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Structure dependence of biological activities for primate cathelicidins‡

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We have analysed the effects of variations in orang-utan (*ppy*), rhesus macaque (*mmu*) and leaf eater (*pob*) monkey orthologues of the human cathelicidin LL-37, on a range of relevant biological activities. These host defence peptides range in cationicity from +4 to +10, and while the more cationic *pob* and *mmu*RL-37 are in a monomeric and unstructured form in bulk solution (F-form), the human and *ppy*LL-37 are in an aggregated/helical form (A-form). The *in vitro* antibacterial activity depended strongly on both the structural form and the charge. F-form peptides were more potent against Gram-positive and -negative bacteria and less salt, medium or serum sensitive than A-form ones. CD studies suggested that A- and F-form peptides interact with LPS in different manners, but the ability to detoxify it did not correlate directly with either the charge or structure. Toxicity towards eukaryotic cells also showed a varied dependence on the peptides' physical characteristics. Haemolytic activity was similar for all the tested peptides while other cytotoxicity assays revealed the highly cationic, F-form *pob*RL-37 as the most toxic, followed by the A-form human LL-37. As shown with the human peptide, toxicity depended markedly on the nature and metabolic state of the target cell. Our results suggest that different evolutionary trajectories for each orthologue lead to distinct sets of physical characteristics, which significantly differentiates their biological activities.

Introduction

The human cathelicidin LL-37 is an important innate immune effector that contributes to our endogenous defences both via a direct antimicrobial activity against a range of micro-organisms [1], and by acting as a multifunctional regulator of cellular immune responses [2,3]. Added to these defence functions, is the ability to mediate healing responses, by stimulating angiogenesis [4] and re-epithelialisation of skin wounds [5], and also to counteract sepsis by binding to LPS [6–8] and neutralising its pro-inflammatory activity. It is the only cathelicidin present in humans, as well as in other primates, and is stored in the granules of cells involved in host defence, such as neutrophils, lymphocytes and macrophages [9,10] as well as in epithelial cells from skin or mucosal surfaces [11,12] that are most exposed to attack by pathogenic micro-organisms. The multiple protective capacities of LL-37 are packed into a relatively small (37 residue) and deceptively simple structure, that of an amphipathic α-helix. As such, it belongs to the vast family of amphipathic, helical antimicrobial peptides (AMPs) produced by organisms ranging from insects to amphibians and mammals [13]. However, unlike most of these peptides that adopt the helical structure only in the presence of biological membranes, LL-37 does so already in bulk solution, under physiological salt conditions. This is due to its high content in both cationic and anionic residues, which stabilise this conformation by forming intramolecular salt-bridges [14]. As the resulting structure is amphipathic, the peptide then readily aggregates to mask the hydrophobic surface (we call this the structured/aggregated or A-form). Recently, we have shown that this characteristic is common also to LL-37 orthologues from other Great and Lesser apes, whereas those from Old World Monkeys do not adopt secondary structure and remain monomeric in aqueous buffer (we call this the unstructured/free or F-form) [15,16]. These latter orthologues tend to be more cationic than the human peptide, so that intramolecular repulsions dominate over attractions and prevent helix formation in bulk solution.

As it has been observed for other AMPs, variation in charged residues during evolution appears to be positively selected [15,17], and in this case it has quite significant structural and functional consequences. We have inferred that A-form orthologues, such as human or orang-utan LL-37 (*ppy*LL-37) readily interact and form complexes with medium components in a manner that apparently reduces the direct antimicrobial activity (we term this a medium segregated or S-form) [15,16], but not the immunomodulatory activities on host cells [18,19]. F-form orthologues, such as rhesus macaque RL-37 (*mmu*RL-37) are less prone to this and present a generally more potent, broad-spectrum and less salt/medium

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Abbreviations used: *DMEM, Dulbecco's modified Eagles medium; FBS, foetal bovine serum; hss, Homo sapiens sapiens; LAL, limulus amoebocite lysate; LDH, lactate dehydrogenase; LPS; lipopolysaccharide; mmu, Macaca mulatta; PBS, phosphate buffered saline; PC, phosphatydilcholine; PG, phosphatidylglycerol; PIL, physiologic ion-like buffer; pob, Presbytis obscura; ppy, Pongo pygmaeus; SPB, sodium phosphate buffer; TFE, trifluoroethanol, TSB, Tryptic soy broth.*

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sensitive antimicrobial activity [15,16]. The differential tendency of primate LL-37 orthologues to assume the two forms also seems to determine their ability to modulate some host-cell responses so that, for example, A-form peptides trigger fibroblast proliferation *in vitro*, while F-form ones do not [20]. In this paper, we further investigate the dependence of biological activities of primate cathelicidins on their physical characteristics, using four orthologues from human, orang-utan, macaque and leaf eater monkey that range in cationicity from +4 to +10, and in conformation from strongly A-form to strongly F-form. In addition, we analyse their *in vitro* antimicrobial activities under different conditions, their toxicity towards eukaryotic cells, and their ability to neutralise the pro-inflammatory activity of LPS. We show that functional characteristics do not depend only on the 3D structure that the peptides adopt, but also on other features, as well as characteristics of the target cells, so that each peptide seems to have evolved specific arrays of biological activities.

Materials and Methods

Peptide Synthesis and Characterisation

LL-37 orthologues were synthesised from human (*Homo sapiens sapiens, hss*), orang-utan (*Pongo pygmaeus, ppy*), rhesus macaque (*Macaca mulatta, mmu*) and leaf eater monkey (*Presbytis obscura, pob*). Fmoc-solid phase peptide synthesises were performed on either a Pioneer (PE Biosystems) or CEM Liberty automated peptide synthesiser, following a protocol described previously [15]. Peptides were cleaved from the resin with a version of the reagent K and the crude peptide purified with a Waters Delta Pak C18 column (15 μm, 300 Å, 25 mm × 100 mm). The quality and correctness of the peptides was confirmed using ESI-MS (Bruker Daltonics Esquire 4000). Peptide solutions were prepared from stock solutions in water, in which the concentrations were independently quantified by (i) the weight of the peptide used, (ii) colorimetric determinations using the bicinconinic acid method (Pierce, Rockford, IL), (iii) phenylalanine absorption at 257 nm ($\varepsilon_{257} = 195.1\ \text{M}^{-1}\ \text{cm}^{-1}$ per residue). CD spectroscopy was performed on a J-715 spectropolarimeter (JASCO Corp., Japan), using 2-mm quartz cells and 20 μM peptide, under various conditions, as described previously [16]. Spectra are the average for at least two independent experiments, each with accumulation of three scans. Buffers used were SPB (10 mM sodium phosphate buffer, pH7); PBS (phosphate buffered saline, SPB + 150 mM NaCl) and PIL (113 mM NaCl; 24 mM $NaHCO_3$; 0.6 mM $MgCl_2$; 1.3 mM $CaCl_2$; 3.9 mM KCl, pH 7.3). Preparation of large unilamellar vesicles (LUVs) has been described previously in SPB [16,21], while for experiments with LPS, solutions of 25 μM endotoxin in water were prepared from a 4 mg ml^{-1} stock solution in SPB, assuming an average MW of 4000 g mol^{-1} [7,22].

Antimicrobial Activity Assays

The concentration and medium dependent antimicrobial activities of the peptides were qualitatively evaluated by following the inhibition of bacterial growth by peptides for 10^6 CFU/ml *Escherichia coli* ML35 or *S taphylococcus aureus* 710A cells placed in a 96 well plate under different medium conditions and peptide concentrations (5–50% v/v TSB, 5% v/v TSB +10% v/v FBS, in 10 mM SPB, 0.5–10 μM peptide). Growth was monitored at 600 nm for 4 h at 37 °C, with a Tecan Sunrise reader. Percent growth inhibition is given as $[(A^0 - A^P)/A^0] \times 100$, where A^0 is the absorption intensity in the absence of peptide at 4 h, and A^P is the absorption intensity in the presence of a given concentration of peptide. Plates were then incubated overnight at 37 °C to determine if long-term growth inhibition occurred, by visual inspection.

Cell Cultures

Primary cultures of human lymphocytes and monocytes were prepared from buffy coat obtained from healthy donors and isolated by Histopaque density gradient centrifugation. Stabilised cell lines such as healthy human adult dermal (haFB), embryonic lung WI38 (ATCC: CCL-75) and NIH3T3 fibroblasts were obtained from the Oncological Reference Centre (CRO) in Aviano, Italy. Neoplastic MOLT4 and NB4 (lymphoma) and MCF-7 (mammalian carcinoma) were from ATCC. The murine macrophage RAW 264.7 cell line was obtained from the Experimental Zooprophylactic Institute of Lombardy and Emilia Romagna regions (Brescia, Italy). Cells were maintained at 37 °C under 5% CO_2 in the appropriate culture medium (RPMI 1640 or DMEM) supplemented with 10% (v/v) foetal calf serum (FCS) and 2 mM glutamine.

Cytotoxic Activity Assays

Haemolysis assays were performed by adding different concentrations of peptides to 0.5% erythrocyte suspensions from healthy donors in PBS, and incubating for 30 min at 37 °C. Lysis was then blocked by adding a five-fold excess of ice-cold PBS and immediately centrifuging at 5000 rpm for 5 min, and haemoglobin absorption in the supernatant measured at 405 nm. Percent haemolysis was calculated as $[(A^P - A^0)/(A^{100} - A^0)] \times 100$, where A^P is the absorption in the presence of peptide, A^0 is that in the absence of peptide (blank) and A^{100} the absorption of the erythrocyte suspension treated with 1% Triton-X to achieve complete lysis.

LDH activity assays were performed on RAW 264.7 cells, seeded in 24-well plates at a density of 0.5×10^6 cells/well and incubated for 24 h at 37 °C in complete RPMI. Cells were then incubated for 2 h at 37 °C with the peptides at 2.5, 5 or 10 μM in complete RPMI. The activity of the cytosolic enzyme LDH was measured in cell-free media and cell lysates using the CytoTox 96 non-radioactive cytotoxicity assay (Promega). LDH activity in the culture media was expressed as percent of total cellular LDH activity.

Permeabilisation to propidium iodide (PI) of peptide-treated NIH3T3 or RAW 264.7 cells was evaluated after 30 min incubation in serum free, standard saline solution [20]. PI staining was assessed by using a flow cytometer equipped with the Cell Quest software and a 488-nm argon ion laser as the excitation source (FACScan, Becton Dickinson). A minimum of 10 000 events per sample were analysed.

Cell viability of cells exposed to LL-37 was determined by methyl thiazole tetrazolium (MTT) standard procedures, after 1 h incubation with 4–40 μM peptide in different media (SPB, PIL and RPMI). After extensive washing with PBS, cells were maintained for further 24 h in complete medium prior to the MTT test.

Inhibition of LPS-induced Activities

A quantitative chromogenic limulus amoebocyte lysate (LAL) assay was performed using the QCL-1000 kit (Lonza). Peptide dilutions were prepared in serum-free RPMI. LPS from *E. coli* O111 : B4 was added to a final concentration of 0.25 ng/ml. Spectrophotometric detection was performed at 405 nm and changes in optical density

(ΔOD) between 0 and 6 min were calculated as described [23]. Percent LPS neutralisation was calculated as: % neutralisation $= [1 - \Delta OD^{peptide}/\Delta OD^{control}] \times 100$.

Nitric oxide (NO) production by RAW 264.7 cells, incubated with complete RPMI and stimulated with 100 ng ml^{-1} LPS from *E. coli* O111 : B4, was monitored in the absence or presence of the peptides. The accumulation in the supernatant of nitrite, a stable end product of NO, was measured after 24 h incubation at 37 °C using the Griess reagent (Molecular Probes). The nitrite concentration was determined with reference to a standard curve generated using known amounts of sodium nitrite. Inhibition of NO production was calculated as: % inhibition $= [1 - NO^{peptide}/NO^{control}] \times 100$.

Results and Discussion

The sequences, physico-chemical characteristics and structural features of the four studied LL-37 orthologues are summarised in Table 1. The human (*hss*) and orang-utan (*ppy*) LL-37 partially structure as α-helices even under relatively low salt conditions (SPB buffer), as determined by CD spectroscopy, and this increases with ionic strength (e.g. in PBS or PIL buffers [16]). The Old World monkey *mmu* and *pob*RL-37, on the other hand, remain unstructured in aqueous solutions under all conditions. All peptides however showed robust and comparable structuring in the presence of helix-favouring conditions such as 50% TFE (Table 1) or SDS micelles [16]. Titration of the peptides in aqueous solution with TFE (Figure 1(A)) reveals that the tendency to fold correlates with an excess of intramolecular salt-bridging attractions to repulsions (Table 1) [15], following the trend *ppy* > *hss* ≫ *mmu* ≈ *pob*. From these data, we deduce that *ppy* and *hss*LL-37 are respectively strongly and moderately A-form (see introduction), while *mmu* and *pob*RL-37 are markedly F-form. All the peptides fold in the presence of LUVs composed of the negatively charged phospholipid phosphatidylglycerol, a model for bacterial membranes, indicating that both types of peptides have a strong ability to interact with and insert into this type of membrane (Table 1). Conversely, LUVs composed of zwitterionic phospholipids (phosphatidylcholine), hardly affect the helix content with respect to aqueous condition, indicating a surface interaction without significant added structuring and insertion, at least for the F-form peptides.

The ability of peptides to inhibit bacterial growth (% growth inhibition) was determined at increasing peptide concentrations, under several different medium conditions, over a period of 4 h, whereas long-term inhibition was checked at 24 h (see Table 2, ○ no growth, ● growth). Taken together, these assays indicate that F-form peptides are generally more potent and less susceptible to both medium and serum components than the A-form ones, following the trend *pob* > *mmu* > *hss* > *ppy*. *pob*RL-37 is capable of inhibiting bacterial growth, on the long term, at quite low concentrations (≤1 μM), regardless of the presence of medium or serum components, whereas *ppy*LL-37 is effective long term only at a ten-fold higher concentration, even under the most permissive conditions (5% v/v TSB). This data suggest that two factors are important in modulating the antimicrobial potency, i.e. charge and conformation. Increased cationicity in general correlates with increased potency of helical peptides [13,25] and this varies dramatically, from +4 to +10, from *ppy* to *pob*. Added to this,

Table 1. Sequence and physico-chemical properties of LL-37 orthologues

Peptide	sequence[a]	MW	q	<H>	μH	A/R	% helix[e] SPB	PBS	TFE	PG^{LUV}	PC^{LUV}
*hss*LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	4493	+6[b]	−1.8[c]	0.59[c]	9/7[d]	30	30	70	60	35
*ppy*LL-37	LLGDFFRKAREKIGEEFKRIVQRIKDFLRNLVPRTES	4506	+4	−1.7	0.59	11/5	50	60	70	75	60
*mmu*RL-37	RLGNFFRKVKEKIGGGLKKVGQKIKDFLGNLVPRTAS	4101	+8	−1.7	0.56	4/5	5	5	65	70	10
*pob*RL-37	RLGNFFRKAKKKIGRGLKKIGQKIKDFLGNLVPRTES	4243	+10	−2.1	0.62	1/9	5	5	65	70	10

[a] Conserved residues in orthologues relative to human LL-37 are shaded grey.
[b] Charge.
[c] Mean hydrophobicity and relative hydrophobic moment, calculated as described in [13].
[d] Intramolecular attractions over repulsions in helix polar sector, derived from Figure 6 in [15].
[e] Estimated for 20 μM peptide in SPB or PBS buffers, 50% TFE or in the presence of LUVs composed of phosphatidylglicerol (PG) or phosphatidyl choline (PC), using the method of Chen *et al*. [24].

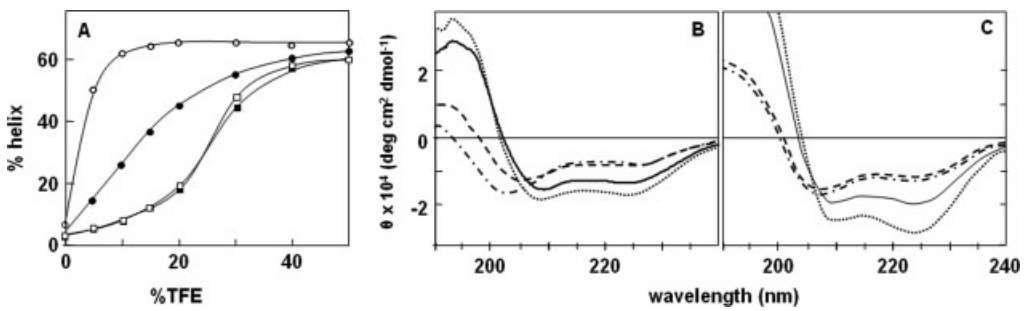

Figure 1. Structuring of LL-37 orthologues in the presence of TFE and LPS. (A) The % helical content in presence of increasing concentrations of trifluoroethanol (TFE) was determined from the molar ellipticity [θ] at 222 nm [24], using 20 μM peptides in SPB; ● *hss*, ○ *ppy*, □ *mmu*, ■ *pob*. (B,C) CD spectra of LL37 orthologues [20 μM] in the presence of LPS in aqueous solution (B) and in SPB buffer (C); (*hss* ———; ppy · · · · ; mmu - - - - ; pob -·-·-·).

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Table 2. Inhibition of bacterial growth by LL-37 orthologues

	% Growth inhibition[a] (*E. coli*)												% Growth inhibition (*S. aureus*)		
	5% TSB			20% TSB			50% TSB			10% FCS			5% TSB		
	μM			μM			μM			μM			μM		
Peptide	0.5	1	5	0.5	1	5	1	2	5	0.5	5	10	2	4	8
*hss*LL-37	100 ●	100 ○	100 ○	10 ●	95 ●	100 ○	10 ●	15 ●	95 ●	<5 ●	100 ○	100 ○	20 ●	70 ●	100 ○
*ppy*LL-37	0 ●	60 ●	90 ○	0 ●	5 ●	95 ●	0 ●	<5 ●	10 ●	<5 ●	100 ○	100 ○	0 ●	0 ●	65 ●
*mmu*RL-37	100 ○	100 ○	100 ○	40 ●	100 ○	100 ○	100 ○	100 ○	100 ○	100 ●	100 ○	100 ○	75 ●	100 ○	100 ○
*pob*RL-37	100 ○	100 ○	100 ○	100 ○	100 ○	100 ○	100 ○	100 ○	100 ○	100 ○	100 ○	100 ○	100 ○	100 ○	100 ○

[a] determined over a period of 4 h as $[(A^a - A^p)/A^a] \times 100$, where *A* is the absorption at 600 nm in the absence (^{a}) or presence (^{p}) of peptide; long-term inhibition was checked after 24 h incubation at 37 °C (○ no growth; ● growth).

structured and aggregated (A-form) peptides seem to be '*stickier*' than the unstructured F-form peptides, and their interaction with medium or serum components could lead to a complexed form (the segregated or S-form), which may be prevented from effectively reaching or interacting with the microbial membrane.

With model amphipathic helical AMPs, we have found that potency is generally lower towards gram-positive bacteria than gram-negative ones [25]. This may be an indication that the thick peptidoglycan layer of the former acts as a more effective barrier to this type of AMP than the outer membrane and thin peptidoglycan layer of the latter. For the LL-37 orthologues, the fact that F-form peptides are more potent against *S. aureus* than A-form ones may indicate a lower tendency to interact with and be sequestered by peptidoglycan components.

It is well known that human LL-37, along with other cationic AMPs, can interact with lipopolysaccharide from the outer membrane of gram-negative bacteria [26], and reduce its pro-inflammatory effects [8,22,23]. It does so presumably by binding strongly to, and sequestering it, and/or by disrupting the structure of LPS micelles [7,22], thus preventing effective interaction with the LPS-binding protein and subsequently to cellular receptors that activate the pro-inflammatory cascade. We have analysed how the structural form or cationicity of LL-37 orthologues affects their anti-endotoxin capacities. To begin with, we have tested the ability of the peptides to interact with LPS by determining its effect on the peptides' structures. In aqueous solution, at a ratio approximately 1 : 1 with LPS, CD spectra (Figure 1(B)) indicate a substantial structuring to a helical conformation for *hss* and *ppy*LL-37, and a lower, albeit significant helical content for *mmu* and *pob*RL-37. The helicity is somewhat increased in the presence of salt (SPB buffer, Figure 1(C)), but still considerably less than for the human and orang-utan peptides, which under these conditions are already partly structured in buffer alone (Table 1). The spectra in SPB for *hss* and *ppy*LL-37 show a change in shape on interaction with LPS, so that the negative maximum at 223 nm now dominates over that at 208 nm, which is consistent with the formation of multimers [16,27]. The A- and F-form peptides may therefore interact differently with LPS, the former in a more structured and aggregated form, and the latter possibly with a combination of monomeric helical and extended conformations.

The LPS detoxification properties of the peptides have been studied by determining their effects on the pro-coagulant activity of LPS, via the LAL assay, and inhibition of NO production induced by LPS in RAW264.7 macrophages, via the Griess assay. In the LAL assay (Figure 2(B)), both A-form peptides (*hss* and *ppy* LL-37) showed a good capacity to reduce the coagulation cascade, whereas the moderately F-form rhesus *mmu*RL-37 acted at 20-fold higher concentrations. The other F-form peptide, the highly

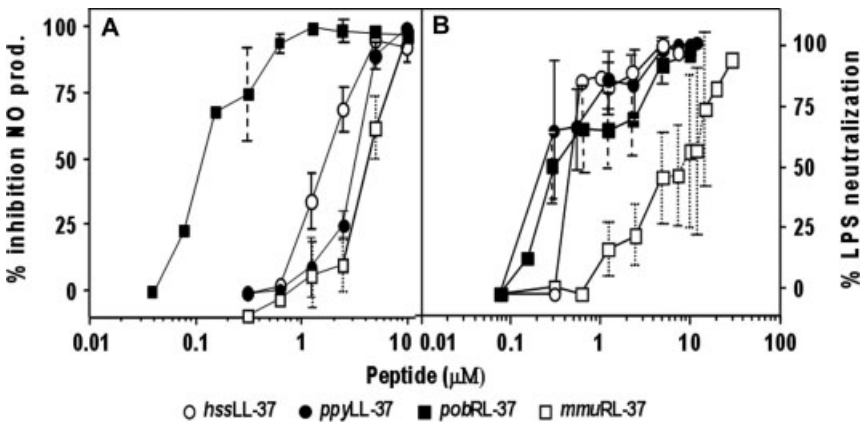

Figure 2. Effect of primate cathelicidins on LPS-induced effects. (A) Nitric oxide generation was determined spectrophotometrically, using the Griess reagent, in RAW 264.7 cells incubated in serum-containing RPMI medium and 100 ng/ml LPS, for 24 h in the presence of peptides. (B) LPS-induced LAL reaction. LPS at 25 ng/ml was incubated in serum-free RPMI medium with increasing peptide concentrations and residual LPS activity determined via the LAL assay. Percentage inhibition of NO production or % LPS neutralisation are reported relative to cells incubated with LPS alone. Results are the mean ± SD of three independent experiments.

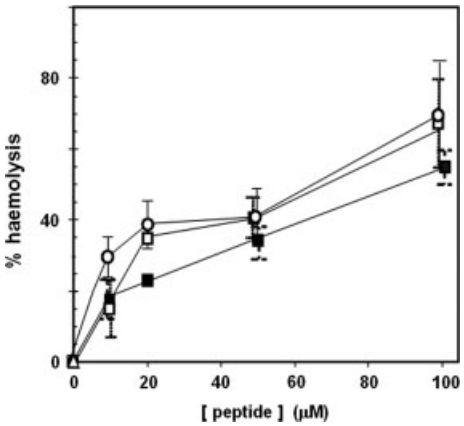

Figure 3. Hemolytic activity of LL-37 orthologues. Percent haemolysis was determined by monitoring haemoglobin release from a 0.5% erythrocyte suspension, at 405 nm, in the presence of peptides at increasing concentrations and relating it to 100% release in the presence of 1% triton-X. Data are mean ± S.D. of at least three independent experiments, using blood from different healthy donors.

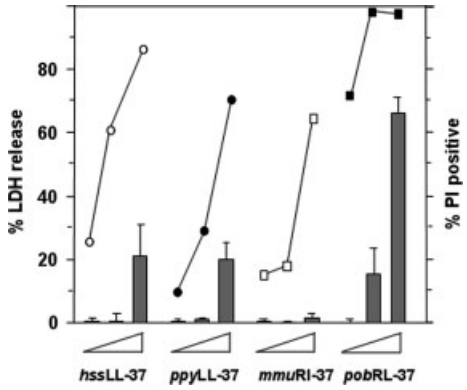

Figure 4. Cytotoxicity of LL-37 orthologues. LDH activity (histograms) was measured in the supernatant of RAW 264.7 cells, treated with increasing peptide concentrations (2.5, 5 and 10 μM) in complete RPMI medium, and expressed as percentage of total cellular LDH activity, after subtraction of the activity detected in the supernatant of untreated cells (2.81 ± 4.87%). Percentage positivity to propidium iodide (plots) was measured in NIH3T3 cells treated with the same peptide concentrations, but in serum free, standard saline solution [20]. Data are mean ± S.D. of three independent experiments.

cationic *pob*RL-37, was however as effective as the A-form peptides in this assay. As shown in figure 2(A), *pob*RL-37 (F-form, +10) was by far the most effective in suppressing LPS-induced NO production in macrophages, followed by *hss*LL-37 (A-form, +6) at significantly higher concentrations, whereas *ppy*LL-37 (A-form, +4) and *mmu*RL-37 (F-form, +8) were relatively inefficient. The LPS-neutralising activity thus does not seem to depend directly on the structural form or charge of the peptides, nor on their ability to interact with LPS (see CD studies), although these likely all contribute in some manner. The discrepancy between the peptides' relative activities in the two assays may in part be explained by *pob*RL-37 being less affected than *hss* or *ppy*LL-37 by the presence of serum in the Griess, while it is not present in the LAL assay, and/or by the different types of LPS/peptide complexes which may form under different conditions, as suggested by the difference in CD spectra (Figure 1). It is interesting to note that whereas the two F-form peptides in any case detoxify LPS at concentrations comparable to their antimicrobial ones, the A-form peptides have detoxifying concentrations that are at least 10-fold lower than the antibacterial ones, under comparable conditions.

The toxic effects of the peptides on eukaryotic cells have been investigated using a range of different assays on primary cells and cell lines. To begin with, we have carried out haemolysis assays on freshly prepared erythrocytes from healthy donors (Figure 3). Haemolysis levels can vary markedly among different donors, but overall, the peptides did not show a significant difference in activities. The A-form *hss*LL-37 was possibly somewhat more haemolytic at lower concentrations, but in the absence of serum, the estimated HC_{50} values for *hss, mmu* and *pob* orthologues (75 ± 25, 70 ± 15, 85 ± 15 μM, respectively) were overlapping. These values are significantly higher than both the *in vitro* antimicrobial and anti-endotoxin concentrations, although measurable haemolysis (15–30%) occurs at 10 μM peptide.

Other assays for cytotoxicity assess damage to the cytoplasmic membrane by monitoring permeabilisation to propidium iodide or release of LDH from the cytoplasm. RAW 264.7 macrophages, cells involved in bacterial clearance, were exposed to increasing concentrations of the peptides and LDH activity measured in the supernatant after 3 h (Figure 4). The *hss* and *ppy*LL37 resulted in significant enzyme release only at high concentrations, and *mmu*RL-37 was not cytotoxic under any of the tested conditions. Significantly, *pob*RL-37 showed the highest cytotoxicity. It should be noted that the LDH assay was carried out in the presence of serum, which might mitigate the cytotoxicity of the A-form peptides but not the F-form *pob* peptide. PI internalisation into RAW cells (not shown) or murine fibroblasts (Figure 4), as determined using flow cytometry, was studied in standard saline buffer, in the absence of serum. This assay confirmed the highest cytotoxicity for *pob*RL-37, and a lower cytotoxicity for *mmu*RL-37 and *ppy*LL-37, while *hss*LL-37 was intermediate. The cytotoxicity of the peptides thus does not seem to depend directly on the structure. It should be noted that CD spectroscopy indicates that the peptides, and particularly F-form ones, do not adopt any secondary structure in the presence of zwitterionic model membranes (Table 1). However, we have shown that they are still capable of efficiently lysing zwitterionic LUVs [16], possibly via some form of surface interaction. Cationicity and possibly other factors, also pertaining to the nature of the target cells, seem to play significant roles.

The influence of specific cellular features (such as surface composition or metabolic state) on cytotoxicity was investigated for the human LL-37 against a number of different normal or transformed cells of human origin, under different conditions. Figure 5 shows the effect of 1 h exposure to the peptide on cell viability as assessed using the MTT assay after 24 h. Under hypotonic conditions (SPB), resting monocytes and lymphocytes are less susceptible than proliferative NB4 cells to the cytotoxic effect of LL-37, which is only partially structured under these conditions. In the presence of PIL buffer, which reproduces physiological mono and divalent salt concentrations, LL-37 is fully A-form, is toxic to NB4 cells and may show some toxicity also towards peripheral blood mononuclear leukocytes (PBML), although statistical significance is not reached. Only in RPMI complete medium one does observe a statistically significant inhibition of all cell types. LL-37 is well structured under these conditions (as assessed by CD spectroscopy at 222 nm, results not shown), and the metabolic activity of cells is increased. It is interesting to note that statistically significant toxicity is observed for monocytes in PIL in the presence of 20% serum, whereas it

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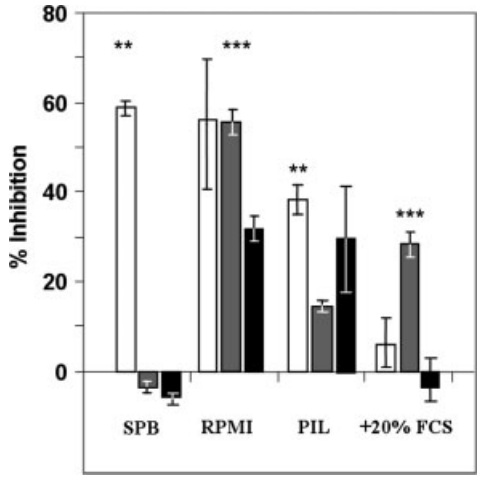

Figure 5. Cytotoxicity of LL-37 under different medium conditions. Human lymphoma NB4 cells (white bars), primary cultures of human monocytes (grey bars) and lymphocytes (black bars) were exposed to 40 μM LL-37 under different medium conditions (SPB, RPMI, PIL, PIL+20 or PIL +50% FCS) for 1 h, washed and incubated in complete medium for 24 h before carrying out the MTT assay. Bars show % inhibition relative to untreated control for the same conditions. Values are the mean ± SEM ($n = 5$). $^{**}p < 0,01$; $^{***}p < 0,001$ (ANOVA test followed by the Student-Newuman-Keuls post-test; Instat software, GraphPad Inc. San Diego CA).

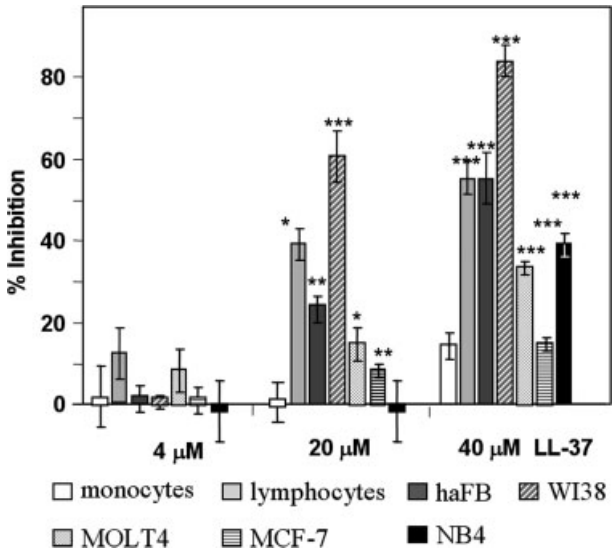

Figure 6. Cytotoxicity of LL-37 towards different cell lines. Human cell lines consisted of fresh primary cultures of human monocytes, lymphocytes, stabilised adult dermal fibroblasts (haFB) or embryonic lung (WI38) fibroblast, solid mammalian tumour (MCF-7) or lymphoma (MOLT4, NB4), and were treated with LL-37 in PIL buffer for 1 h, washed and incubated in complete medium for 24 h before carrying out the MTT assay. Bars show % inhibition relative to untreated control for the same conditions. Values are the mean ± SEM ($n = 5$). $^{**}p < 0,01$; $^{***}p < 0,001$ (ANOVA test followed by the Student-Newuman-Keuls post-test; Instat software, GraphPad Inc. San Diego CA).

decreases for NB4 cells and lymphocytes. From these data it would appear that the metabolic state of the cells is an important factor in determining the activity of LL-37, although the structural form and segregation by binding to serum components likely also play a role.

These studies were extended by investigating the toxic effects of LL-37 towards a panel of primary or transformed cells, in PIL buffer alone, so as to avoid interference from serum or medium effects. LL-37 shows no significant toxicity towards any cell type at 4 μM. At higher concentrations, resting monocytes are the most resistant, with no significant cytotoxicity even at 40 μM peptide, confirming our previous observation (Figure 6). For other cell types, a higher toxicity is evident for fibroblasts, and in particular for fast-dividing embryonic WI38 fibroblasts. In general, our study did not show a greater toxicity of LL-37 towards transformed cell lines.

Conclusions

LL-37 orthologues show a concentration dependent toxicity towards both bacterial and eukaryotic cells, but are generally at least an order of magnitude more effective towards the former. Concerning bacterial cells, F-form peptides are more potent and less salt, medium or serum sensitive than A-form ones. They are also more effective against gram-positive bacteria. This may be because of a lower tendency to be sequestered by medium or serum components, and possibly also to interact with bacterial cell wall components in an unproductive manner. The anti-endotoxin activity of the peptides is more complex, and CD studies indicate that A- and F-form peptides may interact with LPS in different manners. The ability to detoxify the endotoxin however did not seem to depend directly on either the structural form or the cationicity, as the two F-form peptides (*mmu*, +8 and *pob*, +10) place themselves respectively as both most and least effective. Cytotoxicity towards host cells is also complex. While the haemolytic activity is similar for all tested peptides, other assays for cellular permeabilisation reveal the highly cationic, F-form *pob*RL-37 as the most lytic, followed by the A-form human LL-37, so that cationicity and structural form again both seem to be relevant but not determining. The cytotoxicity of human LL-37 also depends markedly on the nature and metabolic state of the target cells, with activated cells appearing to be more susceptible than resting ones. Taken together, our results add to others that just begin to highlight the complexity of the biological activities of primate cathelicidins. Considering the relative simplicity of the *in vitro* assays that are used, it is daunting to consider what may actually be occurring in the much more complex *in vivo* conditions under which these peptides are called on to act. Evidently, these conditions are sufficiently diverse in different primate species to determine evolutionary trajectories for each orthologue, which lead to distinct sets of physical characteristics, which in turn significantly differentiated their biological activities, in a relatively brief span of evolutionary time.

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