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Direct Inactivation of Viruses by MCP-1 and MCP-2, Natural Peptide Antibiotics from Rabbit Leukocytes†

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Six homologous peptides were purified to homogeneity from rabbit granulocytes or alveolar macrophages and tested for their ability to inactivate herpes simplex virus type 1 (HSV-1). Two of the peptides, MCP-1 and MCP-2, showed considerable in vitro neutralizing activity, whereas four structurally homologous peptides (NP-3a, NP-3b, NP-4, and NP-5) were relatively ineffective. Inactivation of HSV-1 by MCP-1 or MCP-2 depended on peptide concentration and on the time, temperature, and pH of the incubation. HSV-2, vesicular stomatitis virus, and influenza virus A/WSN were also susceptible to direct neutralization by MCP-1 or MCP-2, whereas cytomegalovirus, echovirus type 11, and reovirus type 3 were not. We speculate that MCP-1 and MCP-2, peptides that are abundant in rabbit granulocytes and lung macrophages, may contribute to antiviral defenses by mediating the direct inactivation of HSV-1 and selected other viruses.

Rabbit lung macrophages contain substantial concentrations of two unusual peptides, MCP-1 and MCP-2, which display potent in vitro antibacterial and antifungal activity (10, 11, 19, 23). Rabbit granulocytes possess six related peptides (24), two of which (NP-1 and NP-2) are identical to MCP-1 and MCP-2 by amino acid sequence analysis (M. E. Selsted, D. M. Brown, R. J. DeLange, S. S. L. Harwig, and R. I. Lehrer, J. Biol. Chem., in press). The remaining granulocyte peptides, NP-3a, NP-3b, NP-4 and NP-5, have not been detected in alveolar or peritoneal macrophages.

Because granulocytes and mononuclear phagocytes may be involved in antiviral host defenses, as well as participating in antibacterial and antifungal activities, we tested the aforementioned leukocyte peptides for in vitro antiviral activity. In this report, we describe the direct neutralization of herpes simplex virus type 1 (HSV-1) and selected other viruses by MCP-1 (NP-1) and MCP-2 (NP-2) or both.

MATERIALS AND METHODS

Peptides. Rabbit peritoneal exudate granulocytes and alveolar macrophages were processed as previously described to yield the highly purified peptides employed in this study (23, 24; Selsted et al., in press). Because MCP-1 and MCP-2 purified from alveolar macrophages are identical in structure to NP-1 and NP-2 purified from rabbit granulocytes, we will use the terms MCP-1 and MCP-2 to denote the specified peptide, irrespective of its cell of origin. NP-3a, NP-3b, NP-4, and NP-5, absent in alveolar macrophages, were prepared from granulocytes.

Viruses. The viruses listed in Table 1 were purchased from the American Type Culture Collection, Rockville, Md., except for influenza A/WSN, which was a gift from Debi Nayak of the University of California, Los Angeles, and vesicular stomatitis virus strain Indiana, which was provided by the Cetus Corporation, Berkeley, Calif. HSV-1 (McKrae) was obtained from M. Trousdale, Doheny Eye Institute, University of Southern California, Los Angeles.

Except for influenza virus A/WSN, which was grown on MDBK bovine kidney cells, and cytomegalovirus (CMV), which was grown on human embryo lung MRC-5 cells, virus stocks were prepared on monolayers of Vero cells. All cells used in this manner were obtained from the American Type Culture Collection except for MDBK cells, which were a gift from Debi Nayak. The virus-infected cell monolayers were cultured in Eagle minimal essential medium (E-MEM) supplemented with 2% fetal calf serum (Irvine Scientific, Irvine, Calif.), penicillin G (100 U/ml), and streptomycin (100 μg/ml). Stock virus preparations obtained from these monolayers were plaque titered and stored under liquid nitrogen until used.

Direct inactivation. Unless otherwise specified, stock virus preparations were exposed to pure leukocyte peptides for 60 min at 37°C in a final volume of 200 μl. In our earlier experiments, we combined 100 μl of viral stock (in E-MEM with 2% fetal calf serum and antibiotics) with an equal volume of 0.85% NaCl containing 0 to 200 μg of the desired peptide. Such mixtures had a pH of approximately 8.2 after incubation in a 37°C water bath. In subsequent experiments, this procedure was modified to secure better pH control by diluting 20 μl of the virus stock with 160 μl of either Dulbecco phosphate-buffered saline (PBS; pH 7.4) or 0.85% NaCl containing 50 mM Tris (pH 7.25 at 37°C) and adding 20 μl of peptide (typically 1 mg/ml) diluted in 1.2 mM acetic acid. This quantity of acetic acid did not significantly affect the pH. The incubation mixtures were chilled in a melting ice bath, serially diluted 10^2- to 10^6-fold and titered by plaque assay on appropriate monolayers as already noted.

Reduction and alkylation of MCP-1. Lyophilized MCP-1 (1 mg) was dissolved in 2 ml of a reducing buffer containing 6.0 M guanidine hydrochloride, 0.1 M Tris-hydrochloride (pH 8.5), and 0.002 M EDTA. The solution was heated to 50°C for 30 min and brought to room temperature, and 1.0 mg of dithiothreitol was added. This mixture was incubated under N_2 for 4 h at 50°C and then brought to room temperature, and then a twofold molar excess of iodoacetamide (with respect to dithiothreitol) was added to the solution. After a 20-min incubation in the dark, the sample was exhaustively dialyzed in the dark against 0.1% acetic acid in dialysis tubing (Spectrapor 6, Spectrum Industries) with a molecular weight cutoff of 1,000. Five percent of the sample was hydrolyzed

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TABLE 1. Direct inactivation of HSV-1 by rabbit leukocyte peptides MCP-1 and MCP-2[a]

Peptide	pH	n	$\log_{10}$ virus PFU/ml		
			Control	Treated	ΔPFU
MCP-1	8.2	2	6.74 ± 0.04	3.65 ± 0.05	3.09 ± 0.01
	7.4	5	6.17 ± 0.27	4.62 ± 0.27	1.55 ± 0.10
	7.25	2	6.90 ± 0.10	5.35 ± 0.24	1.55 ± 0.14
MCP-2	8.2	3	6.65 ± 0.09	3.68 ± 0.11	2.98 ± 0.07
	7.4	1	6.62	5.36	1.26

[a] Viruses (HSV-1 strain MacIntyre) were incubated for 60 min at 37°C with or without 100 μg of MCP-1 or MCP-2 per ml as described in the text. Viral titers were performed by plaque assays on Vero cells. Results are expressed as mean ± standard error of the mean; *n*, number of experiments.

and subjected to amino acid analysis (23) to determine recovery of peptide after carboxamidomethylation. The remaining sample was lyophilized and stored at −20°C.

RESULTS

Inactivation of HSV-1. Table 1 summarizes our findings in 13 experiments wherein HSV-1 was exposed to 100 μg of MCP-1 or MCP-2 per ml for 60 min at 37°C. Note that MCP-1 treatment reduced the mean number of surviving PFU per milliliter by 97.2%. ($\log_{10}$, 1.55) to 99.9% ($\log_{10}$, 3.09) relative to paired controls incubated without addition of peptide. All of these tests were done on HSV-1 strain MacIntyre that had been grown in Vero cells. However, subsequent studies revealed that HSV-1 strain MacIntyre grown in human GM 2504 fibroblasts (National Institute of General Medical Sciences Human Genetic Mutant Cell Repository, Camden, N.J.) or HSV-1 strain McKrae grown in Vero cells displayed similar sensitivity to MCP-1 as did our standard HSV-1 preparation (data not shown).

Table 2 shows the effects of MCP-1 and MCP-2 against six additional viruses. Although HSV-2, vesicular stomatitis virus, and influenza virus A/WSN were also susceptible to direct inactivation, CMV, reovirus (type 3), and echovirus (type 11) were resistant.

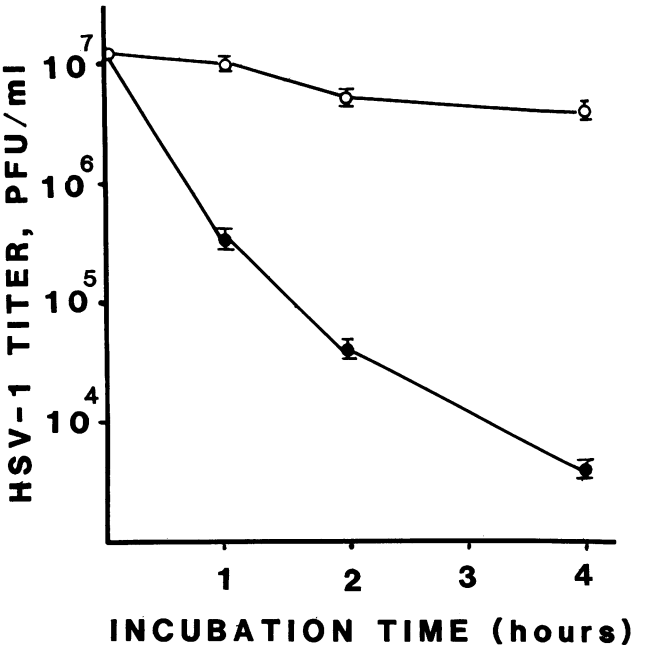

FIG. 1. Susceptibility of HSV-1 to MCP-1. Virus was incubated with MCP-1 (100 μg/ml) at 37°C in Dulbecco PBS buffer (pH 7.4). Symbols: ●, virus incubated with MCP-1; ○, 37°C control. Each data point represents a mean ± standard deviation (horizontal lines).

Modulating factors. The extent of HSV-1 neutralization by MCP-1 or MCP-2 was dependent on incubation time (Fig. 1), peptide concentration (Fig. 2), and incubation temperature

TABLE 2. Direct viral inactivation by MCP-1 and MCP-2[a]

Virus (strain)	Peptide	pH	n	$\log_{10}$ virus PFU/ml		
				Control	Treated	ΔPFU
HSV-2 (MS)	MCP-1	8.2	1	5.11	3.38	1.73
	MCP-1	7.4	1	4.49	3.26	1.23
	MCP-1	7.25	1	5.17	3.49	1.68
	MCP-2	8.2	1	5.00	4.00	1.00
Vesicular stomatitis virus (Indiana)	MCP-1	8.2	1	8.48	6.48	2.00
	MCP-1	7.25	1	8.11	6.08	2.03
Influenza (A/WSN)	MCP-1	8.2	2	8.27	7.42	0.85
	MCP-1	7.25	2	7.52	5.77	1.75
CMV (AD 169)	MCP-1	8.2	1	5.79	5.69	0.10
	MCP-1	7.4	1	5.18	5.00	0.18
	MCP-2	8.2	1	5.87	5.72	0.15
Reovirus type 3 (Dearing)	MCP-1	8.2	1	5.80	5.74	0.06
	MCP-1	7.25	1	5.32	5.11	0.21
Echovirus type 11 (Gregory)	MCP-1	7.25	1	5.68	5.60	0.08
	MCP-2	8.2	1	5.88	6.18	−0.30

[a] Viruses were incubated for 60 min at 37°C with or without 100 μg of MCP-1 or MCP-2 per ml as described in the text. Viral titers were peformed by plaque assays on MDBK cells (influenza virus), MRC-5 cells (CMV), or Vero cells (all others). Data represent mean values derived from serial dilutions performed in triplicate. The mean coefficient of variation (standard deviation ÷ mean) for these data points was 13.6%. *n*, Number of experiments.

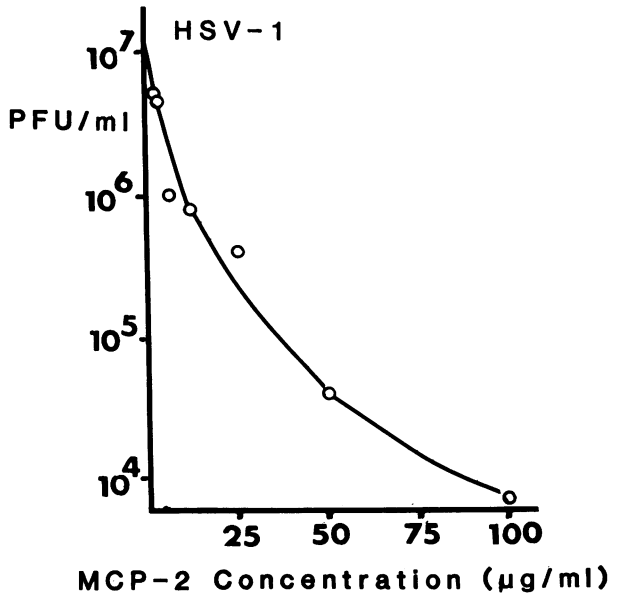

FIG. 2. Activity of MCP-2 against HSV-1. HSV-1 was incubated with 0 to 100 μg of MCP-2 per ml in a 1:1 mixture of normal saline with E-MEM (pH 8.2) for 60 min at 37°C. Surviving PFU were titered on Vero cells.

(Fig. 3). Although we typically terminated our incubations after 60 min, viral inactivation progressed for at least 4 h (Fig. 1). It was abolished by temperatures of ≤15°C and accentuated as the temperature increased from this point. Note that peptide-mediated direct virucidal activity was enhanced at 41°C relative to that at 37°C even when the greater rate of spontaneous thermal inactivation of the control at 37°C is considered. This temperature effect was also studied in temperature shift experiments, wherein HSV-1 was incubated with MCP-2 (100 μg/ml) at 37°C for 0, 10, 30, or 60 min. Paired aliquots were removed at each time; one was chilled in a melting ice bath until the end of the incubation period (60 min), and the other was serially diluted and plated on Vero cell monolayers for plaque assay. After 60 min, the chilled samples were also diluted and subjected to plaque assays. Chilling caused a prompt cessation of the ongoing process of viral neutralization (Fig. 4).

The ability of MCP-1 to inactivate HSV-1 was profoundly influenced by pH (Fig. 5). Excessive spontaneous viral inactivation precluded studies at pH of <4, but little peptide-mediated inactivation was apparent when assays were performed at pH 5 or 6. Although most of our studies were done at pH 7.25 to 7.4, viral inactivation was even more marked at pH 8 or above. To determine whether ionic strength affected the inactivation process, we exposed HSV-1 to MCP-1 (100 μg/ml) in a buffer containing 20 mM sodium phosphate (pH 7.4) and various concentrations of NaCl ranging from 0 to 0.16 M. An equivalent degree of viral inactivation was seen with each of these incubation mixtures, suggesting that direct neutralization was not affected by this range of ionic strength (data not shown). Although phosphate- or Tris-buffered solutions were used in our customary assay systems, direct viral inactivation was also well supported in borate or imidazole buffers (data not shown). Figures 1 through 5 show representative single experiments, each of which was confirmed on two to five occasions.

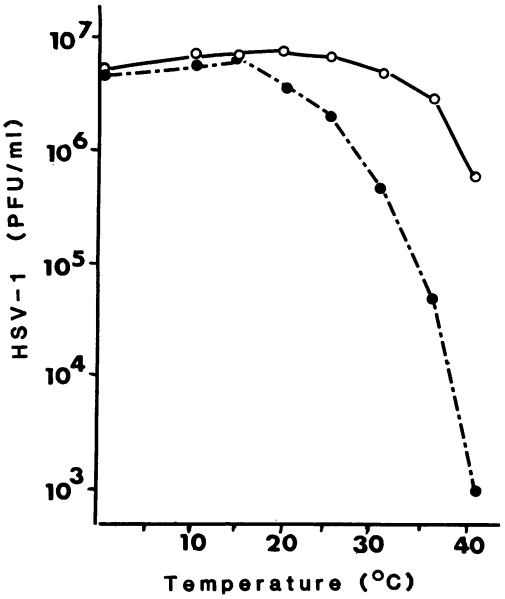

FIG. 3. Effect of temperature on inactivation of HSV-1 by MCP-2. HSV-1 was incubated with MCP-2 (●) (100 μg/ml) or saline (○) for 60 min at the indicated temperatures in 100 μg of a 1:1 mixture of normal saline and E-MEM (pH 8.2). The mixtures were chilled in a melting ice bath, serially diluted, and titered on Vero cells to determine the number of surviving PFU per milliliter.

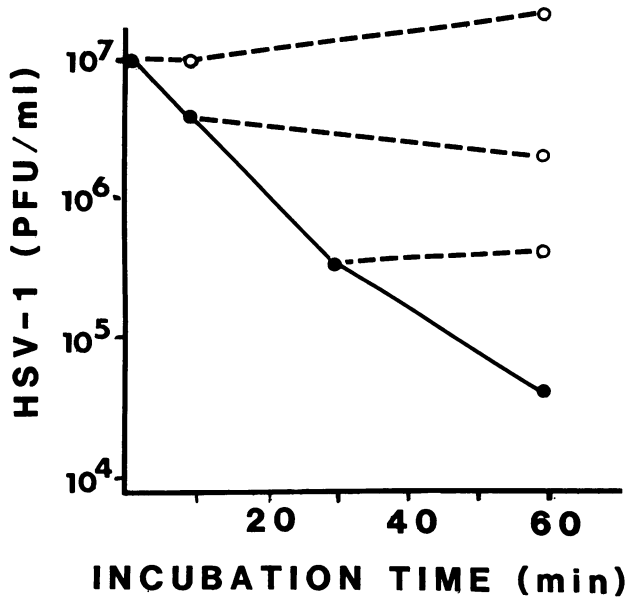

FIG. 4. Effect of temperature shift on inactivation of HSV-1 by MCP-2. The HSV-1 stock virus preparation in E-MEM was mixed with an equal volume (50 μl) of 0.85% NaCl containing MCP-2 (200 μg/ml) and incubated at 37°C. At various times, samples were serially diluted and titered on Vero cells (●) or chilled in a melting ice bath and reserved until the conclusion of the incubation (60 min), when they were also serially diluted and titered on Vero cells (○).

Peptide modification and comparative activity. MCP-1 and MCP-2 are both highly cationic molecules. To determine whether cationic charge alone sufficed to endow these molecules with their direct antiviral activity, we reduced native MCP-2 (which has a net charge of +8 at pH 8) with dithiothreitol and alkylated it with iodoacetamide. Such treatment alters the conformation of MCP-2 by reducing its three disulfide bonds and preventing reformation of the disulfide bridges without affecting net charge. Reduced and alkylated MCP-2 lacked detectable neutralizing activity (data not shown). We also compared the ability of MCP-2 to neutralize HSV-1 with those of NP-3a and NP-3b, each of which also has the same net charge as MCP-2 at or above pH 8 (Selsted et al., in press). Note neither NP-3a nor NP-3b inactivated HSV-1 under such conditions, whereas MCP-1 and MCP-2 were approximately equally active (Fig. 6A). Figure 6B shows a confirmatory experiment run at pH 7.4. Again, NP-3a and NP-3b lacked direct neutralizing activity.

Mixing experiments. Because MCP-1 or MCP-2 was active against the other enveloped viruses in our test panel (Table 1), we wondered whether the evident resistance of CMV arose from extraneous materials (cell debris, defective virions, etc.) in the CMV stock preparation that might compete with infectious CMV particles in binding the peptide. We tested this by exposing a mixture of CMV and HSV-1 stocks to MCP-1, reasoning that if such extraneous materials existed in the CMV preparation, they should prevent the direct inactivation of HSV-1 by the peptide. The much slower growth of CMV relative to HSV-1 made plaque counting of the mixed viral preparations unambiguous in this system.

HSV-1 was mixed with an equal volume of CMV stock or saline (final concentrations: HSV-1, 7×10^6 PFU/ml; CMV, 10^5 PFU/ml) and exposed to MCP-1 (100 μg/ml) for 60 min at 37°C. The mixture with saline yielded 2×10^3 HSV-1 PFU/ml, whereas the mixture with CMV stock yielded 3 ×

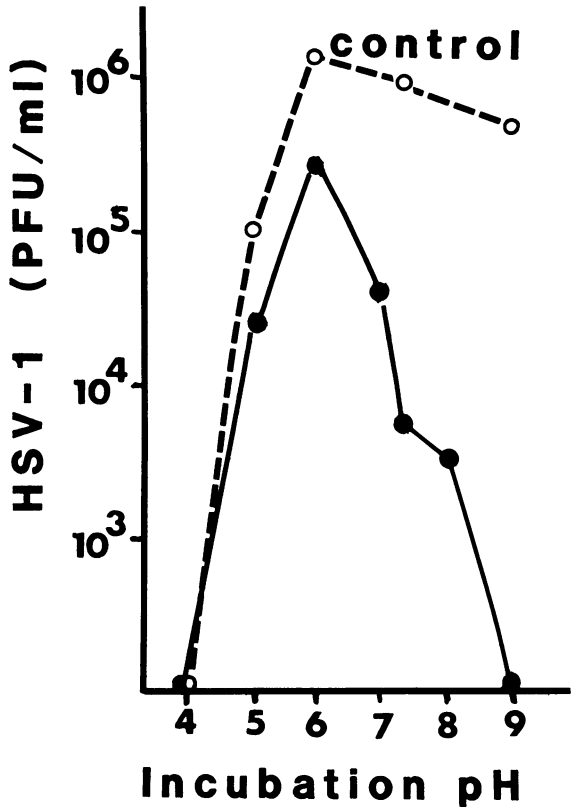

FIG. 5. Effect of pH on inactivation of HSV-1 by MCP-1. MCP-1 (final concentration, 100 μg/ml) was mixed with HSV-1 (final concentration, 10^6 PFU/ml) in solutions containing 0.05 M citrate (final pH, 4.0, 5.0, or 6.0), 0.05 M Tris (pH, 7.0, 8.0, or 9.0), or 0.01 M PBS (pH 7.4). Control mixtures containing HSV-1 (10^6 PFU/ml) but lacking MCP-1 were also prepared. After incubation for 60 min at 37°C, samples were diluted, and PFU per milliliter were determined by titration on Vero cells. Mixtures containing $<10^2$ PFU/ml are plotted at the base line. Symbols: ●, MCP-1-treated HSV-1; ○, saline-treated HSV-1.

10^4 HSV-1 PFU/ml after exposure to MCP-1. These findings suggest that the resistance of CMV to MCP-2 shown in Table 1 is not primarily attributable to interfering or extraneous components in the virus stocks.

DISCUSSION

Granulocytes and mononuclear phagocytes, long recognized to be important effectors of host defenses against bacteria and fungi, have been shown more recently to exert antiviral activity in vitro as well. Bovine and human granulocytes kill virus-infected cells by an antibody-dependent cell-mediated process (4, 7, 18, 27). The antiviral activity of bovine mammary granulocytes against target cells infected by the herpesvirus infectious bovine rhinotracheitis virus both exceeds that of bovine endotoxin-elicited mammary macrophages and is evident at lower antibody concentrations (7). Since granulocytes are known to infiltrate herpetic lesions early (6), they may be especially important to host defenses when antiviral antibody titers are low (7).

The antiviral effect of bovine granulocytes on infectious bovine rhinotracheitis-infected target cells is mediated by release of an acid-stable and trypsin-labile soluble factor which is active on homologous and heterologous target cells (20, 21). Although the specific bovine proteins or peptides responsible for this activity have not yet been identified, the possibility that they may be homologous to MCP-1 and MCP-2 of rabbit leukocytes deserves careful consideration. Both MCP-1 and MCP-2 are acid-stable and trypsin-labile molecules (unpublished data).

Mononuclear phagocytes figure prominently in host defenses against infection by HSV. Replication of HSV (8, 25) or CMV (14, 22) in macrophages is frequently abortive, reflecting the intrinsic antiviral activity of many mononuclear phagocytes. Macrophages have been shown to produce interferon (5, 12) and also to exert direct (2) and antibody-mediated (9) cellular cytotoxicity against virus-infected cells. They can release arginase (28), thus depleting media of arginine, an essential amino acid for productive replication of HSV and other viruses (1, 26). In addition, macrophages can restrict the replication of HSV and other viruses in otherwise permissive cell types (extrinsic antiviral activity) (17).

Extrinsic antiviral activity was manifested by mouse peritoneal macrophages obtained after in vivo administration of *Corynebacterium parvum* or thioglycollate, but was not shown by unstimulated resident macrophages. The augmentation of this effect by macrophage-target cell contact argues against its mediation solely by arginase release and consequent arginine depletion (17, 28). Target cells showed evidence of reduced protein synthesis after exposure to competent macrophage populations (17).

Extrinsic and intrinsic antiviral properties of macrophages appear to arise from different mechanisms. Thioglycollate-elicited mouse peritoneal macrophages are permissive for HSV growth (3, 13) yet exert extrinsic antiviral activity (15, 16). In contrast, resident peritoneal macrophages restrict HSV replication intracellularly, without manifesting extrinsic antiviral activity (17).

Recently, we have described two unusual peptides, MCP-1 and MCP-2, in rabbit macrophages (19, 23). Although they were undetectable in resident peritoneal macrophages, the peptides together constituted 0.5% of the total protein of resident alveolar macrophages and 1.7% of the total protein content of alveolar macrophages elicited by prior in vivo stimulation with complete Freund adjuvant (11). We have recently improved our methods for extracting MCP-1 and MCP-2 from rabbit alveolar macrophages (T. Ganz, M. P. Sherman, M. E. Selsted, R. Condiotti, and R. I. Lehrer, submitted for publication). With these procedures, we have extracted 4.2 ± 0.65 mg (mean ± standard error of the mean; $n = 4$) of MCP-1 from 10^9 resident (unstimulated) alveolar macrophages and a mean of 2.5 mg of MCP-1 from 10^9 peritoneal exudate granulocytes.

When purified to homogeneity, both MCP-1 and MCP-2 contained 33 amino acid residues and were identical except for the substitution of Arg^{13} in MCP-1 for Leu^{13} in MCP-2. The peptides were each arginine and cystine rich, devoid of carbohydrate, and stabilized by three intramolecular disulfide bonds (23). Both MCP-1 and MCP-2 are also found in rabbit granulocytes (24; Selsted et al., in press), where they constitute between 4 to 5% of the total cellular protein content (Selsted et al., unpublished data) and are accompanied by four additional homologous peptides (NP-3a, NP-3b, NP-4, and NP-5) that are absent in alveolar macrophages. Our data establish that MCP-1 and MCP-2 can directly neutralize several viruses in vitro and that HSV-1 strain MacIntyre and vesicular stomatitis virus strain Indiana are particularly susceptible to their action. As judged from our studies with HSV-1, direct inactivation shows marked dependency on temperature and pH but is not appreciably affected by ionic strength. Not all enveloped viruses ap-

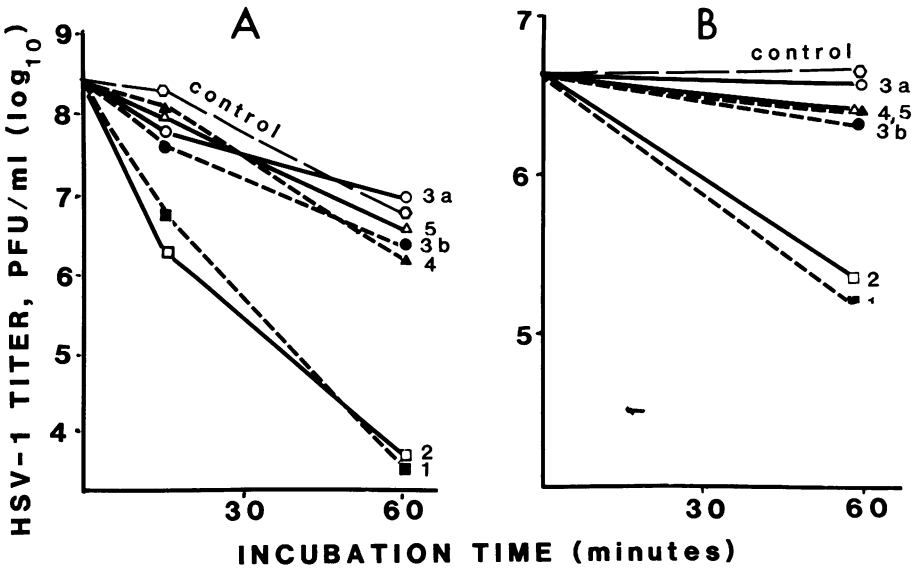

FIG. 6. Activity of rabbit leukocyte peptides against HSV-1. (A) HSV-1 exposed to the indicated peptide (100 μg/ml) in 0.85% NaCl (saline) at 37°C in a final volume of 200 μl at pH 8.2. As a control, HSV-1 was exposed to an equivalent volume of saline and similarly incubated. After 15 or 60 min, samples were removed, serially diluted, and titered by plaque assay on Vero cells. (B) The assay was performed in PBS at pH 7.4. Symbols: ■, MCP-1; □, MCP-2; ○, NP-3a; ●, NP-3b; ▲, NP-4; △, NP-5; ⬡, saline control.

peared to be susceptible to these peptides, for CMV strain AD 169 resisted direct inactivation completely. Neither of the two nonenveloped viruses tested, reovirus type 3 strain Dearing or echovirus type 11, appeared to be susceptible.

Both MCP-1 and MCP-2 are arginine-rich peptides, containing 10 and 9 arginine residues per molecule, respectively. However, it is doubtful that their antiviral activity arises solely from their cationicity, for reduced and carboxamidomethylated MCP-2 lost its ability to neutralize HSV-1, despite maintenance of its net positive charge. In addition, NP-3a and NP-3b lacked the ability to neutralize HSV-1, although each has the same net cationic charge as MCP-2 (Selsted et al., in press).

The observations reported in this manuscript delineate a previously unrecognized host defense mechanism that may equip granulocytes and mononuclear phagocytes to participate in host defenses against certain viruses. The influence of naturally occurring antimicrobial peptides such as MCP-1 and MCP-2 on the pathogenesis of natural and experimental viral infections and their utility as models for new therapeutic agents require further study.

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