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Antimicrobial peptides melittin and cecropin inhibit replication of human immunodeficiency virus 1 by suppressing viral gene expression

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Antimicrobial peptides are effectors of innate immunity, providing their hosts with rapid non-specific defence against parasitic invaders. In this report, the effects are assessed of two well-characterized antimicrobial amphipathic peptides (melittin and cecropin) on human immunodeficiency virus 1 (HIV-1) replication and gene expression in acutely infected cells at subtoxic concentrations. Production of infectious, cell-free virus was inhibited in a dose-dependent manner, with ID_{50} values in the range 0·9–1·5 μM for melittin and 2–3 μM for cecropin. Analysis of the effect of melittin on cell-associated virus production revealed decreased levels of Gag antigen and HIV-1 mRNAs. Transient transfection assays with HIV long terminal repeat (LTR)-driven reporter gene plasmids indicated that melittin has a direct suppressive effect on activity of the HIV LTR. HIV LTR activity was also reduced in human cells stably transfected with retroviral expression plasmids for the melittin or cecropin gene. It is concluded that antimicrobial peptides such as melittin and cecropin are capable of inhibiting cell-associated production of HIV-1 by suppressing HIV-1 gene expression.

Introduction

Animal peptide antibiotics are widespread in nature, occurring in mammals, amphibians and insects (Boman, 1995). These peptides are major constituents of the innate immune system for non-specific defence of the host against microbial parasites. They can be rapidly activated after injury or invasion of the host by microbial agents, combating parasitic growth immediately after infection. Antimicrobial peptides thus provide an important defence mechanism in lower animals and the first line of host defence during the time required for mobilization of specific immunity in vertebrates.

Amphipathic cationic peptides are a major group of antimicrobial peptides and can be divided into four classes according to their structure. Genes for peptides belonging to the α-helix class have been identified in, for example, moths and mammals (cecropins), frogs (magainin) and honey bees (melittin). Like other antimicrobial peptides, they are expressed as parts of protein precursors from which active peptides 20–40 amino acids in length are derived by serial cleavages with cellular proteases. Antimicrobial activities of cationic peptides involve disruption of bacterial membranes (Christensen *et al.*, 1988; Agawa *et al.*, 1991; Hancock, 1997) and possibly other non-membranolytic mechanisms (Boman *et al.*, 1993; Andreu *et al.*, 1992). Interestingly, most cationic peptides do not induce resistance mutants *in vitro* and enhance antimicrobial activity of classical antibiotics in resistant bacteria, thus serving as anti-resistance compounds (Hancock, 1997). In addition to antibacterial activities, amphipathic antimicrobial peptides have also been reported to act against fungi (Ahmad *et al.*, 1995), protozoa (Bevins *et al.*, 1990) and viruses (see below).

Melittin is a 26 amino acid amphipathic α-helical peptide, which is a major component of bee venom (Habermann *et al.*, 1967; Terwilliger *et al.*, 1982; Bazzo *et al.*, 1988). The cecropins are a family of antibacterial peptides 35–39 amino acids in length which occur in a number of insect species and in

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mammals (Boman, 1995). Like melittin, they consist of two α-helices linked by a flexible segment, and contain amphipathic structures. Melittin and cecropin act against a wide range of infectious agents, including Gram-positive and Gram-negative bacteria (Wade *et al.*, 1992). Whereas melittin is lytic for red blood cells at high concentrations, cecropins do not lyse erythrocytes or other eukaryotic cells (Wade *et al.*, 1992; Steiner *et al.*, 1981) and appear to be non-toxic for mammalian cells. Melittin has been reported to inhibit replication of murine retroviruses, tobacco mosaic virus (Marcos *et al.*, 1995) and herpes simplex virus (Baghian *et al.*, 1997), suggesting that melittin also displays antiviral activity. Analogous to antibacterial activity, the antiviral activity of melittin has been attributed to direct lysis of viral membranes, as demonstrated for murine retroviruses (Esser *et al.*, 1979). However, melittin also displays antiviral activity at much lower, non-virolytic concentrations, as shown for T cells chronically infected with human immunodeficiency virus 1 (HIV-1) (Wachinger *et al.*, 1992). In this report, melittin and cecropin A are shown to suppress production of HIV-1 by acutely infected cells. Melittin treatment of T cells reduces levels of intracellular Gag and viral mRNAs, and decreases HIV long terminal repeat (LTR) activity. HIV LTR activity is also reduced in human cells stably transfected with melittin and cecropin genes. These results indicate that antimicrobial peptides such as melittin and cecropin suppress HIV-1 replication by interfering with host cell-directed viral gene expression.

Methods

■ **Compounds.** Melittin (highly purified from bee venom; formula weight 2847) was obtained from ALK Allergologisk Laboratorium and from Bachem. Cecropin A (formula weight 4003) was obtained from Sigma. These compounds were dissolved in medium prior to application to cell cultures.

■ **Cell lines and virus strains.** The following cell lines were used in this study: human T lymphoma cell lines KE37/1 (Popovic *et al.*, 1984*a*), HUT78 (Mann *et al.*, 1989) and chronically infected HIV-1 IIIB (Popovic *et al.*, 1984*b*) and RF-infected (Reitz *et al.*, 1992) derivative cell clones KE37/1-IIIB and HUT78-RF; human embryonic lung fibroblastoid cells LC5 (Mellert *et al.*, 1990) and LC5-CD4, a clonal LC5 cell line transduced with an amphotropic retroviral vector containing the human CD4 receptor gene (Chesebro *et al.*, 1990); and the human EJ bladder carcinoma cell line (Parada *et al.*, 1982).

T lymphoma and fibroblastoid cells were cultured in RPMI 1640 medium supplemented with glutamine (300 mg/l) and foetal calf serum (10% v/v). The EJ cells were grown in DMEM supplemented with 10% foetal calf serum. Frozen or freshly harvested culture supernatants of chronically infected T lymphoma cells served as a source of virus strains HIV-1 IIIB and HIV-1 RF. Virus suspensions were assessed for infectivity by quantifying the induction of HIV-positive syncytia in C8166 T lymphoma cells, as described (Brack-Werner *et al.*, 1992).

■ **Treatment of HIV-1-infected cells with antimicrobial compounds**

(i) Chronically HIV-1-infected T cells. HUT78-RF cell suspensions (100 μl) containing 8×10^4 cells were dispensed into microtitre plates and 100 μl culture medium containing the appropriate concentrations of melittin was added. Infectivity of culture supernatants (100 μl) was assessed 24–48 h later in LC5-CD4 indicator cells (see below).

(ii) Acutely HIV-1-infected cells. For infection of T cells, T lymphoma cells (HUT78 or KE37/1) were exposed to cell-free virus preparations (RF or IIIB) at an m.o.i. of approximately 20 for 2 h at 37 °C. HIV-inoculated cells were dispensed into microtitre plates (2×10^4 cells in 100 μl culture medium per well), cultured for 2 days, and 100 μl melittin-containing growth medium was added to each well. Melittin was replenished at 5 days post-infection (p.i.) by adding 100 μl fresh melittin-containing medium to each well. At 7 days p.i., infectivity of 150 μl culture supernatant was assessed by quantifying the induction of HIV-antigen positive infectious centres in LC5-CD4 cells.

For infection of fibroblastoid cells, LC5-CD4 cells were seeded into microtitre plates ($3–5 \times 10^3$ per well). After 24 h, cell monolayers were exposed to 100 μl virus stock at an m.o.i. of approximately 0·2 for 2–3 h. Virus suspension was removed, cells washed and culture continued with growth medium containing appropriate concentrations of melittin or cecropin. After a further 24 h, cells were assayed for HIV-antigen-positive infectious centres by indirect immunoperoxidase staining with human anti-HIV serum, as described (Mellert *et al.*, 1990).

The metabolic activity of cells was determined by measuring mitochondrial dehydrogenase activity, using an assay based on quantifying reduction of a colourless tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to dark blue formazan (Mosmann, 1983; performed as described by Wachinger *et al.*, 1992).

■ **Western blot analysis for detection of Gag antigen.** Western blot analysis was performed according to standard protocols. Cells were lysed in loading buffer (5 mM Tris–HCl pH 6·8, 2% w/v SDS, 2·5% v/v glycerol, 0·005% w/v bromophenol blue, 2·5% v/v β-mercaptoethanol), and heated at 95 °C for 5 min. Aliquots of cell lysates corresponding to 125 000 lysed cells were loaded on SDS–12% w/v polyacrylamide gels. Immunostaining was carried out with a monoclonal antibody against p24 (Behring) and a peroxidase conjugate of anti-mouse IgG, using an enhanced chemoluminescence detection system (Amersham).

■ **Quantitative RT–PCR analysis of HIV mRNAs in acutely HIV-1-infected T cells treated with melittin.** KE37/1 cells were exposed to cell-free preparations of HIV-1 IIIB, propagated for 2 days and split into subcultures (5×10^4 cells/ml). Subcultures were expanded in the presence of various concentrations of melittin for 3 days (three cultures per melittin concentration), cells were passaged and melittin treatment continued for 2 further days (i.e. melittin treatment for a total of 5 days). Quantitative RT–PCR analysis of HIV mRNAs was performed as described (Neumann *et al.*, 1994). Cellular mRNAs for porphobilinogen deaminase (PBGD) were amplified as control for RNA amounts (Saltarelli *et al.*, 1996; Chretien *et al.*, 1988). Amplification products were quantified by phospho-imaging (FUJI BAS). Levels of HIV-1 transcripts were normalized to PBGD levels. Two separate PCR analyses were performed for each cDNA preparation (12 PCR analyses per melittin concentration).

■ **Assessment of HIV LTR activity in transient transfection assays.** HIV LTR activity in T lymphoma cells was assessed by co-transfection of pHIVLTR-CAT which contains the bacterial reporter gene chloramphenicol acetyltransferase (CAT) under the control of HIV-1 LTR (Ludvigsen *et al.*, 1996) and TAT expression plasmid pSV2tat72 (Frankel *et al.*, 1988) by electroporation at 250 V and 960 μF, as described (Brack-Werner *et al.*, 1992). Electroporated cells were kept in an incubator for 2 h and then split into five subcultures for exposure to different concen-

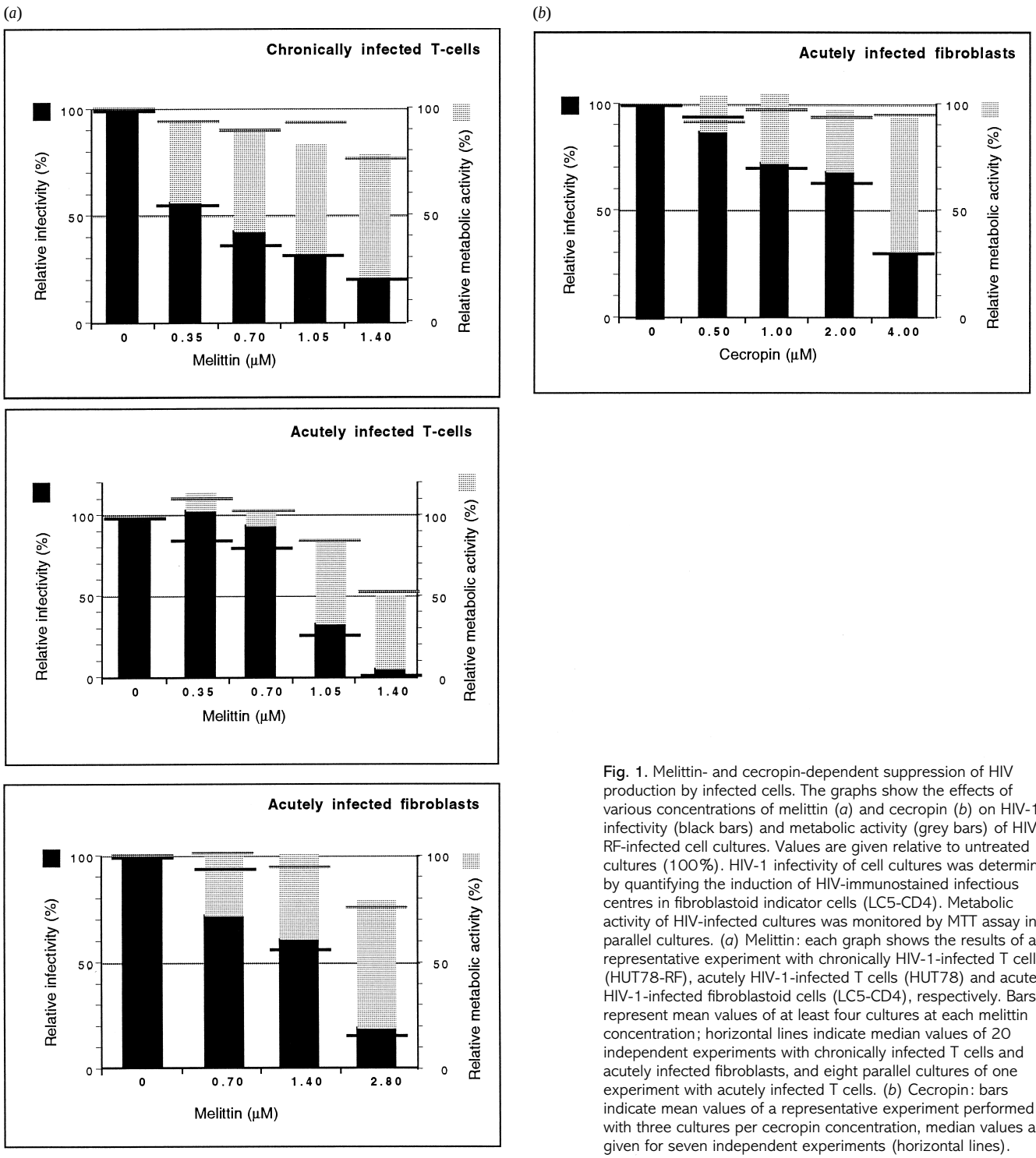

Fig. 1. Melittin- and cecropin-dependent suppression of HIV production by infected cells. The graphs show the effects of various concentrations of melittin (*a*) and cecropin (*b*) on HIV-1 infectivity (black bars) and metabolic activity (grey bars) of HIV-1 RF-infected cell cultures. Values are given relative to untreated cultures (100%). HIV-1 infectivity of cell cultures was determined by quantifying the induction of HIV-immunostained infectious centres in fibroblastoid indicator cells (LC5-CD4). Metabolic activity of HIV-infected cultures was monitored by MTT assay in parallel cultures. (*a*) Melittin: each graph shows the results of a representative experiment with chronically HIV-1-infected T cells (HUT78-RF), acutely HIV-1-infected T cells (HUT78) and acutely HIV-1-infected fibroblastoid cells (LC5-CD4), respectively. Bars represent mean values of at least four cultures at each melittin concentration; horizontal lines indicate median values of 20 independent experiments with chronically infected T cells and acutely infected fibroblasts, and eight parallel cultures of one experiment with acutely infected T cells. (*b*) Cecropin: bars indicate mean values of a representative experiment performed with three cultures per cecropin concentration, median values are given for seven independent experiments (horizontal lines).

trations of melittin for 16–40 h. This procedure (batch-transfection of cells followed by subculture of aliquots of transfected cells with different concentrations of melittin) was carried out to ensure that differences in LTR activity observed with various concentrations of melittin were not due to variations in transfection efficiencies. CAT assays were performed with cell lysates containing equivalent amounts of protein, as described (Brack-Werner *et al.,* 1992). Acetylated and non-acetylated forms of CAT were separated by thin-layer chromatography and quantified by liquid scintillation counting or phospho-imager analysis. Percentage conversions were within the linear range of the CAT assay. LTR activities in melittin-treated cells were standardized to LTR activity in untreated cultures.

EJ cells stably transfected with genes for antiviral peptides were supertransfected with 5 μg pHIVluc (Wintersperger *et al*., 1995) either alone or with 1 μg pSV2tat72. Cell extracts were prepared 48 h post-transfection, and equivalent amounts of protein were used for luciferase assays as outlined previously (Hornsby *et al*., 1992).

■ Establishment of EJ cells stably transfected with prepromelittin and preprocecropin genes, respectively

(i) Expression constructs. The prepromelittin cDNA sequence from *Apis mellifera* was amplified by PCR from plasmid pUM13/4 (Vlasak *et al*., 1983) using primers 1 (5′ ATA**GACGTC**AAGGAAGGAAGCGA-TCGGA 3′; *Aat*II site bold) and 2 (5′ TAT**GGATCC**AACCCTGTT-GCCTCTTACG 3′; *Bam*HI site bold). The preprocecropin sequence of *Hyalophora cecropia* was amplified from plasmid pCP9A1-2 (Lidholm *et al*., 1987) with primers 4 (5′ TAT**GACGTC**TCGTTAGAACGCGGCT 3′; *Aat*II site bold), and 5 (5′ GGC**AGATCT**TAAATGTATCATG-CAAT 3′; *Bgl*II site bold). For generation of retroviral expression constructs, pBag-derived plasmid p125gal (Saller, 1994; Price *et al*., 1987) was digested with *Aat*II and *Bam*HI to remove the *β*-galactosidase gene and the resulting vector fragment ligated to the prepromelittin- or preprocecropin-containing amplification products, respectively. The resulting expression plasmids contain the peptide genes under the transcriptional control of the murine leukaemia virus LTR in addition to a neomycin resistance gene driven by the SV40 early promoter.

(ii) Stable transfection and selection of G418 resistant clones. EJ cells were seeded in a 10 cm tissue culture dish at a density of 2×10^6 cells 1 day prior to lipofection and then lipofected with 4 μg plasmid using the lipofectamine kit from GIBCO BRL-Life Technologies, according to the manufacturer's instructions. Twenty-four h after transfection, the cells were diluted 1:10 and cultured in the usual medium supplemented with 400 μg/ml G418 (GIBCO BRL-Life Technologies). After 14 days, colonies of G418-resistant cell clones were visible and individual colonies isolated.

■ **Statistical analysis.** Testing of monotone dose–response relationship was performed by the exact version of the non-parametric Jonckheere–Terpstra test (StatXact, Cytel Software) which is robust against outliers. Reported probability values are one-sided where the influence of the antimicrobial peptides could be assumed to be in only one direction, otherwise they are two-sided as stated. Values of $P < 0{\cdot}05$ are considered statistically significant.

Results

Melittin- and cecropin-dependent suppression of HIV-1 production

To evaluate antiviral activity of melittin, virus production and metabolic activity of HIV-1-infected cells treated with various concentrations of melittin were compared with untreated cultures (Fig. 1*a*; Table 1). Infectivity of T lymphoma cell cultures (HUT-78) was determined by exposing culture supernatants to human fibroblastoid indicator cells (LC5-CD4) and subsequent quantification of HIV immunostained infectious centres. The compiled results of numerous experiments (median values for $n = 20$ given in Fig. 1*a*) show that production of HIV-1 by chronically HIV-1-infected T cells is decreased by treatment with melittin in a dose-dependent manner, confirming previous results (Wachinger *et al*., 1992). From these data, the ID_{50} was calculated as 0·4 μM (Table 1). The effect of melittin on virus production by acutely infected T cells was assessed by exposure of T lymphoma cells (HUT78) to cell-free preparations of HIV-1 (HIV-1 RF) and subsequent culture in the presence of various concentrations of melittin. Melittin reduced production of HIV in these acutely infected T cells in a dose-dependent manner (ID_{50} approximately 0·9 μM; Table 1). The effect of melittin on HIV production by acutely infected fibroblasts was assayed by directly quantitating infectious centres in LC5-CD4 cultures treated with various concentrations of melittin after exposure to HIV-1. Again, a dose-dependent decrease of the number of HIV-infectious centres was observed, yielding an ID_{50} of 1·5 μM (Table 1). These results demonstrate that the HIV-inhibitory effect of melittin is not T cell-specific. The cells appear to vary somewhat in their tolerance to melittin, the acutely infected T cells being most sensitive to toxic effects of melittin. However, in all cell lines the peptide concentrations required for ID_{50} were well within the non-toxic range (metabolic activity $\geqslant 85\%$ of control cultures, Table 1).

The amphipathic antimicrobial peptide cecropin was also evaluated for its anti-HIV activity. Treatment of acutely HIV-infected fibroblastoid cells with cecropin A resulted in dose-dependent decrease of HIV-infectious centres (Fig. 1*b*) and a calculated ID_{50} in the range 2–3 μM (Table 1). Interestingly, different cecropins varied in their capacity to inhibit HIV infectivity in this assay, cecropin A being more effective than cecropin P1, and cecropin B lacking HIV-inhibitory activity (data not shown).

These results demonstrate that treatment of HIV-infected cells with increasing concentrations of melittin or cecropin suppresses production of infectious HIV. The dose–effect relationship was shown to be highly significant for both peptide compounds ($P < 0{\cdot}0001$; Jonckheere–Terpstra test).

Melittin-induced suppression of HIV-1 intracellular protein and mRNA synthesis

The effect of melittin on intracellular production of HIV-1 Gag proteins was analysed in T lymphoma cells (KE37/1) acutely infected with HIV-1 IIIB and cultured with various concentrations of melittin (Fig. 2). Immunoblot analysis of cell lysates revealed decreased levels of Gag antigen in cells cultured with 1·05 and 1·4 μM melittin, compared to controls without melittin. These results indicate that melittin suppresses intracellular production of HIV structural proteins.

To determine whether melittin affects production of HIV-1 transcripts, HIV mRNAs were assayed in acutely infected T cells cultured with various concentrations of melittin. Previously described RT–PCR technology (Neumann *et al*., 1994) was used, enabling detection and quantification of the individual mRNA species produced in HIV-infected cells by amplification with primer pairs specific for each transcript class (large, intermediate and small).

Table 1. Anti-HIV-1 activity of melittin and cecropin

Peptide	Anti-HIV-1 activity*				Antibacterial activity†	Cytolytic activity‡
	HIV-1 infection	Cells	ID_{50} (μM)	MA at ID_{50} (%)		
Melittin	P	T	0·4	> 90		
	A	T	0·9	> 85	1·5	11
	A	F	1·5	> 90		
Cecropin	A	F	2–3	> 90	0·2	> 200

* Anti-HIV-1 activity was calculated from data presented in Figs 1(*a*) (melittin) and (*b*) (cecropin). T lymphoma cells (T) or fibroblastoid cells (F) were either persistently (P) or acutely (A) infected with HIV-1. Metabolic activity (MA) at ID_{50} is normalized to untreated cell cultures.
† Antibacterial activity is given as the lowest concentration that completely inhibits bacterial (*E. coli*) growth, according to Wade *et al*. (1992).
‡ Cytolytic activity was determined by erythrocyte lysis assay using sheep red blood cells (Steiner *et al*., 1981).

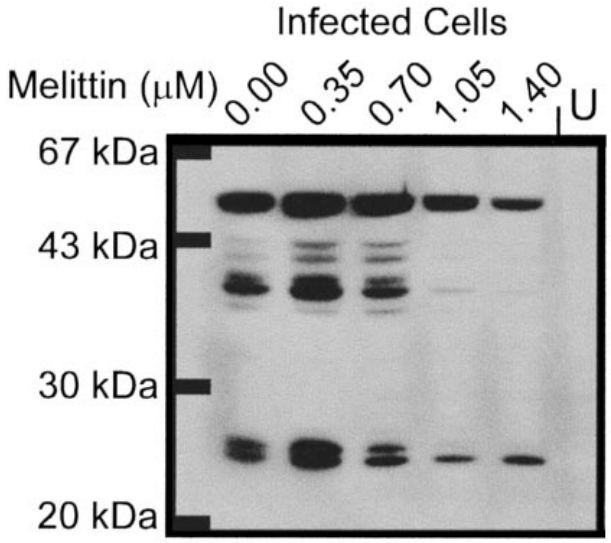

Fig. 2. Effect of melittin on production of cell-associated HIV-1 Gag antigen by acutely infected T cells. Western blot analysis of HIV-1 Gag in cell lysates of acutely HIV-1 (IIIB)-infected T lymphoma cells cultured with the indicated concentrations of melittin for 9 days. Lysates from uninfected T lymphoma cells were analysed as control (U).

Culture of acutely HIV-1-infected T lymphoma cells with various concentrations of melittin did not lead to loss of any HIV-1 transcript species (data not shown), suggesting that melittin does not grossly alter utilization of HIV-1 splice sites. For quantification, HIV-1 mRNAs of each transcript class were normalized to the constitutively expressed form of the single copy gene for PBGD (Chretien *et al*., 1988). No melittin-dependent reduction of mRNA levels of PBGD (Fig. 3*a*) or actin (data not shown) was observed, indicating that melittin does not inhibit expression of cellular housekeeping genes.

Levels of all HIV-1 transcript classes were suppressed in a dose-dependent manner by melittin (Fig. 3*a*). The dose–response relationship was statistically significant for all HIV-1 transcript classes (large, $P = 0{\cdot}0028$; intermediate, $P = 0{\cdot}0034$; small, $P = 0{\cdot}0437$) and for overall HIV-1 transcript levels ($P = 0{\cdot}0045$). Treatment of HIV-infected cells with 1·4 μM melittin led to a reduction of total HIV-1 mRNA levels to approximately 30% of HIV transcript levels in cells cultured without melittin. Synthesis of large and intermediate transcripts appeared to be suppressed to a greater extent than that of small transcripts (Fig. 3*a*). In agreement, the calculated ratio of (large):(intermediate + small) transcripts decreased from 1·9 to 0·7 ($P = 0{\cdot}0078$) and that of (large + intermediate):(small) transcripts decreased from 4·5 to 1·7 ($P = 0{\cdot}011$) between 0 and 1·4 μM melittin.

Suppression of HIV-1 LTR activity by antimicrobial peptides

Melittin-dependent reduction of overall levels of HIV-1 mRNAs suggested inhibition of LTR activity. Therefore, we investigated the effect of melittin on HIV LTR activity by assaying CAT reporter gene activity in T lymphoma cells co-transfected with an HIV-1 LTR-driven CAT plasmid and an HIV-1 *tat*-expression plasmid and subsequently cultured with various concentrations of melittin. Transfection assays were performed with uninfected and HIV-producer T lymphoma cells.

Melittin treatment reduced levels of CAT activity in a dose-dependent manner (Fig. 3*b*). This effect was evident both in uninfected and in chronically HIV-1-infected T cells (data not shown) in several independent transfection experiments. The dose–response relationship was highly significant ($P <$

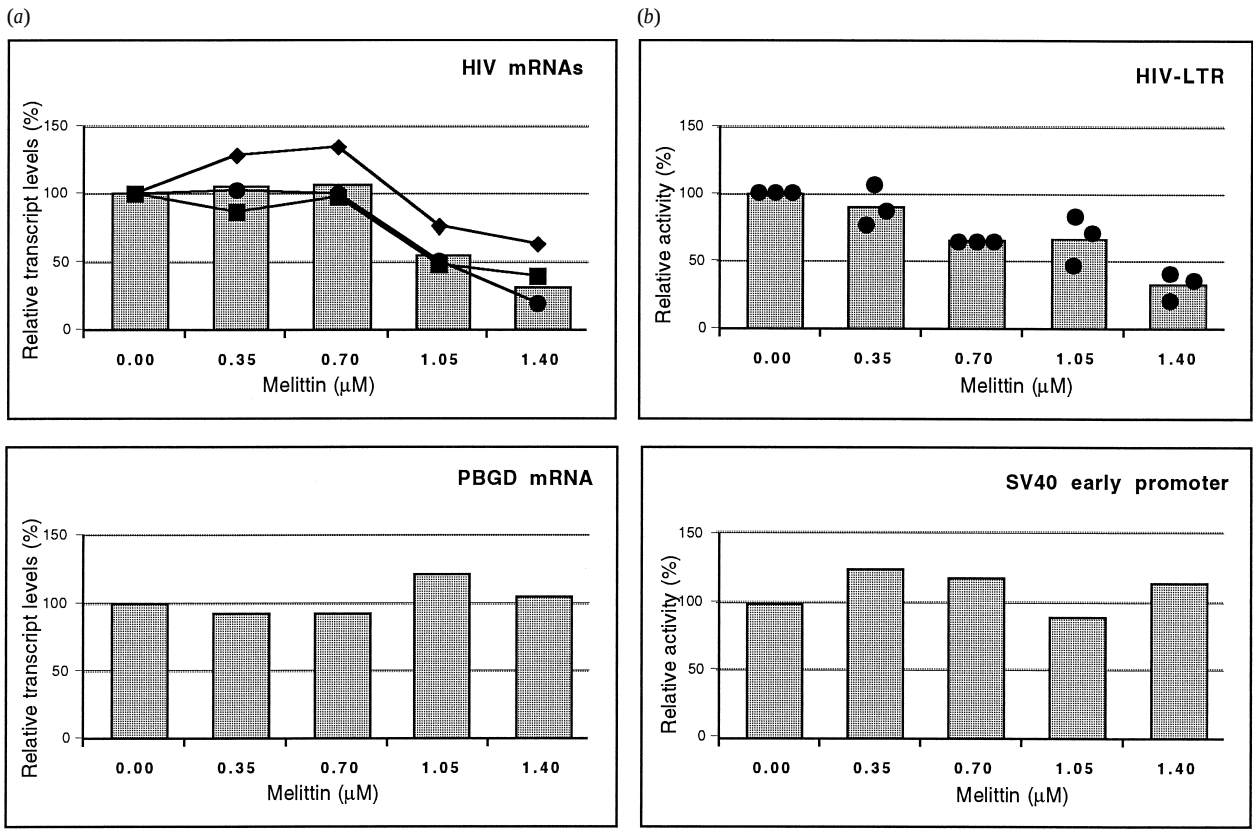

Fig. 3. Suppression of HIV gene expression by melittin. Melittin inhibits HIV-1 mRNA expression (*a*) and HIV LTR activity (*b*) in a dose-dependent manner. (*a*) The top graph shows the effect of melittin on HIV mRNA levels in T lymphoma cells acutely infected with HIV-1 relative to untreated cultures. HIV mRNA levels were determined by quantitative RT–PCR. Average values of relative HIV-1 mRNA levels are shown for total HIV-1 transcripts (bars) and for the three HIV-1 mRNA subclasses (small mRNAs, ◆; intermediate mRNAs, ■; large mRNAs, ●). The bottom graph shows mRNA levels of the cellular housekeeping gene PBGD as control. (*b*) The top graph shows the melittin-dependent decrease of HIV LTR activity in KE37/1 T lymphoma cells transiently cotransfected with an HIV LTR-dependent CAT reporter gene plasmid and a Tat-expression plasmid (pSV2tat72) by electroporation. Subcultures of transfected cells were propagated with various concentrations of melittin. HIV LTR activity was assessed by measuring CAT activity in cell lysates. HIV LTR activity of each melittin-treated subculture was related to untreated cells (%). Bars represent average relative values of three independent transfection experiments. Individual results (●) are indicated. The bottom graph shows the effect of melittin on SV40 early promoter activity as control. Bars represent average relative values of two independent transfection experiments.

0·0001). In transfected cells cultured with 1·4 μM melittin, levels of CAT activity were reduced to about 30% of control cultures without melittin. CAT activity was not reduced significantly in cells transfected with an SV40 early promoter-driven CAT plasmid (Fig. 3*b*), suggesting that melittin does not inhibit the SV40 early gene promoter.

To determine whether genes encoding antimicrobial peptides can direct inhibition of HIV LTR activity in human cells, melittin and cecropin expression plasmids were stably transfected into human cells and HIV LTR activities were assayed in these cells. For this purpose, cDNA encoding prepromelittin or preprocecropin A was inserted into a retroviral expression plasmid. Each construct (including pBag as negative control) was transfected into EJ human bladder carcinoma cells and G418-resistant cell clones were established. Cell clones were assessed for presence of the antimicrobial peptide genes by PCR and for MLV promoter-directed expression of mRNA by S1 analysis (data not shown). HIV-1 LTR activity was analysed by transient supertransfection with an HIV LTR luciferase reporter gene construct. Levels of luciferase activity were compared with a control cell clone containing pBag. To assess the effect of Tat, parallel supertransfections were carried out with and without a *tat*-expression plasmid.

As demonstrated in Fig. 4, HIV LTR activity in cell clones containing antimicrobial peptides (EJppm1, EJppCA10·8) clearly decreased relative to the control cell clone containing the plasmid vector pBag (EJpBag3) or parent EJ cells. Average inhibition was similar for the melittin- and cecropin-containing clones (reduction to $< 20\%$ in three and four independent supertransfection experiments, respectively). The decrease in HIV LTR activity was not significantly affected by expression of Tat, suggesting that the mechanism by which antimicrobial peptides reduces HIV LTR activity is Tat-independent.

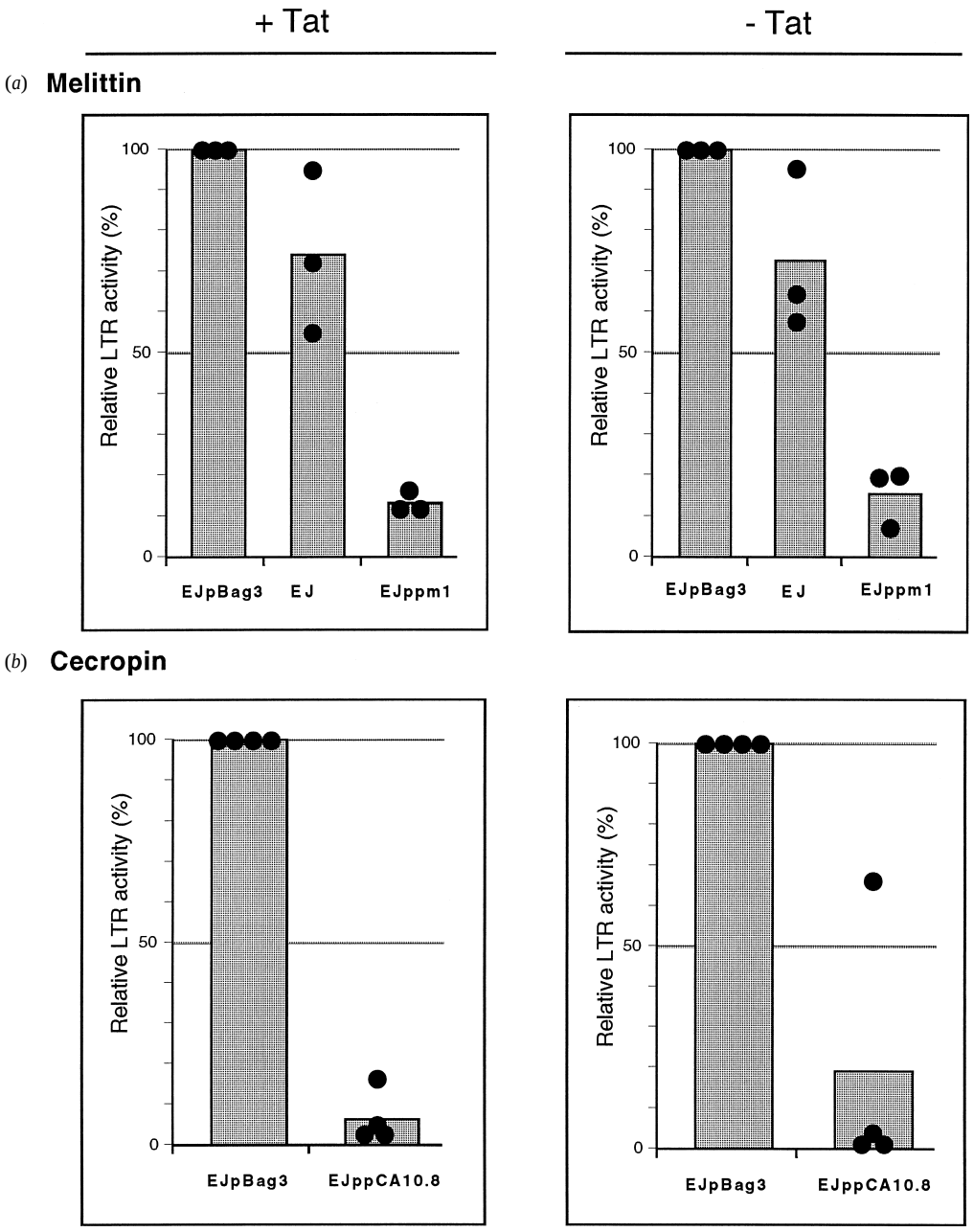

Fig. 4. Reduced HIV LTR activity in human cells stably transfected with genes encoding melittin (*a*) or cecropin (*b*). EJ human bladder carcinoma cells were stably transfected with retroviral expression plasmid pBag, or pBag carrying either the prepromelittin or preprocecropin genes, and G418-resistant cell clones were selected. HIV LTR activity in cell clones was assayed by parallel transient supertransfections of HIV LTR luciferase reporter gene construct with or without *tat*-expression plasmid (+Tat, left graphs; −Tat, right graphs). HIV LTR activities in cell clones containing the prepromelittin gene (*a*; EJppm1) or the preprocecropin gene (*b*; EJppCA10.8) are shown relative to control cells containing the pBag vector (EJpBag3). Relative LTR activities in untransfected parent cells (EJ) are shown in (*a*). Individual results (●) are given for three (*a*; melittin) and four (*b*; cecropin) independent supertransfection experiments. Bars indicate average relative LTR activities.

Discussion

The data presented here demonstrate that melittin and cecropin exhibit a dose-dependent inhibitory effect on production of HIV by infected T cells and fibroblasts *in vitro*. Reduced infectivity is concomitant with decreased intracellular synthesis of HIV gene products and reduced LTR activity. Concentrations at which melittin displays antiviral activity are not cytotoxic (Table 1) and do not reduce infectivity of cell-free virus stocks (Wachinger *et al.*, 1992). These results indicate that the anti-HIV activity of these amphipathic peptides is not due to lysis of cellular or viral membranes. Rather, these

peptides appear to interfere with the cellular capacity to support HIV replication by suppressing HIV transcription and reducing overall levels of viral gene products.

Melittin treatment reduced HIV gene expression without significantly affecting production of the cellular housekeeping gene PBGD or transcriptional activity of the SV40 early promoter (Fig. 3). This suggests that melittin is capable of selectively inhibiting HIV gene expression under conditions where expression of other genes remains unaffected. The decline in overall levels of HIV mRNAs achieved with 1·05 and 1·4 μM melittin paralleled the decline of HIV LTR activities, suggesting a reduction of HIV transcript levels by suppression of HIV LTR activity. Melittin appears to suppress HIV LTR activity in a Tat-independent manner, indicating that melittin influences cellular transcription factors of the HIV LTR. As a membrane-active peptide, melittin is capable of interfering with cellular signal transduction pathways in different ways, including activation of phospholipase A2 (Clark *et al.*, 1987; Sharma, 1993), and decreasing activities of calmodulin (Fisher *et al.*, 1994) and protein kinase C (Gravitt *et al.*, 1994). These properties may change the balance and activities of cellular stimulators of HIV transcription, such as NFκB, AP-1 and NFAT (Makropoulos *et al.*, 1996; Hoover *et al.*, 1996; Hill & Treisman, 1995; Crabtree, 1989; Gaynor, 1992) or induce inhibitory factors, analogous to the interferon-induced cellular inhibitor of HIV transcription (Tissot & Mechti, 1995). In addition, melittin may interfere with post-transcriptional regulation of HIV gene expression, since levels of large and intermediate classes of mRNAs were reduced to a greater extent than small mRNAs (Fig. 3*a*). Involvement of HIV-1 Rev in regulation of large and intermediate mRNAs opens the possibility that melittin may interfere with Rev function. Thus, melittin treatment appears to suppress HIV-1 gene expression at several different levels, suggesting that genes for antimicrobial peptides may be useful for intracellular immunization against HIV. Preliminary observations suggest that melittin inhibits replication of other retroviruses, such as murine leukaemia viruses (Ammentorp, 1994; D. Winder, unpublished results) and feline immunodeficiency viruses (Rauer, 1994). Further studies are required to address the specificity and conditions of inhibition of gene expression of other viruses by antimicrobial peptides.

The function of the innate immune system is to provide immediate defence against infectious invaders and to instruct the acquired immune response for long-range protection (Fearon *et al.*, 1996). In mammals, constituents of innate immunity include the complement system and its activators, such as C-reactive protein, natural killer cells, cytokines and intracellular antimicrobial compounds such as defensins. Innate immunity is important in protecting not only against bacterial pathogens, but also against viruses. This is underlined by the fact that some viruses have evolved mechanisms to at least partially escape initial eradication by innate immunity (Sparer *et al.*, 1996; Smith, 1996). Inhibition of HIV gene expression by melittin and cecropin suggests that the innate immune system may include an antiviral pathway for rapid defence against virus spread by curbing intracellular virus replication. Similar mechanisms could be involved in suppressing HIV production in cells with limited or no HIV replication (Brack-Werner *et al.*, 1992; McCune, 1995; Pomerantz *et al.*, 1992) and in suppression of HIV transcription by a factor produced by $CD8^+$ cells in HIV-infected individuals (Mackewicz *et al.*, 1995), suggesting the natural occurrence of cell-directed anti-HIV mechanisms. Such mechanisms may be especially important in the case of HIV, which has been shown to be protected by serum factors from complement-dependent lysis (Stoiber *et al.*, 1996). Thus, concomitant with immediate defence mechanisms against bacterial invaders, the innate immune system may have evolved multiple pathways for rapid antiviral defences.

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