Killing kinetics of intracellular *Afipia felis* treated with amikacin

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*Afipia felis* is a facultative intracellular bacterium which multiplies in macrophages following inhibition of phagosome–lysosome (P–L) fusion. When *A. felis*-infected cells are incubated for 72 h with various antibiotics, only aminoglycosides are found to be bactericidal. We therefore studied the killing of intracellular *A. felis* by amikacin, and its relationship with the restoration of P–L fusion. Amikacin reduced the number of *A. felis* from $8.5 \times 10^5$ to $3.5 \times 10^2$ cfu/mL within 94 h. P–L fusion was restored after 30–40 h of incubation with amikacin. Both mechanisms may participate in the intracellular killing of bacteria.

Introduction

*Afipia felis* was first described in 1991 as a putative, aetiologic agent of cat-scratch disease (CSD) following its isolation from lymph nodes of patients with CSD. Subsequently another bacterium, *Bartonella henselae*, has been identified as the agent of most cases of CSD, although a role for *A. felis* continues to be postulated.

*Afipia felis* is a Gram-negative, facultative, intracellular bacterium which multiplies in macrophages following inhibition of phagosome–lysosome (P–L) fusion. This inhibition is mediated by a factor that is inactivated by proteinase K digestion. When *A. felis*-infected cells are incubated for 72 h with various antibiotics, only aminoglycosides have been shown to be bactericidal. In this report we have investigated the respective roles of the microbicidal activity of amikacin and of the cell in the killing kinetics of intracellular *A. felis*. We have established the bactericidal kinetics of amikacin against intracellular *A. felis* and studied its relationship with the restoration of P–L fusion.

Materials and methods

**Bacterial strains**

Two strains of *A. felis* (ATCC 49714, ATCC 49716) obtained from the American Type Culture Collection were cultivated on buffered charcoal–yeast extract (BCYE) agar for 72 h at 30°C.

**Antibiotics**

Amikacin (Bristol-Meyers Squibb, Paris, France) was used at a single concentration that corresponded to the lower MIC breakpoint (16 mg/L). Aliquots of a stock solution (10 mg/mL) of amikacin were prepared and frozen at 80°C until used.

**Cell line**

The P388D1 murine macrophage-like cell line (clone 3124 from the European Collection of Cell Cultures) was grown in shell vials (3.7 mL; Bibby Sterilin, U K) and in 25 cm² flasks (Corning, U K) in minimal essential medium (MEM) supplemented with 10% fetal calf serum and 2 mM L-glutamine.

**Kinetics of the amikacin bactericidal effect on intracellular *A. felis***

Shell vials containing $8 \times 10^4$ P388D1 cells were inoculated with $10^2$ cfu of *A. felis* suspended in 1 mL culture medium (i.e. inoculum 125 cfu/cell) and incubated at 37°C for 48 h in a 5% CO₂ atmosphere. The cells were then washed twice with phosphate-buffered saline (PBS), and fresh cell culture medium containing amikacin 16 mg/L was overlaid on to them. A nontoxic-free controls were prepared using the same procedure. *A. felis*-infected shell vials were incubated at 37°C in a 5% CO₂ atmosphere for 4, 8, 12, 18, 30, 40, 50, 70 and 94 h. At each incubation time, titration of surviving intracellular bacteria was carried out as...
Our results therefore suggest that very low cells, amikacin cells. The atmosphere and assessed by lysosome cells were infected and were determined after 5 days of incubation at 30°C. To assess any potential variation in the antibiotic susceptibility of different A. felis strains, two isolates (ATCC 49714 and ATCC 49716) were tested. The same protocol was used with an inoculum of either agar-grown or cell-grown A. felis. Each strain was tested three times.

Results and discussion

In summary, the supernatant of each shell vial was removed, centrifuged at 10,000 g for 10 min, and the resulting pellet was suspended in fresh culture medium. The infected monolayer was harvested with PBS-EDTA, disrupted by three freeze-thaw cycles in liquid nitrogen, pelleted at 10,000 g and suspended in fresh culture medium. Pellets from cell lysates and from the supernatant were mixed and centrifuged at 10,000 g to remove all remaining antibiotic. The pellet was then suspended in 1 mL of culture medium, different dilutions of which were plated on to BCYE agar. The number of cfu was determined after 5 days of incubation at 30°C. To assess any potential variation in the antibiotic susceptibility of different A. felis strains, two isolates (ATCC 49714 and ATCC 49716) were tested. The same protocol was used with an inoculum of either agar-grown or cell-grown A. felis. Each strain was tested three times.

Restoration of P–L fusion

Flasks (25 cm²) containing P₃₈₈-D₁ cells were infected and treated using the same procedure as above (inoculum 125 cfu/cell). A. felis-infected cells incubated without amikacin were used as an antibiotic-free control. As a positive control for P–L fusion, uninfected cells were incubated for 30 min with 1:174 v/v polystyrene latex beads (LB) (10% solids, 0.803 µm diameter; Sigma, St Louis, MO, USA) in culture medium at 37°C. These cells were then washed and incubated with or without amikacin. All flasks were incubated for 30 or 40 h at 37°C in a 5% CO₂ atmosphere and assessed by lysosome labelling using the following two methods. Ferritin labelling: secondary lysosomes of cells were labelled with cationized ferritin (Sigma) and processed for transmission electron microscopy (TEM) as previously described. Acid–phosphatase (AcPase) cytochemistry: carried out as previously described.

Samples were examined on a JEM 1200 EX electron microscope (JEOL, Kyo kyo, Japan) at 80 kV. The P–L fusion ratio was defined as the ratio between the number of labelled vacuoles containing bacteria or LB and the total number of vacuoles containing bacteria or LB after incubation with or without amikacin. At least 200 observations were recorded per experiment. For AcPase cytochemistry, two independent series of experiments were carried out. The P–L fusion ratios were compared by a Chi-squared test. Differences were established as significant if P < 0.05.

In A. felis-infected cells, the presence of amikacin had a progressive and profound effect on the number of surviving intracellular bacteria. When P₃₈₈-D₁ cells infected with agar-grown A. felis (ATCC 49716) were treated with amikacin, the number of intracellular bacteria (cfu/mL) decreased progressively from $8.5 \times 10^5 \pm 4 \times 10^5$ to $3.5 \times 10^2 \pm 2.4 \times 10^2$ cfu/mL after 94 h of incubation. Similar killing kinetics were obtained with an inoculum of cell-grown A. felis ATCC 49716 and with A. felis ATCC 49714 (Figure 1). No comparable changes were observed in untreated controls that showed a stationary phase growth with the different strains (Figure 1). The inability of A. felis to grow in cell-free MEM had been previously verified. Short incubation times with amikacin were sufficient to observe a marked decrease of the number of living intracellular bacteria. When A. felis ATCC 49714 was used as inoculum, the initial bacterial count decreased from $1.45 \times 10^6$ to $7.2 \times 10^5$ cfu/mL after only 4 h with amikacin, to $5.9 \times 10^5$ cfu/mL after 8 h, to $3.5 \times 10^5$ cfu/mL after 12 h, and to $2.7 \times 10^5$ cfu/mL after 18 h. In Staphylococcus aureus-infected P₃₈₈-D₁ cells, amikacin could not be detected in cells after 4 h of incubation, using a methodology that had a threshold of detection of 0.18 mg/L. Our results therefore suggest that very low concentrations of amikacin can inactivate intracellular A. felis. This observation is not altogether surprising, as very low MICs were observed with aminoglycosides tested on A. felis in vitro. However, why amikacin is effective on bacteria within phagosomes, when it is thought to accumulate largely within lysosomes, is still unclear. Amikacin is thought to be internalized by the cell through pinocytosis and to reach phagosomal bacteria by fusion of

![Figure 1. Effect of amikacin on A. felis-infected P₃₈₈-D₁ cells. Agar-grown A. felis ATCC 49714 and agar-grown or cell-grown A. felis ATCC 49716 were used to infect P₃₈₈-D₁ cells. The bactericidal effect of amikacin on A. felis-infected P₃₈₈-D₁ cells was evaluated by comparison of the cfu obtained in the cell lysates after antibiotic exposure with the cfu obtained with the untreated control.](image-url)
Intracellular killing of *A. felis* with amikacin

*...*pinocytotic endosomes with phagosomes, as reported for gentamicin in *Listeria monocytogenes*-infected macrophages.8

*A. felis* lives within cells in a phagosome following inhibition of P–L fusion (Figure 2). As the presence of amikacin has a rapid and profound effect on the number of intracellular *A. felis*, we were interested to know when P–L fusion was restored in infected cells incubated with this antibiotic. After 30 and 40 h of incubation within amikacin, the P–L fusion ratios in *A. felis*-infected cells (31% and 22%, respectively) were significantly lower than the bead-positive cell controls (Figure 2) (70.5% and 69%, respectively) when assessed by either cationized ferritin (P < 10⁻⁷) or after AcPase cytochemistry (P < 10⁻⁷) methods (Table). We therefore confirmed with both labelling methods that *A. felis* lives in a phagosome and inhibits P–L fusion.4 After 30 h of incubation with amikacin, the P–L fusion ratio in infected cells was not significantly different from that obtained in untreated infected cells. We obtained the same result with both labelling methods (Table). The P–L fusion ratio of vacuoles containing LB was also not significantly different with or without amikacin. We therefore conclude that the presence of amikacin has no effect on the P–L fusion ratio of vacuoles containing *A. felis* or LB after 30 h incubation. After 30 h incubation with amikacin, although the number of living intracellular *A. felis* had already decreased compared with the antibiotic-free control, the

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**Figure 2.** Enlarged views of P₃₅D₁ cells thin sections stained for AcPase cytochemistry or cationized ferritin after infection with *A. felis* (AF) or latex beads (L) and incubation with or without amikacin. (a) Positive control for P–L fusion: a vacuole containing a latex bead fuses with cationized ferritin-labelled lysosomes (arrow) after 30 h of incubation without amikacin. Bar: 0.5 μm. (b) *A. felis* enclosed in a cationized ferritin (F) positive vacuole of phagolysosome (PL) after 30 h of incubation without amikacin. Bar: 0.4 μm. (c) *A. felis* enclosed in an AcPase-negative vacuole of phagosome (arrow) after 40 h of incubation without amikacin. Bar: 1 μm. (d) *A. felis* enclosed in an AcPase-positive vacuole (PL) after 40 h of incubation with amikacin. The AcPase reaction product (PA) (arrow) appears as discrete patches. Bar: 0.4 μm.
Amikacin, an antibiotic that inhibits protein synthesis, may therefore lead to the restoration of P–L fusion by blocking the synthesis of the protein factor of killing of intracellular A. felis and leads to the restoration of P–L fusion after 40 h. Both mechanisms may participate in the killing of intracellular A. felis.

In conclusion, amikacin is rapidly effective against intracellular A. felis and leads to the restoration of P–L fusion after 40 h. Both mechanisms may participate in the killing of intracellular A. felis.

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References


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