BACTERIAL VIRULENCE

Effect of monensin on the invasiveness and multiplication of *Legionella pneumophila*

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Summary. The polyether antibiotic monensin exhibited bacteriostatic activity against a clinical isolate of *Legionella pneumophila* *in vitro*. Experiments designed to test the effect of the compound on the invasiveness and multiplication of *L. pneumophila* in HeLa cells showed that, in the presence of the antibiotic, legionellas that penetrated the cells did not multiply. However, monensin did not alter the characteristics of phagosomes that contained ingested legionellas. In the presence of monensin, infected cells exhibited extensive vacuolation and a noticeable reduction in the number of intracellular micro-organisms was evident a few hours after infection.

Introduction

Legionella pneumophila is a facultative intracellular pathogen that replicates within a wide variety of cultured human and animal cells,^{1–6} and soil and fresh water protozoa.^{7–9} In cultured non-lymphoid tumour cells, the penetration of *L. pneumophila* takes place by a process that is best described as induced uptake,⁶ and this is considered to be a virulence-associated property.¹⁰ After uptake, the avoidance of digestion by lysosomal enzymes is achieved by the prevention of phagosome-lysosome fusion, probably induced by a bacterial cytotoxin.¹¹ Replication of micro-organisms is accomplished within cytoplasmic vacuoles; transmission electronmicroscopy of intracellular *L. pneumophila* shows the micro-organisms residing in a ribosome-studded phagosome, a structure unique among intracellular bacteria.¹² Various inhibitors of eukaryotic metabolism have been used to identify the processes involved in the intracellular replication of *L. pneumophila*. Among these, two typical lysosomotropic agents, ammonium chloride and chloroquine, showed contrasting results.¹³ Whereas chloroquine prevented almost all intracellular multiplication in HeLa cells, and caused extensive vacuolation, disruption of calcium metabolism and destruction of monolayers, ammonium chloride had no effect on the multiplication of legionellas. However, Byrd and Horwitz¹⁴ observed inhibition of multiplication of *L. pneumophila* in human monocytes by both chloroquine and ammonium chloride.

Monensin is a carboxylic ionophore that affects receptor-mediated endocytosis¹⁵ by alkalinisation of the content of lysosomes or pre-lysosomal compartments, or both. This effect is a consequence of the insertion of the ionophore into lysosomal membranes.¹⁶ Because of its lipophilic nature, monensin can insert into cellular membranes to cause a marked slowing of intracellular transport of newly synthesised secretory proteins, proteoglycans and plasma-membrane glycoproteins. The major site of this inhibition is within the Golgi complex, and in this respect the action of monensin is unique.¹⁶ Monensin also exhibits antibiotic activity by affecting the flux of sodium and potassium ions across the bacterial cell membrane.^{17,18} It is one of several polyether antibiotics used commercially to improve feed efficiency in ruminants.^{19–23} These agents are mainly active against gram-positive micro-organisms. Gram-negative bacteria are generally ionophore-resistant because of the outer membrane which serves as a protective barrier.²² Nothing is known about the effect of monensin on *Legionella*, and the present study was undertaken to examine the activity of the antibiotic *in vitro*, and on the penetration and multiplication of *L. pneumophila* in HeLa cells.

Materials and methods

Bacterial strain

A virulent clinical isolate of *L. pneumophila* serogroup 6, strain Monza 3/1386,²⁴ which was subcultured only twice after isolation, was used. The strain was stored as a stock culture at –70°C in skimmed milk, and subcultured once on Buffered

Charcoal-Yeast Extract Agar with α -ketoglutarate 0.1% (BCYE- α -agar; Oxoid)²⁵ before use.

Cells

HeLa S3 cells were grown at 37°C in Eagle's Minimal Essential Medium (MEM) (Flow Laboratories) containing NaHCO₃ 1.2 g/L, fetal calf serum (Flow Laboratories) 10% and 2 mM glutamine. The same medium containing only fetal calf serum 2% was used to maintain the cells.

Antibacterial activity of monensin

Monensin (Sigma) was dissolved in ethanol to achieve a concentration of 1 mM and stored at -20°C.

The minimal inhibitory concentration (MIC) was determined by preparing serial two-fold dilutions of monensin covering the range 50–0.2 μ M in Yeast Extract Broth (Difco) supplemented with Legionella growth supplement (Oxoid). A standardised *L. pneumophila* suspension was added to each dilution to obtain a final concentration of *c.* 10⁵ cfu/ml. After incubation for 48 and 72 h at 37°C in CO₂ 2.5% in air, tubes were examined for bacterial growth. The lowest concentration of monensin that inhibited visible growth was taken as the MIC. Dilutions without visible growth were plated on BCYE- α -agar to determine the minimal bactericidal concentration (MBC).

Cell toxicity test

To determine the cytotoxic effect, HeLa cells (4 × 10⁵ cells/ml) were seeded in growth medium in 96-well plates (0.1 ml/well) and incubated with various concentrations of monensin in Eagle's MEM for 1 h at 37°C in a moist air atmosphere containing CO₂ 5%. After a further incubation at 37°C for 48 h in drug-free medium, cells were inspected to detect changes in morphology such as swelling, granularity, rounding or floating. The viability of the cells was determined by neutral red uptake after incubation for 48 h.

Ingestion assay

A modification of the techniques described by Finlay and Falkow²⁶ and Harley and Drasar¹³ was used. For the assay, HeLa cells were suspended in antibiotic-free complete Eagle's MEM, plated in 24-well culture dishes (Corning, NY, USA) at 2.2 × 10⁵ cells/well and maintained at 37°C in a moist air atmosphere containing CO₂ 2.5%. After incubation for 24 h, cell monolayers were washed twice with antibiotic-free complete Eagle's MEM and 0.25 ml of a bacterial suspension in the same medium (4 × 10³ cfu/ml) were added to the cells. After centrifugation for 1 h at 4000 g, the plates were incubated for 1 h at 37°C. The cells were then washed five times with

Eagle's MEM supplemented with gentamicin 50 μ g/ml and left in the same medium for 1 h at 37°C to kill non-internalised bacteria. Cells were then washed twice with antibiotic-free complete Eagle's MEM and disrupted by addition of sterile double-distilled water (0.2 ml/well). The number of viable bacteria in the lysate was counted as described below.

Effect of monensin

Monensin was tested at concentration of 12.5 and 25 μ M. The compound was added to the cells at various times, from 1 h before infection to 6 h after the addition of bacteria to the HeLa cells. In control experiments, the infected cells were exposed to concentrations of ethanol equivalent to those present in the dilutions of monensin. In some experiments, monensin was removed from the monolayers immediately before the infection. In others, monensin was either added to the cells with the inoculum and removed by washing after the infection or added to the maintenance medium at different times after the infection and kept in contact with the cells for 48 h. In these experiments appropriate controls were included in which non-infected cells were maintained in the presence of monensin for 48 h.

Bacterial counts

The numbers (cfu) of viable legionellas in the inoculum and in the cell lysates were assessed by plating the samples on BCYE- α agar and counting the number of colonies developing after incubation for 72 h at 37°C in a moist air atmosphere containing CO₂ 2.5%.

Electronmicroscopy

HeLa cells were washed twice and then fixed for 60 min at 4°C with glutaraldehyde (Electron Microscopy Science, Fort Washington, PA, USA) 2.5% in 0.1 M sodium cacodylate buffer (BDH Limited, Poole), pH 7.3. Glutaraldehyde was then removed, and the cell monolayers were washed five times with cacodylate buffer. Cells were fixed for 45 min at 4°C in osmium tetroxide (Agar Scientific Ltd, Cambridge) 1% in zetterquist.

Monolayers were dehydrated in graded ethanol solutions (from 70% to 100%) and removed from the wells by overnight agitation at 18–20°C with propylene oxide (Fluka Buchs, Switzerland). Samples were embedded in Epon 812 (Fluka Buchs), and ultra-thin sections were mounted on formvar-coated copper grids. The sections were stained with a saturated solution of uranyl acetate (Fluka Buchs) in ethanol 50% for 3 min, followed by lead hydroxide (ICN Costa Mesa, CA, USA) for 10 min. Electronmicrographs were taken with a Hitachi H-7000 electronmicroscope.

Fig. 1. Effect of monensin on invasiveness of *L. pneumophila* (expressed as percentage yield of viable bacteria relative to a monensin-free control with ethanol 2.5%): ■, control with ethanol 1.25%; ▨, monensin 12.5 µM added with the bacterial inoculum (NP); ▤, cells pre-incubated with monensin 12.5 µM before infection (P); □, control with ethanol 2.5%; ▩, monensin 25 µM added with the bacterial inoculum (NP); ▥, cells pre-incubated with monensin 25 µM before infection (P).

Results

Susceptibility test

MICs of monensin for the virulent strain of *L. pneumophila* tested were 0.78 and 1.56 µM after 48 and 72 h, respectively. The MBCs were substantially greater than the MICs, with 5–10% survival even at 50 µM of the drug. Higher concentrations of monensin were cytotoxic.

Effect of monensin on invasiveness of *L. pneumophila* in HeLa cells

Pre-incubation of cells with monensin caused a decrease in the number of legionellas entering the cells; the difference was more evident when the monensin was used at a concentration of 25 µM (fig. 1). The effect was compared to the appropriate controls in which cells were added with an equivalent amount of ethanol. Ethanol alone at a concentration of 2.5% caused a higher penetration rate of legionellas into cells, probably due to a perturbation of the cell membrane.

Multiplication of *L. pneumophila* in HeLa cells in the presence of monensin

The presence of ethanol in the culture medium allowed the micro-organisms to multiply without affecting bacterial growth. When monensin was added at the time of the infection and removed with the inoculum after 2 h, the growth rate was the same as the controls (fig. 2), indicating that the continued presence of the drug was required to inhibit multiplication of *L. pneumophila*. Addition of monensin to the cells either at the time of the infection or 3 and 6 h post-infection caused a marked decrease in the number of legionellas as measured by the viable count of bacteria.

Control experiments in parabolic chambers con-

Fig. 2. Multiplication of *L. pneumophila* in the presence of monensin (counts expressed as number of viable legionellas in the cell lysate of a single well): ● antibiotic-free control; ○ monensin 25 µM added with the inoculum and removed with the bacteria after 2 h; □ monensin 25 µM added 3 h after infection; ▲ monensin 25 µM added 6 h after infection; ■ monensin 25 µM added with the inoculum and maintained throughout the experiment (48 h).

firmed the inability of *L. pneumophila* to multiply in conditioned medium in the presence of cells separated by a 0.45 µm filter or in MEM alone^{1,5} (data not shown).

Electronmicroscopy

Electronmicroscopy of HeLa cells treated with monensin, or with ethanol at the concentrations used to dilute monensin, showed the complete integrity of cells and the absence of any morphological alteration.

During the first hours of infection, HeLa cells showed vacuoles containing the micro-organisms (figs. 3a and b) which multiplied within the vacuoles (fig. 3c). Later, before lysis of the cell, the cytoplasm appeared to be completely altered with several micro-organisms in each cell (fig. 3d).

Fig. 3. Intracellular multiplication of *L. pneumophila* in HeLa cells: **a**, *L. pneumophila*-containing vacuoles lined with ribosomes (arrow); **b**, mitochondria closely apposed to the vacuolar membrane (large arrow)—smooth vesicles are apparently fusing with or budding off from the vacuolar membrane (thin arrow); **c**, replication within vacuoles surrounded by ribosomes (arrow); **d**, late stage of intracellular development—organisms occupy the entire cell cytoplasmic space. **a**, **b**, 3 h, **c**, 24 h, **d**, 48 h after infection. Bars, 1 μ m.

In the presence of monensin, the cycle of multiplication of legionellas in HeLa cells was dramatically interrupted. Intracellular micro-organisms were observed only on rare occasions and visible legionellas were mainly outside the extensively vacuolated cells (figs. 4a, b, c and d).

Discussion

Although the activity of monensin is exerted mainly on gram-positive and anaerobic bacteria, the clinical isolate of *L. pneumophila* tested in the present study appeared to be as susceptible as these other micro-organisms.^{23, 27} In keeping with the results obtained by Nagaraja and Taylor²⁸ with other carboxylic ionophores, monensin was found to behave as a bacteriostatic compound within the range of concentrations used, as it was impossible to determine an MBC value. However, concentrations equal to or greater than the MIC caused a fall in the counts of *L. pneumophila* compared to controls.

L. pneumophila enters human mononuclear phagocytes by a process called "coiling phagocytosis",²⁹ and multiplies within an anomalous membrane-bound vacuole studded with ribosomes. Vacuoles usually contain one bacterium, and only occasionally two. Often it is possible to observe smooth vesicles and mitochondria in close proximity to these structures.³⁰ Vacuoles containing *L. pneumophila* do not fuse with lysosomes.³¹

A similar interaction between *L. pneumophila* and host cell macrophages has also been observed with differentiated HL-60 cells, a promyelocyte cell line readily maintainable in culture, although the receptors mediating adherence were different.³²

Various studies have described the growth of *L. pneumophila* in several types of non-professional phagocytic cell (MRC-5, HeLa, Hep-2, McCoy, Vero, L929) in tissue culture.^{4-6, 10, 33} Infected cell monolayers allowed bacterial multiplication and showed cytopathic changes varying from lysis of the cells to a more chronic type of infection. Intracellular replication was

Fig. 4. Infected HeLa cell monolayers in the presence of monensin: **a**, **b**, cells showing extensive vacuolation and extracellular bacteria; **c**, organism inside a vacuole studded with ribosomes (arrow)—note the extensive vacuolation; **d**, mitochondria in proximity to the *L. pneumophila* vacuole. **a**, 3 h, **b**, 4 h, **c**, 6 h, **d**, 10 h after infection. Bars, 1 μ m.

similar for each cell type with the presence of vacuoles lined with cell ribosomes and adjacent to mitochondria and other cell structures.⁶ However, engulfment of *L. pneumophila* by non-professional phagocytes is different, since coiling phagocytosis is not observed. Expression of the uptake into HeLa cells induced by *L. pneumophila* appears to be a virulence-associated property as it has been reported that virulent strains entered cultured HeLa cells 1000 times more efficiently than isogenic avirulent isolates.¹⁰ The present studies were performed in HeLa cells with a virulent clinical isolate of *L. pneumophila*. This strain entered HeLa cells more efficiently than the Philadelphia 1 reference strain (data not shown). In order to improve the attachment efficiency, which is low, even with virulent strains,⁶ the bacterial inoculum was centrifuged on to cell monolayers.

The results obtained demonstrate that *L. pneumophila* failed to replicate in HeLa cells in the presence of monensin. Furthermore, the number of legionellas decreased during the incubation period by a factor of

100 or more compared to controls that grew exponentially. Monensin also caused a fall in viable count when it was added 3 and 6 h after the infection, confirming that the antibacterial effect continued after bacterial division had started. In these experiments the inoculum of legionellas was relatively low, in order to emphasise possible effects of the antibiotic. Pre-incubation of cells with monensin did not modify the multiplication of internalised legionellas (data not shown). However, in experiments performed without pre-incubation with the drug, monensin, which penetrates cells within 1–2 min of addition,¹⁵ produced a bactericidal effect on internalised bacteria. The disappearance of legionellas was confirmed by transmission electronmicroscopy 3, 4, 6 and 10 h after infection of tissue culture cells.

In the absence of monensin, *L. pneumophila* multiplied 100-fold (fig. 2). Transmission electronmicroscopy performed at 3, 24 and 48 h after infection revealed actively multiplying intracytoplasmic organisms in membrane-bound vacuoles lined with ribo-

somes. In the late stages of infection dilated vacuoles contained many legionellas (fig. 3).

In contrast, electronmicrographs of infected HeLa cells treated with monensin showed very few bacteria inside cells with several vacuoles. The presence of an intensive vacuolation in eukaryotic cells treated with monensin has been explained as the result of a dramatic dilatation of the normally compressed cisternae of the Golgi complex, probably due to a perturbation of the intracellular ion levels.¹⁶ Intact micro-organisms were present outside the cells (fig. 4) but, when legionellas were intracellular, the structure of the phagosome with ribosomes and mitochondria was similar to the controls without monensin.

Certain features of the infection of cells by *L. pneumophila* are also seen with other intracellular pathogens such as *Toxoplasma gondii* and *Chlamydia* spp.^{34,35} The vacuole structure containing the micro-organisms and the disposition of cytoplasmic organelles close to the vacuole itself are also seen with these organisms.³⁰ In addition, like legionellas, these intracellular parasites inhibit phagosome-lysosome fusion.^{34,35}

The mechanism by which monensin exerts a bactericidal effect on intracytoplasmic legionellas is not clear. The common property shared by this drug with all the lysosomotropic agents is that of increasing the pH of acidic compartments such as phagosomes. This

prevents some micro-organisms from escaping from acidic phagosomes;³⁶ however, this mechanism cannot be effective in the case of *L. pneumophila* which is capable of multiplying in the phagosome consequent to the inhibition of phagosome-lysosome fusion. The fall in viable count of intracellular legionellas found after exposure to monensin is unlikely to be due to the penetration of gentamicin into cells, despite the high concentrations used (50 mg/l), because it was not observed in appropriate controls.

It has been reported that intracellular multiplication of *L. pneumophila* is dependent upon the availability of iron in monocytes³⁷ and HL-60 cells.³² Lysosomotropic bases interfere with normal iron metabolism by raising endocytic and lysosomal pH;³⁸ in fact chloroquine and ammonium chloride inhibit intracellular multiplication of *L. pneumophila* in human monocytes.¹⁴ Since monensin is a carboxylic ionophore which shares with weak bases the ability to increase the pH of cell compartments, it is possible that the inhibition of multiplication of legionellas in HeLa cells is also related to altered iron metabolism.

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