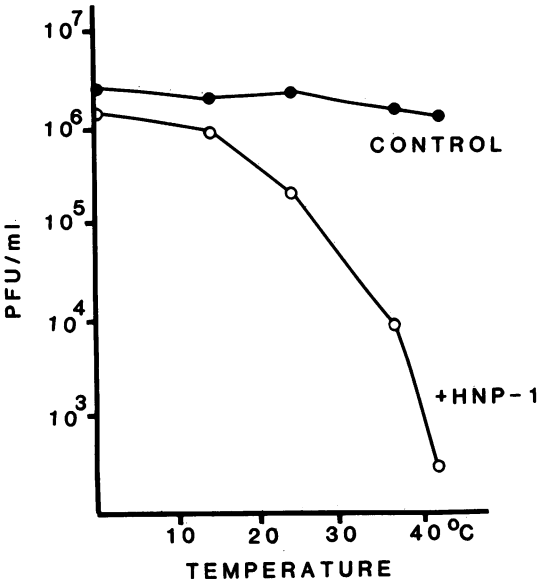

FIG. 2. Effect of temperature on inactivation of HSV-1 by HNP-1. HSV-1 was incubated with (○) or without (●) 25 μg of HNP-1 per ml in PBS, pH 7.4, for 60 min at the indicated temperatures. Surviving PFU/ml were then determined.

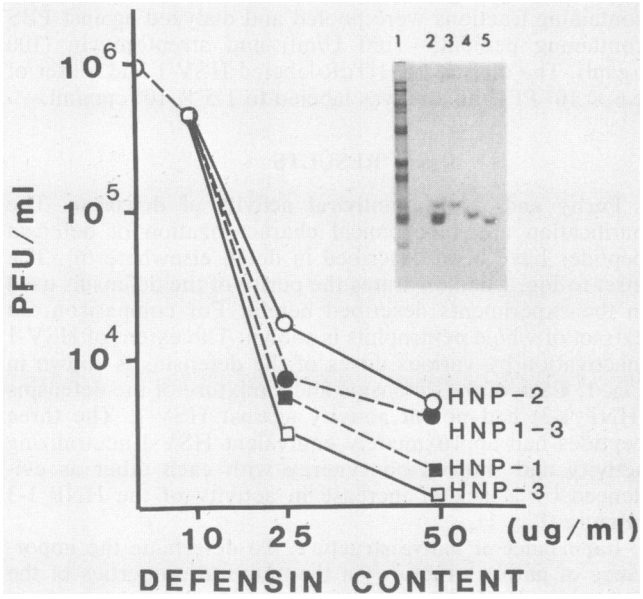

FIG. 1. Comparison of the anti-HSV-1 activities of HNP-1, HNP-2, HNP-3, and HNP 1-3 (a mixture of the three defensins). HSV-1 was incubated with the indicated concentrations of defensins in PBS, pH 7.4, at 37°C for 60 min, and surviving PFU/ml were determined. Inset: acid urea-polyacrylamide gel electrophoresis of human neutrophil peptides. Lanes: 1, crude PMN extract equivalent to 4×10^5 cells; 2, 6 μg of an HNP 1-3 mixture; 3, 2 μg of HNP-1; 4, 2 μg of HNP-2; 5, 2 μg of HNP-3. The gel was stained with Coomassie brillant blue.

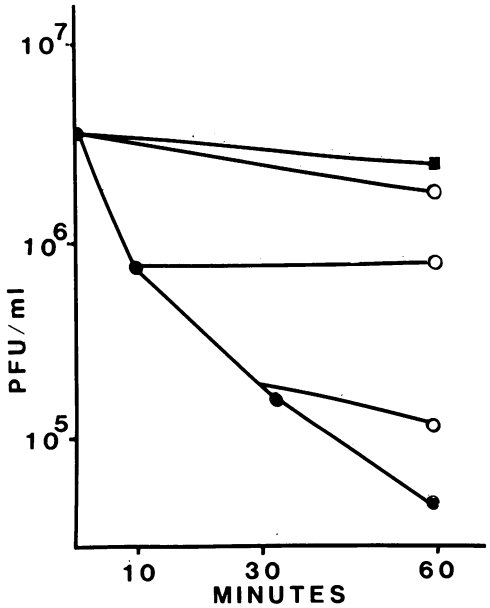

FIG. 3. Effect of temperature shift on inactivation of HSV-1 by HNP-1. HSV-1 was incubated with (●, ○) or without (■) 25 μg of HNP-1 per ml at 37 or 0°C. At the indicated times, samples were serially diluted and their titers were determined on Vero cells (●), or they were chilled in a melting ice bath and reserved until the conclusion of the 60-min incubation period, when they were also serially diluted and their titers were determined on Vero cells (○).

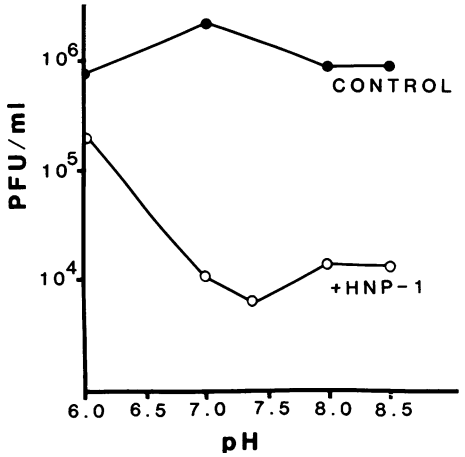

FIG. 4. Effect of pH on inactivation of HSV-1 by HNP-1. HSV-1 was incubated with (○) or without (●) 25 μg of HNP-1 per ml in 20 mM sodium phosphate buffers prepared at pHs 6.0, 7.0, 7.4, 8.0, and 8.5. After 60 min at 37°C, the mixtures were diluted and the number of PFU/ml was determined.

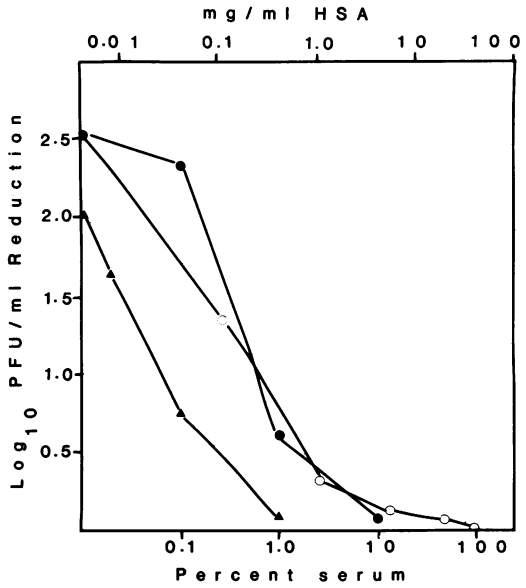

FIG. 5. Inhibition of HNP-1-mediated HSV-1 inactivation by FCS, human serum, or human serum albumin (HSA; Sigma Chemical Co., St. Louis, Mo.). HSV-1 was incubated with or without 25 μg of HNP-1 per ml in PBS, pH 7.4, containing the indicated amount of FCS (▲), human serum (anti-HSV-1 antibody negative; ●), or HSA (○). After 60 min at 37°C, the mixtures were diluted and the PFU/ml were determined. Log_{10} PFU/ml reductions were obtained by subtracting the value of the treated sample from that of the control sample without HNP-1 at the same serum or HSA concentration. The control log_{10} PFU/ml values were 6.29 and 6.30 for mixtures containing 0 or 1% FCS; 6.36, 6.45, and 6.36 for mixtures containing 0, 1.0, and 10% human serum; and 6.36, 6.49, and 6.51 for mixtures containing 0, 1.0, and 40 mg of HSA per ml, respectively.

lability of HSV-1. To ascertain the effect of ionic strength on the inactivation process, HSV-1 was treated with 25 μg of HNP-1 per ml in a buffer containing a fixed concentration (8.75 mM) of Tris hydrochloride (pH 7.4) and concentrations of NaCl ranging from 0 to 0.88 M. The extent of HSV-1 inactivation was not affected by NaCl concentrations ranging from 0 to 0.16 M. Higher NaCl concentrations inhibited the anti HSV-1 activity of HNP-1 only slightly (data not shown). The lack of marked effect of ionic strength on antiviral activity contrasts with the dependence on low ionic strength for optimal microbicidal activity against certain bacteria and fungi (unpublished data). Thus, the exact nature of HNP interactions with viruses and higher microorganisms may be different.

Effect of serum. When HNP-1 (500 μg/ml) was mixed with FCS or human serum (each at 2%), a precipitate formed. This precipitate was stable and could be washed by repeated cycles of pelleting and suspension in PBS. When washed pellets were analyzed on gradient sodium dodecyl sulfate-polyacrylamide gels, they were found to consist of HNP-1 and various serum proteins, most notably albumin (data not shown). When FCS, human serum, or human serum albumin was added to mixtures of HSV-1 and HNP-1 it inhibited the inactivation process (Fig. 5), presumably by binding the defensin molecules and reducing their availability for binding to the virus (vide infra).

Absence of cell-mediated antiviral activity. In the aforementioned experiments, direct viral neutralizing activity of defensins was tested by incubating HNPs with cell-free virus then diluting out those mixtures serially 10-fold for plaque assay titration on cell monolayers in medium containing 2% FCS. Such experiments would reveal any potential cell-mediated antiviral effects caused by defensins only if such effects could occur both in the presence of 2% FCS and at defensin concentrations from 10- to 10,000-fold lower than the concentrations used in the direct neutralization assays. To exclude the possibility that cell-mediated effects contributed to the reduction in PFU/ml seen in the previous experiments, monolayers of Vero cells were treated with HNP-1 before, during, or after infection with HSV-1 in the presence of 2% FCS. The ability of HSV-1 to grow in the treated cells was assessed by determining the PFU/ml yield of HSV-1 10.5 h after infection. Table 2 shows the lack of any anti-HSV-1 effects under these conditions. To test whether HNP-1 could directly inactivate HSV-1 in the presence of tissue culture cells and 0.15% FCS, the following experiment was done. Vero cells were inoculated with HSV-1 in the presence or absence of HNP-1 in MEM containing 0.15% FCS. After 90 min, the monolayers of cells were rinsed and MEM containing 2% FCS was added. The cells were harvested 10.5 h after infection, and viral yields were determined. The results of this experiment demonstrated that HNP-1 could effectively inactivate HSV-1 to reduce viral yield in the presence of tissue culture cells (Table 3). Finally, to test the possibility that HNP-1 may have some species-specific, interferonlike, cell-mediated antiviral activity, a human cell line, MRC-5, was treated with the peptide before and after infection with HSV-1. These cells were treated in the absence of FCS to avoid any potential inhibition of activity. Pretreatment of cells for 15 or

TABLE 2. HNP-1 treatment of Vero cells in the presence of 2% FCS: effect on HSV-1 yield

Duration (h) of exposure to HNP-1			HNP-1 concn (μg/ml)	HSV-1 yield (log_{10} PFU/ml)
Before inoculation	During inoculation	After inoculation		
0	0	0	0	7.30
24	0	0	25	7.41
0	1.5	0	25	7.18
0	0	10.5	25	7.23

2 h before infection with up to 10 μg of HNP-1 per ml for 15 h or up to 50 μg of HNP-1 per ml for 2 h had no effect on HSV-1 yield at 22 h after infection (log_{10} PFU/ml controls, 8.20 and 8.29; HNP-1 treated, 8.26 to 8.30). Treatment of cells for 4 h after infection with up to 50 μg of HNP 1 per ml also had no effect on viral yield (log_{10} control PFU/ml, 8.23; HNP-1 treated, 8.15 to 8.34). Prolonged treatment (24 h and greater) of uninfected MRC-5 cells with 50 μg of HNP-1 per ml in the absence of FCS led to increased cellular rounding and cell death.

HNP-1-binding properties and effect on viral morphology. HSV-1 particles remained intact after incubation with up 100 μg of HNP-1 per ml as judged by electron microscopy. No change in the proportion of enveloped to naked forms was observed (data not shown). After [^{125}I]HNP-1 was mixed with a semipurified preparation of [^{3}H]TdR-labeled HSV-1 at 37°C, the iodinated peptide cosedimented with HSV-1 in a density gradient (Fig. 6). When a similar incubation was carried out at 4°C, substantially less HNP-1 associated with HSV-1. Although we cannot rigorously exclude the possibility that HNP-1 binds to nonviral or noninfectious components in virus preparation which are equal in density to HSV-1, these results are consistent with the possibility that HNP-1 exerts its antiviral effects on HSV-1 by binding directly to virus particles. Moreover, the inhibition of binding at 0°C could explain the lack of direct viral neutralization at that temperature (Fig. 2 and 3). when we mixed an aqueous suspension of HNP-1 with a suspension of phosphatidylserine in chloroform-methanol, greater than 95% of the HNP-1 was found in the organic phospholipid phase. No HNP-1 was found in organic solvent in the absence of phosphatidylserine (data not shown). These data support the hypothesis that HNP-1 has the ability to bind to some phospholipids and suggest that binding to viruses may be mediated by the lipid envelope.

DISCUSSION

Each of the human defensins, HNP-1, HNP-2, and HNP-3, had potent neutralizing activity against HSV-1. HNP-1 also showed activity against HSV-2, vesicular stomatitis virus, influenza virus, and, at high concentrations, cytomegalovirus. HNP-1 did not inactivate the two nonenveloped viruses tested, echovirus and reovirus. The difference in susceptibility to inactivation between enveloped and naked viruses suggests the lipid envelope may be a site of interaction between the defensin and the virion. The marked effect of temperature on the inactivation and binding of HSV-1 by HNP-1 is consistent with but does not prove the possibility that the viral envelope and its fluidity may be important for HNP antiviral effects. Similar temperature dependency has been reported for the inactivation of certain enveloped viruses by long-chain saturated monoglycerides and alcohols (22) and amphotercin B methyl ester (10). Although we have not tested the ability of HNP-1 to bind to viruses other than HSV-1, we have observed significant binding of selected defensins to tissue culture cells, as well as to *Candida albicans* (14; unpublished data). The human defensins exert microbicidal effects against various bacteria and fungi in addition to viruses. The ability of defensins to interact with such diverse organisms could arise from their ability to interact with and perturb lipid membranes.

Inhibition of defensin-mediated antiviral activity by serum and serum proteins may place important limitations on the in vivo efficacy of defensins during viral infections. Defensins may exert their antiviral effects intracellularly after the phagocytosis of viral particles or by being secreted to the local extracellular milieu. Conceivably, high local defensin concentrations might overcome the inhibitory effects of any serum components present at the site of an infection or inside a phagolysosome. The mean volume of a PMN is 485 μm^3 (3). Since 10^9 PMN contains approximately 5 mg of defensins (6) in a total cellular volume of 0.485 ml, the mean cellular concentration of defensins in PMN would exceed 10 mg/ml if the peptides were homogeneously distributed instead of being concentrated in the cytoplasmic granules. The sequestration of defensins in granules would allow for even higher local concentrations to be achieved by degranulation

TABLE 3. HNP-1 treatment of Vero cells in the presence of 0.15% FCS: effect on HSV-1 yield

Duration (h) of exposure to HNP-1			HNP-1 concn (μg/ml)	HSV-1 yield (log_{10} PFU/ml)
Before inoculation	During inoculation	After inoculation		
0	0	0	0	7.53
0	1.5	0	10	7.30
0	1.5	0	25	5.43
0	1.5	0	50	2.00

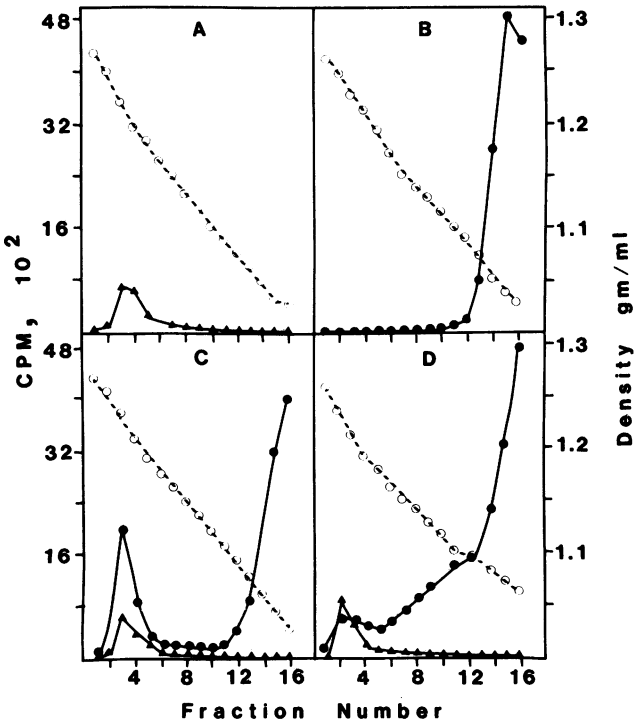

FIG. 6. Binding of HNP-1 to HSV-1. The following mixtures were prepared in PBS buffer, pH 7.4, and incubated for 60 min (panel): A, [^{3}H]TdR-labeled HSV-1 at 37°C; B, 1 μg of [^{125}I]HNP-1 at 37°C; C, [^{3}H]TdR-labeled HSV-1 and 1 μg of [^{125}I]HNP-1 at 37°C; D, [^{3}H]TdR-labeled HSV and 1 μg of [^{125}I]HNP-1 at 4°C. At the end of the incubation period, the mixtures were chilled on ice, layered onto Nycodenz density gradients, and centrifuged at 38,850 × *g* for 3 h at 4°C. Fractions were collected dropwise and counted with a Beckman LS1800 scintillation counter. A computer-aided calculation method was then applied to resolve counts per minute attributable to ^{3}H and ^{125}I separately. Symbols: ▲, counts per minute attributable to [^{3}H]TdR-labeled HSV-1; ●, counts per minute attributable to [^{125}I]HNP-1. The density (○) of each fraction was determined by measurement of the refractive index with comparison to Nycodenz standards of known density.

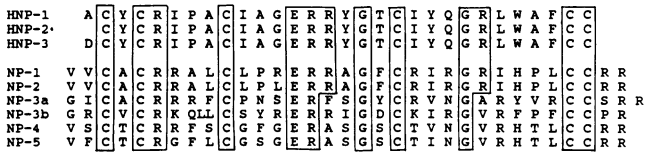

FIG. 7. Sequence comparison of human and rabbit defensins. Each sequence is represented by the single-letter amino acid symbol. The sequences were aligned to show the structural homology conferred by the 11 residues common to both the human and rabbit peptides and the additional residues conserved among the antiviral peptides. A, alanine; R, arginine; N, asparagine; D, aspartic acid; C, cysteine; Q, glutamine; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; V, valine. This figure was based on sequences published in reference 4. NP-1 and NP-2 are identical in amino acid sequence to MCP-1 and MCP-2, respectively (23).

into confined spaces such as endocytic or phagocytic vacuoles. Since the daily production of PMNs in man is estimated at 0.6×10^9 to 4.0×10^9 PMN/kg (29), a 70-kg person synthesizes at least 217 to 1,120 mg of defensins daily.

We have previously reported the direct virus neutralizing activity of MCP-1 and MCP-2, two defensin peptides derived from rabbit granulocytes and lung macrophages. The spectrum of antiviral activity of these two defensins was very similar to that of the human peptides. Four other rabbit defensin peptides, NP-3a, NP-3b, NP-4, and NP-5, showed little or no ability to directly inactivate HSV-1 (12), despite their substantially greater net cationic charge. The sequences of these peptides and those of the human defensins are shown in Fig. 7. The peptides vary from 29 to 34 amino acid residues in length and share a core of 11 conserved amino acids, including all six cysteinyl residues. An additional two arginine residues are conserved among the antiviral peptides. Also, at seven of the unmatched residues between the HNPs and MCPs 1 and 2 there are conservative substitutions of nonpolar amino acids for other nonpolar amino acids (positions 4, 8, 9, 11, 12, 26, 28, and 29; with the amino terminus of the rabbit defensins as position 1). Given their similarities in sequence and antiviral activity, these peptides are likely to show similar structural features. We expect that three-dimensional structural analyses of the defensins, now well advanced, will allow us to ascertain the essential features required for antiviral activity.

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