The antimicrobial peptides lactoferricin B and magainin 2 cross over the bacterial cytoplasmic membrane and reside in the cytoplasm

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Abstract The localization of immunolabelled antimicrobial peptides was studied using transmission electron microscopy. *Staphylococcus aureus* and *Escherichia coli* were exposed to lactoferricin B (17–41), lactoferricin B (17–31) and D-lactoferricin B (17–31). *E. coli* was also exposed to cecropin P1 and magainin 2. The lactoferricins were found in the cytoplasm of both bacteria. In *S. aureus* the amount of cytoplasmic lactoferricin B (17–41) was time- and concentration-dependent, reaching a maximum within 30 min. Cecropin P1 was confined to the cell wall, while magainin 2 was found in the cytoplasm of *E. coli*. The finding of intracellularly localized magainin is not reported previously.

Key words: Antimicrobial peptide; Lactoferricin; Magainin; Cecropin; Cytoplasmic membrane; Cytoplasm

1. Introduction

Cationic antimicrobial peptides have been isolated from many different organisms during the last decade [1]. They vary in primary and secondary structure, but have some common properties. They are short, most of them consisting of less than 45 amino acids, amphipathic, and carry a net positive charge [2]. Many of these peptides have been extensively studied in order to elucidate their antimicrobial mode of action. The peptide action on the bacterial cytoplasmic membrane is thought to be responsible for their bactericidal effect on susceptible bacteria. Different models for this action have been proposed. They might form pores [3], or act by thinning the membrane [4] or by destabilizing the membrane bilayer [5]. The net result of these models is increased permeability of the membrane and lysis of the bacterial cell.

Cecropin P1 (Cec P1) is a 31 amino acid long basic polypeptide isolated from pig small intestine [6]. It is most active against Gram-negative bacteria [5]. It kills bacteria by immediate lysis [7]. Magainins are 23 amino acid long antimicrobial peptides isolated from frog [8]. These peptides kill bacteria by permeabilizing their membranes. D-Analogues of cecropins and magainins retain their antibacterial effect, and hence their mode of action is not thought to be receptor-mediated [9,10].

Intracellular targets are reported for other antimicrobial peptides. Buforin II binds to DNA and RNA [11], indolicidin inhibits DNA synthesis and to a lesser extent RNA synthesis [12], and cecropin PR39 inhibits DNA and protein synthesis [7]. These peptides also have effects on the outer and inner membranes of bacteria. It is not known whether their bactericidal mode of action is due to their membrane effects, their effects on intracellular targets, or a combination of these effects [13].

Lactoferricin B (Lfcin B) is generated by gastric pepsin cleavage of bovine lactoferrin [14]. It consists of 25 amino acids, corresponding to residues 17–41 of bovine lactoferrin. It has a broad-spectrum antibacterial effect [15]. The precise mode of action is not fully elucidated, but human lactoferricin is shown to cause depolarization and loss of integrity of the cytoplasmic membrane, loss of the pH gradient and to exert a bactericidal effect on *Escherichia coli* [16].

We have previously identified LPS and TA as the initial binding sites for Lfcin B [17]. Lfcin B neither lyses bacteria nor causes a major leakage from liposomes [18]. It depolarizes membranes of susceptible bacteria and induces fusion of negatively charged liposomes [18]. Kinetic studies have shown that the lactoferricins have a slow bactericidal action [19], which might indicate an intracellular target. Based on these findings we wanted to visualize the possible finding of Lfcin B (17–41) in the cytoplasm by use of transmission electron microscopy (TEM). Truncated derivatives of Lfcin B are shown to have antibacterial effect [20]. A D-analogue of a truncated derivative is shown to have a better effect than the corresponding L-analogue against *E. coli* [21]. These derivatives were included in this study to see if they behaved like the mother peptide.

Our studies of the initial binding of Lfcin B (17–41), magainin (Mag) 1 and Cec P1 indicate that Lfcin B (17–41) acts more like magainins than cecropins [17]. Even though the presently known acting mechanisms of Mag 2 and Cec P1 are limited exclusively to the cytoplasmic membrane, we wanted to examine if a cytoplasmic phase is a property of these cationic antimicrobial peptides.

2. Materials and methods

2.1. Bacterial strains

We used *Staphylococcus aureus* ATCC 25923 and *E. coli* ATCC 25922. The bacteria were stored at −70°C until use. The bacteria

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Abbreviations: Lfcin B, bovine lactoferricin; Cec P1, cecropin P1; Mag 2, magainin 2; BPW, Bacto peptone water; MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; CFU, colony-forming units; TEM, transmission electron microscopy

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were grown in 2% Bacto peptone water (BPW) (Difco 1807-17-4) pH 6.8 at 37°C to the exponential growth phase.

2.2. Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

MIC and MBC were determined as described by Vorland et al. [22]. This is a standard microdilution technique using a final inoculum of 1×10^6 colony-forming units/ml (CFU/ml). All assays were performed in parallel and repeated at least three times.

2.3. Peptides

Lfcin B (17–41) was prepared by pepsin digestion of bovine lactoferrin by the Centre for Food Technology, Queensland, Australia. Two derivatives, Lfcin B (17–31) and D-Lfcin B (17–31), were synthesized using fluorenylmethoxycarbonyl chemistry, and were analyzed and purified by HPLC [21]. All these peptides have a purity higher than 95%. Cec P1 (Prod. no. C 7927), with a purity of at least 95%, and Mag 2 (Prod. no. M 7402), with a minimum purity of 97%, were purchased from Sigma. The peptides were dissolved in double distilled water and stored at −20°C until use.

2.4. Antibodies

Polyclonal antibodies to Lfcin B (17–41), Lfcin B (17–31), D-Lfcin B (17–31), Cec P1 and Mag 2 were raised by and purchased from Pharos SA, Eurogentech, Belgium.

2.5. Exposure to peptides and fixation

S. aureus and *E. coli* were grown to mid-logarithmic growth phase in 2% BPW pH 6.8 at 37°C. They were diluted in 2% BPW to a concentration of 2×10^6 CFU/ml. Equal volumes of bacteria solution and peptide solution (diluted in double distilled water) were mixed, giving a final bacteria concentration of 1×10^6 CFU/ml. The final peptide concentrations used correspond to subMIC, MIC and subMBC of the different peptides against the bacteria. SubMIC was defined as 80% of the MIC value, and subMBC as 80% of the MBC value. Time studies with different peptide concentrations were performed for Lfcin B (17–41) against *S. aureus* and *E. coli* and also for MIC of Cec P1 against *E. coli*. In the rest of the studies the bacteria were exposed to subMIC of the peptides for 30 min.

The solutions containing bacteria and peptides were placed in a shaking water bath at 37°C. At the different exposure times, the solutions were centrifuged for 10 min at $1700\times g$, and the supernatant discarded. The samples were resuspended in 600 μl fixation buffer (8% paraformaldehyde in 200 mM HEPES, pH 7.2) and stored overnight at 4–8°C.

2.6. Specimen preparation

The fixed solution was transferred to an Eppendorf tube and centrifuged for 20 s. The fixation buffer was discarded, and the pellet resuspended in 50 μl 10% fish gelatin (37°C), and stored on ice for 2 h. The sample was then transferred to an Eppendorf tube containing 1 ml 2.3 M sucrose, and stored at 4°C for at least 4 h. The pellet was cut into small pieces and mounted onto a specimen stub. Excess sucrose was removed before the specimen was quickly frozen in liquid nitrogen.

Ultrathin sections of the specimen were cut on an RMC MT-7 ultramicrotome. The sections were submerged in 1% fish gelatin overnight, then rinsed 2×1 min in phosphate-buffered saline (PBS) before being immunolabelled.

2.7. Immunolabelling and examination by TEM

Antibodies to the different peptides were diluted to final working solutions in 1% fish gelatin. The concentrations of these working solutions were optimized for the individual peptide antibodies.

The sections were immunolabelled with the antibodies for 20 min. After rinsing in PBS for 2×1 min, protein A–gold 10 nm (diluted to a working solution of 1:65) was added, and incubated with the specimens for 20 min. This was followed by 5×2 min rinsing in PBS and 5×1 min rinsing in ultra-pure water. The specimens were contrasted with a mixture of uranyl acetate and methylcellulose 1:10 for 6 min, and then dried.

Different control procedures were performed to exclude the possibility of non-specific binding during the labelling procedure. Control of non-specific binding of primary antibody to bacteria was performed by immunolabelling bacteria unexposed to peptide. Control of non-specific binding of protein A–gold antibodies was checked by excluding the primary antibody in the labelling procedure. Control of antibody binding to peptide was done by incubating peptide in excess with the primary antibody before the labelling procedure.

The sections were examined with a JEOL JEM 1010 transmission electron microscope, and on micrographs taken on Kodak Electron Microscope film No 4489.

To determine intracellular peptide content, immunogold-labelled particles, representing peptides, were counted in at least eight cells, and the means of these counts were calculated. An exception to this was for the truncated peptides against *E. coli*, where five cells were counted. We studied micrographs taken at 20K magnification of the preparation. When a complete section of a bacterium was seen in the micrographs, the gold particles in the bacterium were counted. All bacterial cells of which we had a complete section were counted. Cells containing no immunolabelling were also counted (as 0 particles), if we had complete sections of the cells.

2.8. Statistics

Kruskal–Wallis non-parametric one way analysis of variance was used for the Lfcin B (17–41) time studies, while the Mann–Whitney non-parametric test was used for comparison of the D- and L-forms of Lfcin B (17–31).

3. Results

3.1. MIC/MBC values

The MICs and MBCs of Lfcin B (17–41), Lfcin B (17–31) and D-Lfcin B (17–31) against *S. aureus* and MICs for the same peptides against *E. coli* were as shown in [21]. Against *E. coli* the MIC of Mag 2 was 40 μg/ml and the MIC of Cec P1 was 4 μg/ml.

3.2. Detection of peptides

No non-specific binding of antibodies was detected in the different control procedures (Fig. 1). Background labelling was a minimal problem. Because no non-specific binding was observed, immunogold-labelled particles represent the presence of peptides.

3.3. S. aureus

At the MIC of Lfcin B (17–41), most of the labelled peptide was in the cytoplasm after 15 min of exposure (Fig. 2). Only small amounts were associated with the cell wall. At 30 min, the peptide content in the cytoplasm was much higher than observed after 15 min. After 1 h of exposure, the mean num-

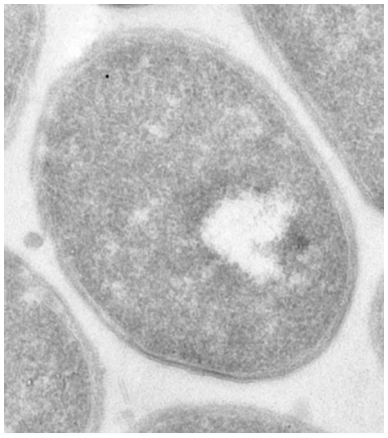

Fig. 1. TEM of *E. coli*. The cell is a negative control, not exposed to peptide. It is labelled with antibodies to Mag 2, visualized with gold-marked antibodies.

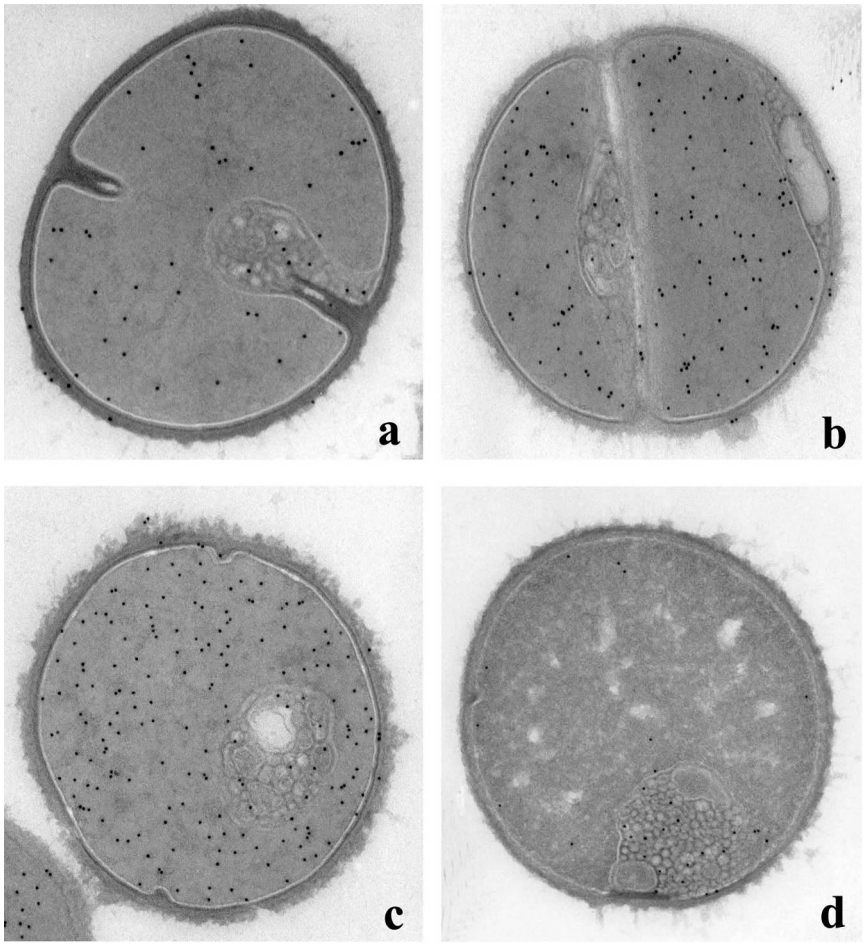

Fig. 2. TEM of *S. aureus*. The cells are exposed to MIC of Lfcin B (17–41) for 15 min (a), 30 min (b), 1 h (c) and 2 h (d). Lfcin B (17–41) is visualized with gold-marked antibodies.

ber of gold particles was in the same range, but with great intercellular variation. This variation was also observed after 2 h of exposure. Some of the cells were almost empty, while others had higher peptide content. At 2 h the mean number of gold particles was lower than at 30 min exposure time. The differences in intracellular peptide labelling between the different time points were highly significant ($P < 0.001$), with the greatest differences between 15 and 30 min and between 30 min and 2 h.

At subMIC and subMBC, results corresponding to those seen for MIC were observed. They were, however, more pronounced for subMBC than for MIC than for subMIC, indicating a concentration and time dependence.

Because only small amounts of truncated lactoferricins were available, they were only studied after 30 min exposure to subMIC of the peptides. For Lfcin B (17–31) small amounts of peptide were detected in the cytoplasm, and no peptide seemed to be associated with the cell wall. The mean labelling of intracellular D-Lfcin B (17–31) was significantly higher ($P < 0.001$), although intercellular differences were seen.

3.4. *E. coli*

After 15 min exposure of *E. coli* to MIC of Lfcin B (17–41), almost no peptide labelling was observed in the cytoplasm. The amount of cytoplasmic peptide labelling increased after 30 min exposure. After 1 and 2 h of exposure, the amount was less than after 30 min exposure. The observed differences in this time study were marginally significant ($P = 0.025$), with the greatest difference between 15 and 30 min. When *E. coli* was exposed to subMIC of Lfcin B (17–41), the same variation in bacterial number over time was observed as with the MIC exposure.

The localization of the truncated peptides was investigated after 30 min exposure to subMIC of the peptides. Only small amounts of cytoplasmic Lfcin B (17–31) immunolabelling were seen. At the same time, the cells exposed to D-Lfcin B (17–31) contained significantly larger amounts of peptide labelling in the cytoplasm ($P = 0.008$) (Fig. 3).

E. coli was exposed to Mag 2 at subMIC for 30 min. Gold particles were found scattered in the cytoplasm, and small amounts were observed in the cell wall (Fig. 4). After 30 min exposure of *E. coli* to subMIC of Cec P1, most of the detected peptide was in the cell wall. After exposure to MIC of Cec P1 up to 2 h, the labelled peptide was still localized in the cell wall of *E. coli*. Very limited amounts were observed in the cytoplasm (Fig. 5).

4. Discussion

As observed by TEM, Lfcin B was recovered in the cytoplasm of both *S. aureus* and *E. coli*.

For *S. aureus* exposed to Lfcin B (17–41), the passage of

peptide into the cells appeared to be time- and concentration-dependent. More peptide reached the cytoplasm faster if the concentration was higher. There was a maximum peptide content at 30–60 min, followed by a decrease in cytoplasmic peptide content thereafter.

The cytoplasmic content of Lfcin B (17–41) was generally lower in *E. coli* than in *S. aureus*, and the time and concentration relation was not shown so convincingly for *E. coli*. The MIC of this peptide is the same for the two bacteria, whereas the MBC for this peptide is higher for *E. coli* than for *S. aureus* [21]. Stronger binding of a greater quantity of added Lfcin B (17–41) to the cell wall of *E. coli* may be an explanation, and higher peptide concentration and/or longer exposure time may therefore be needed to reach a corresponding cytoplasmic peptide content.

The observed decrease in cytoplasmic peptide content over time may be caused by several mechanisms: enzymatic degradation of the peptide, passive leakage due to cell damage, or active efflux due to a pump.

We have shown that protease inhibitors increase the effect of Lfcin B (17–41) [23]. Enzymatic degradation of the peptide is henceforth feasible.

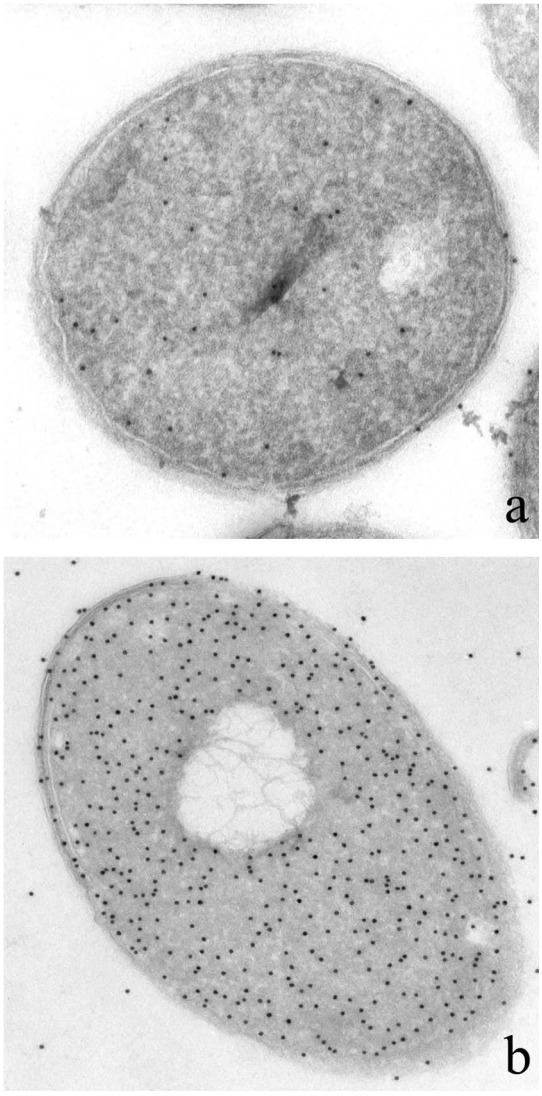

Fig. 3. TEM of *E. coli*. The cells are exposed for 30 min to subMIC of Lfcin B (17–31) (a) and of D-Lfcin B (17–31) (b). Lfcin B (17–31) and D-Lfcin B (17–31) are visualized with gold-marked antibodies.

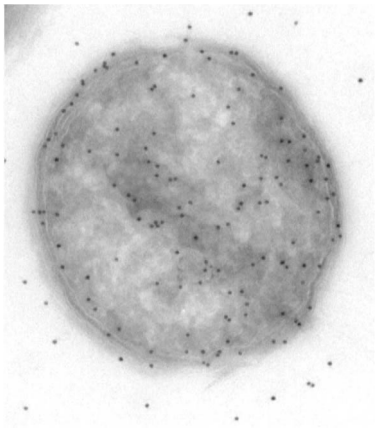

Fig. 4. TEM of *E. coli*. The cell is exposed for 30 min to subMIC of Mag 2. Mag 2 is visualized with gold-marked antibodies.

Passive leakage of molecules of this size due to cell damage is not very probable, since the cells are at most exposed to subMBC of the peptide, which does not induce that much damage to the cell in the time span used [19].

An mtr efflux system in gonococci is shown to modulate the effect of the antibacterial peptides protegrin-1 and LL-37 [24]. A similar mechanism may explain our observed results.

For both bacteria exposed to the truncated lactoferricins, the cytoplasmic content of the L-form was considerably lower than of the D-form. This is probably due to enzymatic degradation of the L-form in the cell wall [23].

Mag 2 was found in the cytoplasm, although to a more limited extent than Lfcin B. Cec P1 was almost exclusively associated with the cell wall in spite of increasing concentration and exposure times.

These findings may be consistent with what is known of the acting mechanism of these peptides. The action of magainin on the membrane is thought to be due to the formation of a dynamic peptide–lipid supramolecular pore, which allows the mutually coupled transbilayer transport of ions, lipids and peptides per se [3]. During this process the magainin is translocated to the inner leaflet of the cytoplasmic membrane [3]. Our findings could be consistent with a further release from the inner leaflet into the cytoplasm. To our knowledge, this is the first time magainin is observed intracellularly in the cytoplasm. Park et al. have earlier shown that Mag 2 does not penetrate the cell membrane, but is associated with it [25]. This was done using a technique where the peptide was biotin-labelled before it was mixed with the bacteria. The biotin labelling of the peptide might induce a change in the configuration of the peptide. This might change the property of the peptide, and it might behave in another manner than the naked peptide. In our experiment the bacteria were exposed to naked, unlabelled peptide. This might explain why our results differ from those found by Park et al. [25].

The cecropins are thought to act on the membrane by a carpet mechanism. They disintegrate the membrane without forming transmembrane pores [5]. Our findings are consistent with an acting mechanism confined to the cytoplasmic membrane.

In conclusion, this study has shown that lactoferricins were detected in the cytoplasm in great numbers in *E. coli* and *S.*

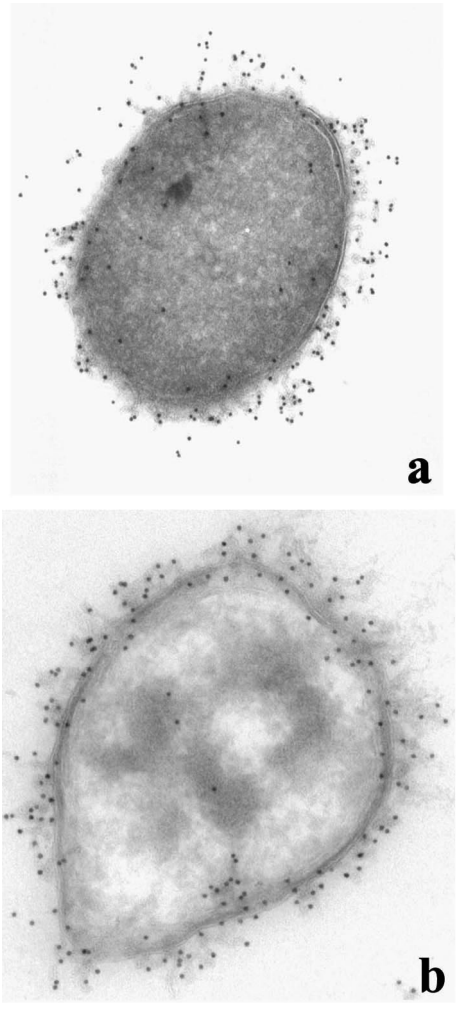

Fig. 5. TEM of *E. coli*. The cells are exposed to MIC of Cec P1 for 30 min (a) and 2 h (b). Cec P1 is visualized with gold-marked antibodies.

aureus. Cec P1 was almost exclusively cell wall-associated in *E. coli*. Mag 2 was clearly detected in the cytoplasm of *E. coli*, a finding that has not been reported earlier. The decrease in cytoplasmic content of Lfcin B (17–41) over time might be due to its degradation or to an efflux pump.

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