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Selective Killing of Vaccinia Virus by LL-37: Implications for Eczema Vaccinatum¹

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Possible bioterrorism with smallpox has led to the resumption of smallpox (vaccinia virus) immunization. One complication, eczema vaccinatum, occurs primarily in patients with atopic dermatitis (AD). Skin lesions of patients with AD, but not psoriasis, is deficient in the cathelicidin antimicrobial peptide (LL-37) and human β -defensin-2 (HBD-2). We hypothesized that this defect may explain the susceptibility of patients with AD to eczema vaccinatum. The Wyeth vaccine strain of vaccinia virus was incubated with varying concentrations of human (LL-37) and murine (CRAMP) cathelicidins, human α -defensin (HBD-1, HBD-2), and a control peptide. Outcomes included quantification of viral PFU, vaccinia viral gene expression by quantitative real-time RT-PCR, and changes in virion structure by transmission electron microscopy. CRAMP knockout mice and control animals were inoculated by skin pricks with 2×10^5 PFU of vaccinia and examined daily for pox development. Physiologic amounts of human and murine cathelicidins (10–50 μ M), but not human defensins, which had antibacterial activity, resulted in the *in vitro* reduction of vaccinia viral plaque formation ($p < 0.0001$), vaccinia mRNA expression ($p < 0.001$), and alteration of vaccinia virion structure. *In vivo* vaccinia pox formation occurred in four of six CRAMP knockout animals and in only one of 15 control mice ($p < 0.01$). These data support a role for cathelicidins in the inhibition of orthopox virus (vaccinia) replication both *in vitro* and *in vivo*. Susceptibility of patients with AD to eczema vaccinatum may be due to a deficiency of cathelicidin. *The Journal of Immunology*, 2004, 172: 1763–1767.

Viral infections occur after entrance of virions into host cells by a variety of mechanisms, including endocytosis of nonenveloped viruses and fusion with the cell membrane by enveloped viruses (1). One primary barrier to the infection is epithelial keratinocytes in the skin. Alterations in skin barrier function are seen in atopic dermatitis (AD).³ This finding may contribute to infection with bacteria and selected viruses, including herpesviridae (HSV, varicella-zoster virus) and vaccinia virus. However, it is unlikely that a defect in the physical barrier alone accounts for the remarkably increased susceptibility of AD patients to recurrent skin infections. Patients with plaque psoriasis, a common Th1-mediated inflammatory skin disease also associated with skin barrier dysfunction, do not have increased susceptibility to microbial skin infection (2).

Resolution of infection and protection against reinfection with viruses depend on cooperation between innate and adaptive immune processes. These processes include antimicrobial proteins, complement activation, macrophages, NK cells, numerous cytokines, CTLs, specific Abs (3), and γ/δ T cells (4). In addition to the modified skin barrier in AD, alterations in cellular immunity have been described in this disease (5). These alterations are possible candidate mechanisms for the serious consequences of herpes viruses and vaccinia virus in this skin disease. Goodyear et al. (6) had previously observed increased quantities of HSV when cultured on skin explants obtained from patients with AD compared with skin from normal individuals. Thus, these experiments were performed in the absence of an adaptive immune response. Eczema vaccinatum is a complication of smallpox vaccination seen within 10 days after virus inoculation during primary immunization, also suggesting an important role for local, innate immune responses in restricting vaccinia viral replication (7).

Two major classes of antimicrobial peptides are produced by mammalian skin: β -defensins and cathelicidins (8, 9). Both families of compounds have antimicrobial activities against bacterial and fungal pathogens. A third class, α -defensins (e.g., human neutrophil peptide-1 (HNP-1)), found in human neutrophils and mucosal epithelial cells, also inhibits virus replication, particularly enveloped viruses, including HSV1, HSV2, CMV, vesicular stomatitis virus, and influenza A/WSN (10). Cathelicidins derived from bovine (11) and porcine (12) neutrophils also have antiviral (HSV) activity *in vitro* along with peptides of varying physical and chemical structures. However, the effects of antimicrobial peptides on vaccinia virus have not been studied. The mechanism of action for these cationic antimicrobial peptides is hypothesized to involve disruption of the microbial membrane and/or penetration of the microbial membranes to interfere with intracellular functions. Keratinocytes are primary producers of these peptides in the skin after injury or an inflammatory skin response (8). However, neutrophils

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³ Abbreviations used in this paper: AD, atopic dermatitis; EEV, extracellular enveloped virion; HBD-2, human β -defensin; HNP-1, human neutrophil peptide-1; IMV, intracellular mature virion; MBC, minimum bactericidal concentration; TSB, tryptic soy broth.

infiltrating into the pustules of smallpox could also play a role in limiting viral invasion by the production of antiviral molecules such as α -defensin. Based on our recent observation that keratinocytes in the inflammatory skin lesions of patients with AD were deficient in the cathelicidin LL-37 and human β -defensin-2 (HBD-2) relative to psoriasis (13), we examined the effects of cathelicidins, α -defensins, β -defensins, and control peptides on vaccinia virus replication *in vitro*. The physiological role of cathelicidin deficiency in *in vivo* pox formation was further investigated using *Cnlp*^{-/-} mice, which lack CRAMP, the murine homologue of LL-37.

Materials and Methods

Virus source and culture

The Wyeth strain, currently the vaccine strain available in the U.S., was obtained from the Centers for Disease Control and Prevention. HeLa S3 (American Type Culture Collection, Manassas, VA; CCK-2.2) human adenocarcinoma cells were grown to confluence in RPMI 1640 medium supplemented with 10% FCS for propagation of vaccinia virus. The cells were rinsed and overlaid with RPMI 1640-2.5% FCS, then inoculated with 5×10^6 PFU/T-175 flask and incubated at 37°C in 5% CO₂ for 3 days. Virus was harvested after disruption of cells, yielding infectious virions in the form of intracellular mature virions (IMV) (14).

Animals

BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *Cnlp*^{-/-} (CRAMP knockout) mice were generated on the 129/SvJ background as previously described (15). 129/SvJ and *Cnlp*^{-/-} mice were obtained from R. L. Gallo (Veterans Affairs Medical Center, San Diego, CA). All protocols are directed by the guidelines stated in institutional animal care and use committee-approved protocols. This institution has an animal welfare assurance number (A3026-1) on file with the Office of Protection from Research Risks.

Before inoculation, mice were briefly anesthetized by injecting ~250–300 μ l of 2.5% avertin (2,2,2-tribromoethanol and tertiary amyl alcohol) into the peritoneal cavity using a 25-gauge needle and a tuberculin syringe. Once anesthetized, mice were inoculated with 2×10^5 PFU of vaccinia virus by scarification with 15 pricks into the skin of the dorsal thorax region. The use of C57/BL6J and BALB/c mice in these studies allowed for comparison with previous reports. 129/SvJ wild-type animals were compared with the homozygous CRAMP knockout (*Cnlp*^{-/-}) mice.

Peptide preparations

Human and murine cathelicidins (LL-37 and CRAMP) and the control peptide 8044 (GLNGPDIYKGYQFKSVEFD) were synthesized via solid phase t-BOC chemistry using standard methodology and purified to homogeneity via HPLC by the Molecular Resource Center at National Jewish Medical and Research Center. Peptide 8044 was chosen from a library of existing peptides for use as a control for the antimicrobial peptides tested in these studies based on size, overall hydrophobicity, and charge similarities with test peptides, yet it had no sequence identity with the test peptides. HNP-1 was purified to homogeneity as assessed by acid-urea PAGE from bronchiectatic sputum using fast protein liquid chromatography and HPLC as previously described (16). HBD-1 and HBD-2 were produced from baculovirus-infected insect cells and purified by HPLC to homogeneity. HBD-1 and HBD-2 were gifts from Dr. J. Lubkowski (National Cancer Institute, National Institutes of Health, Bethesda, MD). The purities of HBD-1 and HBD-2 were confirmed by HPLC, which showed single peaks eluting at 31.3 and 37% acetonitrile, respectively, on a gradient of 0–41% acetonitrile in 0.1% trifluoroacetic acid. The identities of LL-37 and HNP-1 were confirmed by mass spectroscopy. The concentrations of antimicrobial and control peptides used in these experiments ranged from 0–50 μ M, which corresponds to 0.20–2.25 μ g/ml of LL-37, 0.19–2.0 μ g/ml CRAMP, 0.16–1.7 μ g/ml HNP-1, 0.2–2.2 μ g/ml HBD-2, and 0.1–1.1 μ g/ml 8044.

The biological activity of each peptide was tested against *Escherichia coli* ML-35p in 0.01 \times tryptic soy broth (TSB) buffer containing 10 mM NaPO₄, pH 7.4. Briefly, peptide was added to bacterial cultures and incubated for 2 h at 37°C. After the brief incubation, cultures were diluted and plated to determine the CFU. The minimum bactericidal concentration (MBC) was determined for each peptide as the concentration that reduced the number of colonies by at least 99.9% relative to the starting culture. Mean MBC were determined from three independent experiments for each peptide.

Antiviral assays

BS-C-1 (American Type Culture Collection; CCL-26), African green monkey kidney cells (2×10^5 /well), were seeded in 24-well tissue culture plates in MEM-10% FCS and penicillin/streptomycin and allowed to grow overnight before the supernatant was removed and replaced with MEM-2.5% FCS for virus incubation. BS-C-1 cells were used for the quantitative estimates because they present uniform plaques (17). HeLa S3 cells are routinely used for preparations of virus stock because they give consistently high yields of virus, but, due to their rounded morphology, do not present uniform plaques.

Peptides were diluted to the proper concentrations in 0.01 \times tryptic soy broth containing 10 mM sodium phosphate buffer, pH 7.4. Virus diluted in the same buffer was added to the peptides, and they were incubated for 24 h at 37°C. Twenty microliters of the peptide/virus mixture was added to the cells in 0.5 ml of MEM-2.5% FCS and allowed to infect for 24 h for RNA analysis or 48 h for plaque development. For the plaque assay, the medium was removed, and wells were overlaid with 0.5 ml of 4% buffered formalin and allowed to fix for 10 min at room temperature. The formalin was removed, and 0.5 ml of 0.1% crystal violet in PBS was added to the wells for 5 min at room temperature. Wells were then aspirated and air-dried for visualization of plaques. We found the most accurate results with the virus alone, forming 50–80 plaques/well.

Electron microscopy

Virus stock (10^8 PFU) was concentrated by ultracentrifugation at 50,000 rpm at 4°C for 60 min in a TL-100 rotor. The medium was removed, and the pellet was washed with 1.0 ml of 0.01 \times TSB/10 mM phosphate buffer and spun again. The final pellet was resuspended in a 0.2-ml final volume in buffer containing peptides at final concentrations of 5, 25, and 50 μ M. The mixture was incubated at 37°C for 24 h, followed by the addition of an equal volume of 3% glutaraldehyde. The samples were stored at 4°C until transmission electron microscopy with a Phillip's CM-10 could be performed at the Electron Microscopy Laboratory at National Jewish Medical and Research Center.

Vaccinia gene expression

Vaccinia gene expression was evaluated using quantitative real-time RT-PCR. Briefly, BS-C-1 cells were cultured in 24-well plates at a concentration of 2×10^5 cells/well. Twenty-four hours after culturing, virus and peptide were added to corresponding wells and allowed to incubate for an additional 24 h. RNA was isolated from cultured cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's guidelines. Real-time PCR was performed using an ABI 7700 Sequence Detection system (PE Applied Biosystems, Foster City, CA). The primer sequences that were used to assay for the vaccinia gene transcripts are: forward, 5'-GCCAATGAGGGTTCGAGTTC-3'; and reverse, 5'-CAACATCCCGTCGTTTCATCA-3'. This region of the genome encodes a subunit of a DNA-directed RNA polymerase expressed within 2 h of viral entry (18). The TaqMan probe was purchased from PE Applied Biosystems; it was 5' labeled with FAM and 3'-labeled with TAMRA. Amplification reactions were performed in MicroAmp optical tubes (PE Applied Biosystems) in a 25- μ l volume containing 2 \times TaqMan Master Mix (PE Applied Biosystems), 900 nM forward primer, 900 nM reverse primer, 200 nM probe, and the template RNA. Thermal cycling conditions were 50°C for 2 min and 95°C for 10 min for one cycle. Subsequently, 40 cycles of amplification were performed at 94°C for 15 s and 60°C for 1 min. To quantitatively express the levels of vaccinia virus in BS-C-1 cells, a standard curve was generated using cDNA from purified vaccinia virus.

Statistical analyses

Statistical comparisons of the reduction in PFU and mRNA were made using the Excel program for two-tailed *t* test. Analyses of viral structural alterations were performed using Fisher's exact test (StatXact 4; Cytel, San Diego, CA).

Results

The biological activity of all antimicrobial peptides used in these studies was confirmed against *E. coli* ML35p, and the MBC values for each peptide are displayed in Table I. HBD-2 exhibited the lowest MBC of 0.10 μ M and therefore displayed the greatest antimicrobial activity against ML35p. HNP, LL-37, and HBD-1 exhibited MBC values of 8.42, 0.35, and 16.02 μ M, respectively. The negative control peptide 8044 did not exhibit antimicrobial activity against ML35p.

Table I. Minimum bactericidal concentrations against *E. coli* ML35p for antimicrobial peptides^a

Peptide	Minimum Bactericidal Concentration (μM)
HNP	8.42 ± 0.43
HBD-1	16.03 ± 1.20
HBD-2	0.10 ± 0.02
LL-37	0.35 ± 0.01

^a Control peptide 8044 did not possess antimicrobial activity against ML35p.

Although relatively low concentrations of HNP, HBD-1, and HBD-2 were sufficient to exhibit antibacterial activity against ML35p, concentrations as high as 50 μM did not inhibit vaccinia virus replication (Fig. 1). In contrast, a concentration-dependent inhibition ($p < 0.001$) of viral replication was observed in tissue culture cells after infection with a virion preparation, consisting primarily of IMV, that had been preincubated with human (LL-37) and murine (CRAMP) cathelicidins. Significant reduction in viral replication by LL-37 and CRAMP was observed with concentrations as low as 25 μM . The control peptide 8044 possessed no antiviral activity against vaccinia virus.

In previous reports the salt concentration and incubation time with target organism were shown to impact the function of antimicrobial peptides in in vitro experiments (10). Fig. 2 shows the effects of varying salt concentration and exposure time on LL-37 activity, the only human peptide shown to inhibit viral replication.

The salt concentration influenced viral replication primarily at 2 h, with enhancement of the effect at the higher salt concentrations. There was no effect of either variable at 4 h, whereas at 8 h, the two highest salt concentrations were slightly more effective than with no salt. Similar findings were seen at each salt concentration at 24 h.

Quantification of vaccinia mRNA in tissue culture cells by real-time PCR under the conditions used for the plaque assays showed inhibition of viral mRNA expression (Fig. 3). The amount of viral mRNA in the tissue culture cells decreased in proportion to the concentrations of LL-37 and CRAMP. Although LL-37 is minimally expressed in normal skin keratinocytes (19), it is abundantly

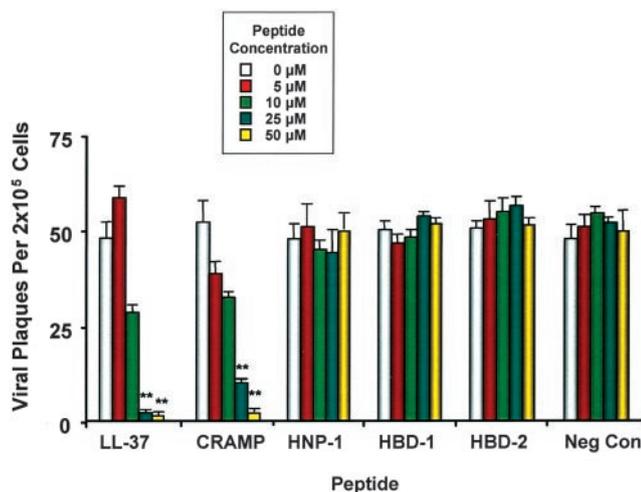


FIGURE 1. PFU of vaccinia virus after incubation with antimicrobial and control peptides. The p values compare vaccinia alone with a reduction in virus at varying concentrations of the individual peptides. **, $p = 0.0041$, $p < 0.0001$, and $p < 0.0001$, for LL-37 at 10, 25, and 50 μM , respectively; and $p = 0.0113$, $p = 0.0002$, and $p < 0.0001$ for CRAMP at 10, 25, and 50 μM , respectively. These data are representative of six experiments with four replicates per condition.

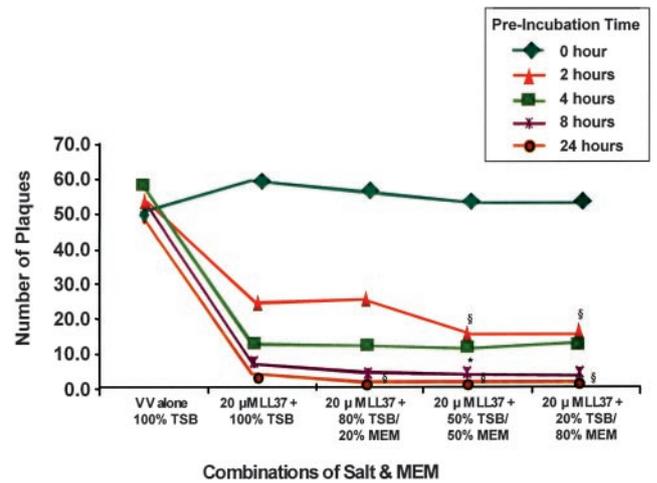


FIGURE 2. PFU of vaccinia virus (0.1 PFU/cell) after incubation with LL-37 at different salt concentrations for varying time periods. All conditions used 20 μM LL-37 for comparison of salt and time conditions; 0.01 \times TSB and 10 mM NaPO_4 contains 0 mM NaCl, whereas 20% MEM contains 20.68 mM NaCl, 50% MEM contains 51.70 mM NaCl, and 80% MEM contains 82.75 mM NaCl. Statistical differences from the 20 μM LL-37 plus 100% TSB condition (used in other experiments) at each time point were determined using a two-tailed t test and the permutation test for the 24 h point. §, $p = 0.03$; *, $p < 0.05$.

expressed after viral infection (20), and the effective concentrations were equivalent to tissue levels found in human psoriatic skin (13, 19) or normal skin after injury (21). Electron microscopy of virions exposed to LL-37, α -defensin HNP-1, and control peptides demonstrated a concentration-dependent effect of only LL-37 on the structure of the IMV, including loss of integrity of the double-layered external envelope as well as that of the internal structure (Fig. 4). In contrast, α -defensins and control peptides did not have an effect on virion structure. Examination of untreated virions did not identify similar structural changes, thus lessening the possibility that apparent alterations in structure are due to sectioning artifacts. A dose-dependent effect of in vitro incubation of LL-37 on

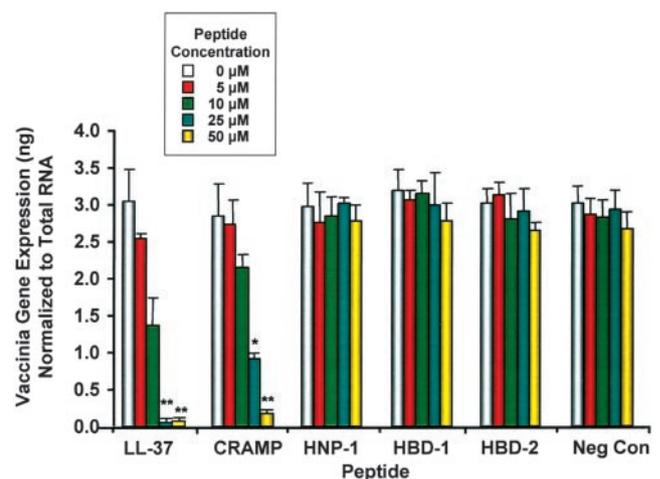


FIGURE 3. Quantity of vaccinia mRNA in tissue culture cells after incubation of virus before culture with antimicrobial and control peptides. The p values compare vaccinia mRNA as measured by real-time PCR to reduction in viral message at varying concentrations of the individual peptides. *, $p < 0.05$; **, $p < 0.001$. These data represent two separate experiments with four replicates per condition.

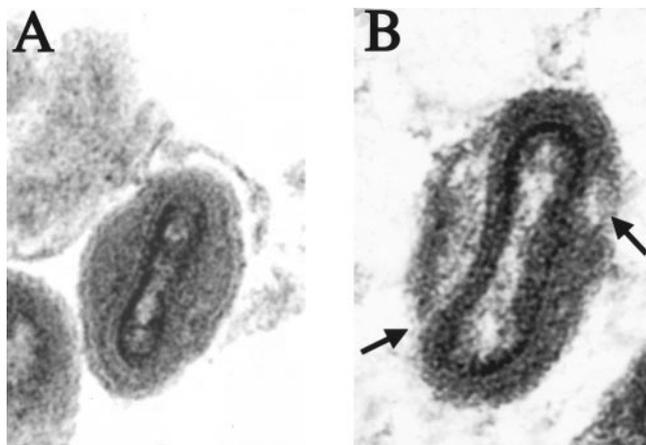


FIGURE 4. Transmission electron micrographs ($\times 27,500$ magnification) of vaccinia virions after 24 h of incubation with control peptide (A) or with LL-37 at a $25\text{-}\mu\text{M}$ final concentration (B). Arrows in B identify disruptions of the internal and outer bilayers of the IMV.

the numbers and percentages of altered virions was found (see Table II).

Previous studies addressing the effect of the tissue culture cells on in vitro activity have incubated antimicrobial peptides with cells before adding viruses to the system (10). Although these types of experiments yield valuable information, they cannot be easily controlled for the multiple variables at play in vivo. The physiological role of cathelicidin in vivo can be evaluated using a cathelicidin-deficient murine model and monitoring pox lesion development in inoculated skin. We inoculated 2×10^5 PFU of the virus by scarification with 15 pricks into the skin of five C57BL/6, four BALB/c, six 129/SVJ wild-type, and six homozygous CRAMP knockout ($\text{Cnlp}^{-/-}$) mice on the 129/SVJ background. $\text{Cnlp}^{-/-}$ mice develop normally and have normal skin morphology, but have increased susceptibility to infection by group A *Streptococcus* (15). Four of six homozygous $\text{Cnlp}^{-/-}$ animals demonstrated 2-mm pox lesions by day 10, whereas only one (BALB/c) of 15 control animals had a lesion, and this was <1 mm in diameter ($p < 0.01$, by Fisher's exact test). Upon comparison with the 129/SVJ wild-type control, $\text{Cnlp}^{-/-}$ mice exhibited significant differences ($p < 0.03$) in lesion size and number. A typical pox lesion is displayed in Fig. 5. This value is conservative, because the results of only four of the animals were available. As normal mice rarely demonstrate skin lesions using the scarification method, and reports of intradermal inoculation of the Wyeth strain into ear pinna of BALB/c yielded minimal or no lesions (22), the observed inconsistency in appearance of pox lesions in the control animals is not unexpected. The other two $\text{Cnlp}^{-/-}$ animals died within 2 days of inoculation. Histological changes in the livers and lungs were typical of septic shock (data not shown). Vaccinia virus

Table II. Numbers and percentages of altered virions after exposure to LL-37^a

LL-37 (μM)	Altered Virions/ Virion Number	Percent (%)
0	1/23	4
5	19/28	67
25	27/30	90
50	39/41	95

^a HNP-1 ($25\ \mu\text{M}$) and control peptides did not alter virion structure.

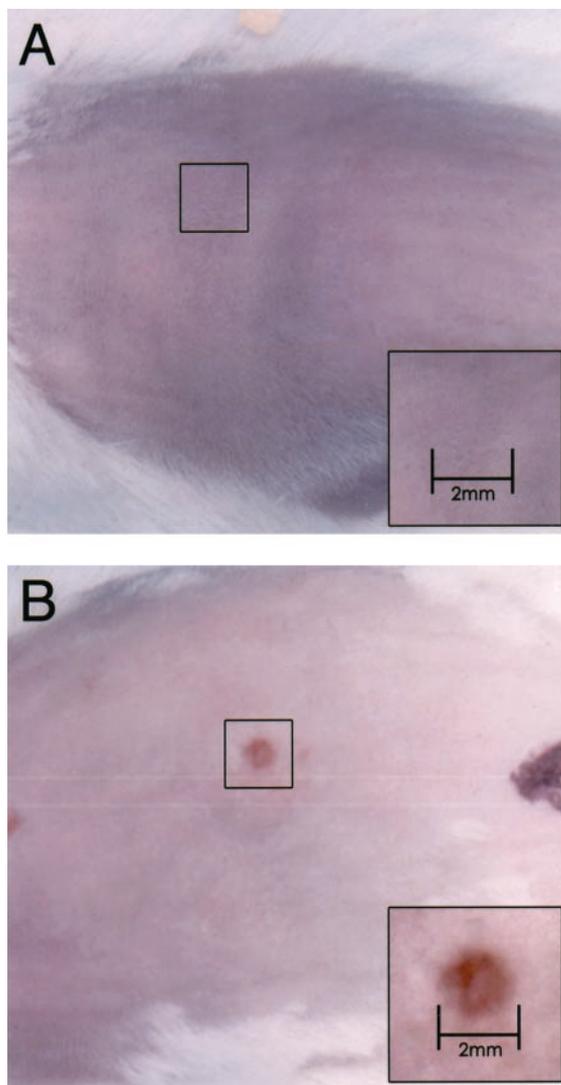


FIGURE 5. Generation of vaccinia pox lesion in CRAMP knockout mice. A, Inoculation in a representative control mouse; 10 days after scarification, the reaction was negative. B, Inoculation in a representative CRAMP KO mouse; 10 days after scarification with vaccine virus, the animal developed a pox lesion. The boxed area represents the inoculation site on the dorsal region of the mouse. Bars = 2 mm.

RNA was present in the lungs and skin, the only tissues so studied, of each of the expired animals using the methods described above.

Discussion

As long as there is a need for protection against smallpox as a weapon of bioterrorism, evaluation of the host responses that contribute to control of orthopox virus infections, in general, and vaccinia virus, in particular, is an important goal. Furthermore, smallpox vaccination was recently required in response to an outbreak of monkeypox in midwestern United States (23). Although the live virus smallpox vaccine is highly effective, it has the dubious distinction of having one of the highest rates of vaccine-associated adverse events. Many of these adverse events may relate to a failure of the host to control vaccinia virus replication and dissemination. Furthermore, there is no effective antiviral agent that can be used therapeutically against vaccinia infection.

The current study is the first to identify human and murine cathelicidins as innate antimicrobial peptides capable of interfering in vitro and in vivo with replication of vaccinia virus. LL-37 and

CRAMP were effective at concentrations at least 1 log lower than that required for killing of *S. aureus* (21). Despite demonstrating biological activity against *E. coli*, the α -defensin, HNP-1, and the β -defensins, HBD-1 and HBD-2, did not inhibit viral replication.

These findings show that not all cationic and membrane active peptides have antiviral activity, as LL-37 and CRAMP, but not HBD-2, was able to inhibit vaccinia replication. This relative specificity suggests that specific structural elements of the cathelicidins are required for the observed effects. It also highlights the need for a family of antimicrobial peptides with overlapping activities to control the complex galaxy of microbes humans can encounter.

The mechanisms by which many small cationic human antimicrobial peptides kill bacteria and fungi require binding of the peptide to the microbial cell membrane, after which the proton gradient and membrane integrity are lost (24). However, there are no published reports describing the mechanism by which viruses are disrupted by human antimicrobial peptides. IMV of vaccinia have a double-layer membrane composed of endoplasmic reticulum-derived membrane cisternae, and as they migrate through the cell they acquire a double-layer outer envelope consisting of a cellular cisterna known as a wrapping membrane and become known as intracellular enveloped virions. Egress from the cell is accompanied by fusion of the outermost layer with the plasma membrane, yielding a three-layer outer membrane on extracellular enveloped virions (EEV) (25). Both IMV and EEV forms are infectious, with the EEV being the most efficient in cell entry (26). Our current electron microscopy studies suggest that LL-37 has direct effects on the integrity of the vaccinia viral membrane structure.

Due to their increased risk of eczema vaccinatum, patients with AD cannot be inoculated with the smallpox vaccine unless there is imminent risk of exposure to smallpox. To determine the potential in vivo significance of LL-37 deficiency, we studied Cnlp knock-out mice, which are known to lack expression of CRAMP, a close murine orthologue of human LL-37. Importantly, these mice generated a significantly greater number of pox skin lesions than wild-type isogenic control mice. The two mice that did not generate pox skin lesions died within 2 days of septic shock following smallpox vaccination. The recent reports of human deaths after vaccination and the deaths of two Cnlp^{-/-} animals reiterate the importance of testing the roles of antimicrobial peptides as components of innate defense in experimental animals, followed by application of these experimental findings to their roles in human disease. These in vitro and in vivo observations suggest that the increased susceptibility of AD patients to eczema vaccinatum may be due to a deficiency of cathelicidin (13).

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