EFFECT OF ENDOCYTOSIS INHIBITORS ON *COXIELLA BURNETII* INTERACTION WITH HOST CELLS

E.TUJULIN[1,3], A. MACELLARO[1], B. LILLIEHÖÖK[2], L. NORLANDER[1*]

[1]Department of Microbiology, [2]Department of Biomedicine, Division of NBC Defence, Defence Research Establishment, S-901 82 Umea, Sweden; [3]Department of Pathology, Swedish University of Agricultural Sciences, P.O. Box 7028, S-750 07 Uppsala, Sweden

Received April 7, 1998; accepted May 12, 1998

Summary. – The obligate intracellular rickettsia *Coxiella burnetii* has previously been reported to reach the intravacuolar compartment of host cells by phagocytosis. With the aim to further examine the mechanisms of *C. burnetii* internalisation, macrophage monolayers were treated with well characterised inhibitors of endocytosis. The treatment with two general inhibitors, colchicine and methylamine, resulted in a pronounced dose-dependent decrease of radiolabelled phase II rickettsiae retained from the intracellular fraction. A third inhibitor used, amiloride, has been reported to reduce effectively clathrin-independent pinocytic pathways. The internalisation of *C. burnetii* was shown to be substantially reduced also by amiloride and the effect was dependent on its concentration. The passive role of *C. burnetii* in the internalisation was verified by using heat-killed *C. burnetii*. Host cells treated with either of the three inhibitors (amiloride, colchicine and methylamine) showed a similar reduction of intracellular *C. burnetii* after exposure to killed as well as live organisms.The data presented indicate that different endocytic mechanisms, pinocytosis as well as phagocytosis, may mediate the uptake of *C. burnetii* by a host cell.

Key words: Coxiella burnetii; internalisation; endocytosis

Introduction

Many bacteria possess specific components for invasion of host cells and enter by mechanisms requiring participation of both the host and bacterium, while others completely rely on cellular functions, namely endocytosis (Moulder, 1985). Endocytic mechanisms, phagocytosis and pinocytosis, are cellular phenomena with several common features used by most eukaryotic cells to efficiently internalise and transport various molecules into the cells (Goldstein *et al.*, 1979). Endocytic processes occur at high rate and include the formation of plasma membrane vesicles and vacuoles. Phagocytosis is an efficient process of internalisation of a variety of microbes (Ofek *et al.*, 1992). Four morphologically distinct pinocytic pathways have been characterised, i. e. the clathrin-dependent pinocytosis (coated pits) and the formation of non-coated pinocytic vesicles, macropinosomes and caveolae (Lamaze and Schmid, 1995; Watts and Marsh, 1992). Some of these pathways have been described as ways of internalisation of bacterial species. E.g., members of the obligate intracellular species *Chlamydiaceae* are reported to exploit at least two pinocytic entry mechanisms in addition to phagocytosis (Hodinka and Wyrick, 1986; Hodinka *et al.*, 1988). One of the entry mechanisms is mediated by coated pits, another proceeds via an inducible pinocytic pathway independent of clathrin (Reynolds and Pearce, 1990). Recently, the existence of a macropinocytic uptake process was described for invasive *Salmonella typhimurium* (Alpuche-Aranda *et al.*, 1994; Francis *et al.*, 1993). Rather than generating a localised membrane response close to the bacterium, it stimulates a larger portion of the macrophage

*Corresponding author.

Abbreviations: DMSO = dimethyl sulphoxide; MEM = Minimal Essential Medium; PBS = phosphate-buffered saline

surface. A bacterium that contracts the macrophage surface is caught as the membrane ruffles close to form an intracellular vesicle.

The caveolar vesicles, which are approximately 95 nm in diameter, have never been associated with bacterial transport and can hardly be expected do so due to their small size (Anderson, 1993).

The Q fever agent *C. burnetii* is an obligate intracellular rickettsia. The organism infects a number of cell types including monocytes, macrophages and a variety of transformed cells, where the bacteria grow and develop exclusively within the acidic phagolysosomes (Baca and Paretsky, 1983; Hackstadt and Williams, 1981). The ability to invade and subsequently grow within eukaryotic cells is a key virulence factor for organisms like *C. burnetii*, which require an intracellular environment for their multiplication.

C. burnetii has previously been reported to reach its internal niche by phagocytosis (Brezina and Kazár, 1965; Wisseman *et al.*, 1967; Burton *et al.*, 1971; Kazár *et al.*, 1975; Kishimoto and Walker, 1976; Baca *et al.*, 1993). It does not actively promote its entry, but rather enters passively (Brezina and Kazár, 1965; Wisseman *et al.*, 1967; Kazár *et al.*, 1975; Baca *et al.*, 1993).

There are several inhibitory substances available for studies of general uptake processes in eukaryotic cells. The cytochalasins effectively inhibit phagocytosis by blocking elongation of actin filaments by interacting with the polymerising ends of the filaments (Casella *et al.*, 1981). Colchicine is another substance reported to interfere with endocytosis. It acts on the polymerisation of microtubuli by binding to and inducing a local unfolding of the growing ends (Sackett and Varma, 1993). Another inhibitor, methylamine, inactivates the membrane enzyme transglutaminase, which catalyses cross-linking of proteins (Kaplan and Keogh, 1981). The enzyme is reported to have a significant role in the membrane cross-linking and receptor-mediated endocytosis (Davies *et al.*, 1980; Lorand *et al.*, 1976). A third substance, which has been used in studies of cell pathways of nutrient uptake, is amiloride (West *et al.*, 1989). It blocks the Na^+/H^+ exchange and is also reported to interfere with a number of receptors and the formation of pinosomes but not coated pits (Hewlett *et al.*, 1994).

The aim of this report was to evaluate the internalisation of *C. burnetii* in the presence or absence of the inhibitors methylamine, colchicine and amiloride. Phase II organisms were used as a model because of their efficiency in internalisation. The effect of the inhibitors suggests that, in addition to phagocytosis, the uptake of *C. burnetii* by macrophages may be mediated by a pinocytic process.

Materials and Methods

C. burnetii Nine Mile strain in phase II, kindly provided by L. Mallavia, Pullman, WA., USA, was used in the study. It was grown in the BGM cell line (Flow Laboratories) in Minimal Essential Medium (MEM) supplemented with Earle's salts, 2 mmol/l L-glutamine, 0.2 % $NaHCO_3$, 5% heat-inactivated foetal bovine serum (Nordcell) and 1% non-essential amino acids (Sigma). Confluent cell layers were infected with *C burnetii* and incubated at 37°C. Fresh medium was added after 20 – 24 hrs. *C. burnetii* was collected from the medium of actively growing cultures after 7–8 days by differential centrifugation. An initial centrifugation at 1,500 x *g* for 8 mins at 4°C removed the cell debris, while a second centrifugation at 16,500 x *g* for 30 mins at 4°C pelleted the rickettsiae. Mycoplasma testing of infected cell cultures was periodically performed using the Mycoplasma PCR Primer Set (Stratagene) and standard techniques.

P388D1 cells The mouse macrophage-like cell line P388D1 (ATCC TIB 63) was cultured in RPMI-1640 medium supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml) and 5% (v/v) heat-inactivated Myoclone Super Plus foetal bovine serum (Gibco) in a humified 6% CO_2 atmosphere at 37°C. Two days prior to the infection, the cells were scraped off, washed and diluted to 1 x 10^6 cells per flask in medium without antibiotics.

Labelling of C. burnetii. A modification of an *in vitro* acidic activation buffer system was used for labelling of the rickettsiae (Thompson *et al.*, 1990). Extracellular *C. burnetii* was isolated as described above, washed twice and finally resuspended in buffer A (10 mmol/l NaCl and 20 mmol/l KH_2PO_4 pH 6.8) to a concentration of 10^{10} organisms per ml. After 3 hrs at room temperature, the suspension was supplemented with buffers B and C. The final concentration of buffer B (pH 4.5) was 20 mmol/l KH_2PO_2, 100 mmol/l KCl, 10 mmol/l NaCl and 20 mmol/l $MgCl_2$ and buffer C was 250 mmol/l sucrose, 5 mmol/l glucose, 5 mmol/l glutamate, 80 mmol/l glycine and 0.1 mmol/l each of alanine, valine, leucine, isoleucine, proline, serine, threonine, phenylalanine, tyrosine, tryptophane, lysine, arginine, histidine, aspartate, asparagine, cysteine and glutamine. After the addition of [^{35}S] L-methionine (specific activity 1,000 Ci/mmol) to final activity of 250 µCi/ml, the mixture was incubated at 37°C overnight. The labelled rickettsiae were washed twice in phosphate-buffered saline (PBS) and aggregates were eliminated by running the suspension through a syringe. The number of rickettsiae was initially estimated by spectrophotometry at A_{565} and a standard curve was constructed by measuring the turbidity of serial dilutions of an enumerated rickettsial suspension (Waag *et al.*, 1991). The titers of the rickettsial suspensions used in the experiments were verified by a titration method using shell vial cultures (Schneider, 1989).

Labelled rickettsiae (6 x 10^8) were washed in PBS, resuspended in 0.5 ml 0.1 mol/l phosphate buffer pH 5.5, applied on a sucrose density gradient (25 – 60%) (Wachter *et al.*, 1975) and centrifuged at 105,000 x g for 40 hrs in a Beckman XL90 ultracentrifuge. The absorbance (A_{280}) and radioactivity (cpm) were measured in each

fraction (1 ml). The rickettsiae were heat-killed by incubation in PBS at 65°C for 1 hr (Ransom and Huebner, 1951; Williams, 1991).

Studies of uptake. Cell layers in 25 cm^2 tissue culture flasks were pre-incubated with the inhibitors methylamine, colchicine or amiloride for 15 mins at 37°C and the labelled rickettsiae (0.2 – 1.4 x 10^6 cpm per 1 x 10^8 rickettsiae) were added at multiplicity of infection of 100 rickettsiae per cell. After incubation at 37°C, the non-bound rickettsiae were removed, the cell layers were washed three times with 5 ml of cold PBS and scraped off into 1.5 ml of PBS. Total cell-associated rickettsiae were determined by liquid scintillation counting (Beckman LS 5,000 CE) in one third of the suspension. Cells in the rest of the suspension were lysed by intensive shaking for 3 mins and centrifuged for 8 mins at 1,500 x *g* to remove cell debris. The rickettsial content of the supernatants (intracellular fraction) was determined by liquid scintillation counting and expressed in cpm, which was taken for rough estimate of the amount of internalised rickettsiae.

Inhibitors. Stock solutions were prepared as follows: 200 mmol/l colchicine was dissolved in culture medium containing 0.4% dimethyl sulphoxide (DMSO), 400 mmol/l methylamine was made in cultute medium and 100 mmol/l amiloride was prepared in 0.4% DMSO (all inhibitors were from Sigma). The cell viability after treatment with the inhibitors was estimated by the trypan blue exclusion and MTT tests (Bagge Hansen *et al.*, 1989; Philips, 1973). The uptake of trypan blue by dead cells is due to a reduced permeability barrier. The MTT test of mitochondrial enzyme activity involves conversion of a tetrazolium salt (MTT) to a coloured product, the concentration of which is spectrophotometrically (A_{565}) measured.

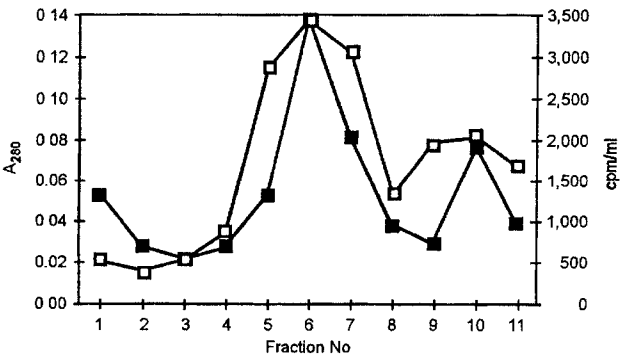

Fig. 1
Sucrose density gradient centrifugation of [^{35}S]-methionine-labelled *C. burnetii*
Density gradient made from 25 – 60% sucrose, centrifugation at 105,000 x g for 40 hrs, Beckman XL 90 ultracentrifuge, 1 ml fractions. A_{280} (□); cpm (■)

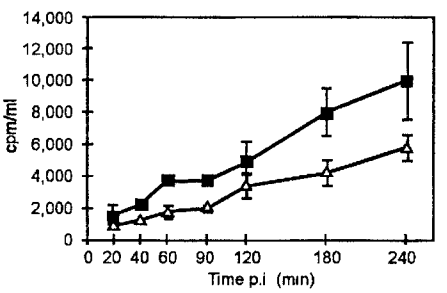

Fig. 2
Kinetics of binding and internalisation of [^{35}S]-methionine-labelled *C. burnetii* by P388D1 cells
Means of triplicate samples ± SD from 3 experiments, cell-associated (■) and intracellular (Δ) radioactivity.

Results

Model system

We studied the uptake of *C. burnetii* by P388D1 cells over a period of 4 hrs using [^{35}S]-methionine-labelled phase II rickettsiae. The rickettsiae used in these experiments were collected from the supernatants of continously growing cells and labelled in a cell-free *in vitro* system. The rickettsiae separated by sucrose density gradient were shown to consist of a heterologous population with [^{35}S]-methionine incorporated in the small cell fractions (peak 1) as well as the large cell fractions (peak 2) (Fig. 1) (Wachter *et al.*, 1975).

The incubation was interrupted after various intervals and the total cell-associated rickettsiae and rickettsiae in supernatants of lysed cells were determined. The cell-associated rickettsiae were detectable within 20 mins and increased steadily during the incubation period (Fig. 2). The internalised rickettsiae (intracellular fraction) showed a minimal initial increase during the first 40 mins. Later on, there was a reproducible increase of radioactivity of the intracellular fraction.

Inhibition experiments

Cultures of P388D1 cells were infected with radiolabelled *C. burnetii* in the presence of the endocytosis inhibitors colchicine, methylamine or amiloride, respectively. After 3 hrs of incubation at 37°C, the infection was interrupted and the intracellular fraction of labelled rickettsiae was estimated. Colchicine-treated cells showed a marked reduction of the intracellular fraction and the effect was dose-dependent. The treatment with low concentrations (1.25 – 2.5 mmol/l) caused a 67 – 50% reduction of the intracellular fraction. At the highest concentration used in these experiments, i. e. 10 mmol/l, this fraction was reduced to 30% of the control (Fig. 3).

A strong dose-dependent inhibition of internalisation of *C. burnetii* was observed also in methylamine-treated cells (Fig. 3). The concentrations ranged from 5 to 40 mmol/l

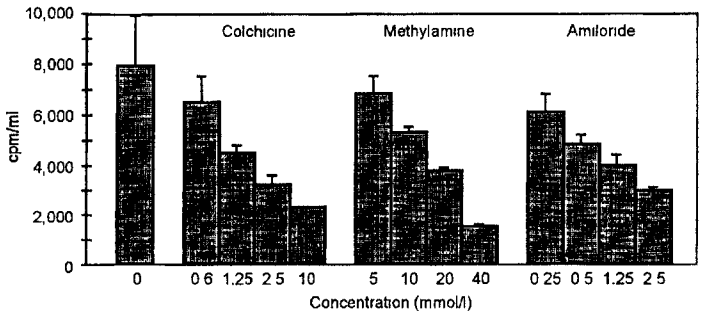

Fig. 3
Effect of endocytosis inhibitors on the internalisation of *C. burnetii*
P388D1 cells pre-incubated with colchicine (0.6 – 10 mmol/l), methylamine (5 – 40 mmol/l) or amiloride (0.25 – 2.5 mmol/l) for 15 mins, and then infected with the rickettsiae for 3 hrs. Means of triplicate samples ± SD from 3 experiments.

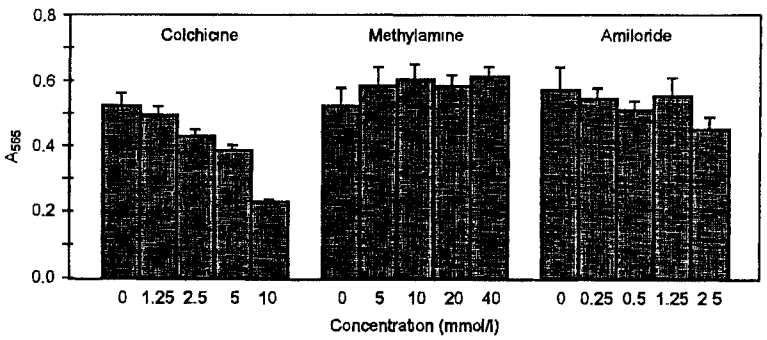

Fig. 4
Effect of endocytosis inhibitors on mitochondrial enzyme activity of uninfected P388D1 cells
Cells incubated with colchicine (1 25 – 10 mmol/l), methylamine (5 – 40 mmol/l) or amiloride (0.25 – 2.5 mmol/l) for 3 hrs. Mitochondrial enzyme activity determined by the MTT test (A_{565}). The bars represent means of duplicate samples ± SD.

and at the highest concentration the reduction of intracellular rickettsiae was approximately to 20% of the control.

Treatment of P388D1 cells with amiloride in concentrations of 0.25 – 2.5 mmol/l also caused a substantial dose-dependent reduction of the uptake (Fig. 3). At 2.5 mmol/l, amiloride inhibited the *C. burnetii* internalisation to about 40% of the control (Fig. 3).

Effect of inhibitors on uninfected cells

The effect of the three inhibitors on uninfected P388D1 cells was monitored in parallell cultures. The cell layers were visually examined for changes in cell form and adherence, and the cell viability was determined by the trypan blue and MTT tests. For comparison, non-treated cells were all adhering to the surface and approximately 20% of them were rounded up. The effect of colchicine was most apparent. The examination showed that 5 mmol/l colchicine had a negative effect on the cell morphology with approximately 55% of the cells rounded up. After 3 hrs of treatment with 10 mmol/l colchicine, 98% of the cells were still adhering to the surface, but almost all of them were rounded up. Approximately 3% of the cells were stained by trypan blue. The MTT test showed only slightly affected cells at low concentrations but a 50% reduction of mitochondrial enzyme activity in cells treated with 10 mmol/l colchicine (Fig. 4). There was a dose-dependent reduction in the activity at increased concentrations of colchicine.

The effects of methylamine and amiloride on the cells in general were not as pronounced as that of colchicine. The cells incubated with methylamine at concentrations up to 40 mmol/l retained the mitochondrial activity as monitored by the MTT test (Fig. 4). The activity of the cells incubated with lower concentrations of amiloride was the same as that of non-treated cells and only a slight reduction was observed at higher concentrations, i. e. to 80% of the control at 2.5 mmol/l amiloride (Fig. 4).

Furthermore, the effects of methylamine and amiloride on the cells was not clearly visible. At 2.5 mmol/l amiloride, the highest concentration used, all the cells were still adhering to the surface and only about 30% of them were

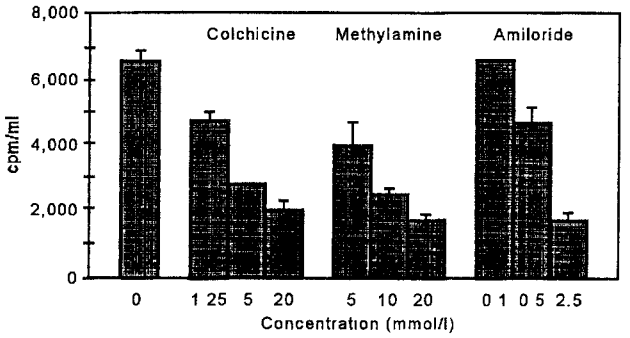

Fig. 5
Effect of endocytosis inhibitors on internalisation of non-viable *C. burnetii* in P388D1 cells
Cells monolayers pre-treated with colchicine (1.25 – 20 mmol/l), methylamine (5 – 20 mmol/l) or amiloride (0.1 – 2.5 mmol/l), and then infected with heat-killed rickettsiae. Means of triplicate samples ± SD from 2 experiments.

rounded up. None of the concentrations used caused an uptake of trypan blue. At lower concentrations of methylamine, 30 to 50% of the cells had affected the cell morphology, and at the highest concentration approximately 85% of the cells were rounded up. However, they were all adhering to the surface and only about 3% of them had increased membrane permeability.

Passive mode of entry

The passive mode of entry of *C. burnetii* into cell was verified in this experimental system. *C. burnetii* was heat-killed at 65°C for 1 hr and used inhibition experiments with P388D1 cells. It was found that the percentage of the non-viable rickettsiae in the intracellular fraction was reduced by the three inhibitors in a dose-dependent manner (Fig. 5).

Discussion

Cells maintain a high endocytic rate as a consequence of the fact that most internalised membrane components are not degraded but recycled back to the cell surface (Kaplan and McVey Ward, 1990). As a result, there are always suitable binding sites available on the cell surface for bacteria, which use endocytosis for a passive uptake.

The members of the intracellular species *Chlamydiaceae* are reported to be effectively internalised and utilise both phagocytic and pinocytic modes of entry (Reynolds and Pearce, 1990; Moulder, 1991). One type of pinocytosis is mediated by coated pits, while the other occurs via inducible clathrin-independent pinosomes.

C. burnetii show many similarities with the *Chlamydiaceae* species; they are both intracellular organisms that alternatively exist in a metabolically inactive, infectious form and a metabolically active, intracellular growth form (Baca and Paretsky, 1983; Moulder, 1991). Both *Coxiella* and *Chlamydia* species are reported not to expend energy in gaining entrance to their host cells, i. e. they are solely depending on host cell mechanisms for their internalisation (Moulder, 1991; Baca *et al.*, 1993).

An endocytic mode of internalisation of *C. burnetii* by mouse fibroblast cells was first demonstrated by electron microscopic studies (Burton *et al.*, 1971). It was later shown that phagocytosis of *in vivo* labelled *C. burnetii*, isolated from the intracellular compartments of host cells, was inhibited by high concentrations of cytochalasins (Baca *et al.*, 1993).

In this paper, we have further examined the pathways of internalisation of *C. burnetii* by using well-known inhibitors of endocytosis. The rickettsiae used in the study were collected from culture supernatants containing a variety of sizes of the rickettsia (Heinzen, 1997) The presence of small as well as large sizes of the labelled rickettsiae was verified. This size spectrum of *C. burnetii* organisms extends from particles that might be predicted to enter the host cell by pinocytosis to those for which phagocytosis seems the most plausible mode of entry (Pratten and Lloyd, 1986). It might be of advantage for an obligate intracellular organism like *C. burnetii* to use passively various efficient pathways of internalisation and thus to ensure an efficient multiplication.

The experimental system used in this study was based on the content of radioactivity in the intracellular fraction of cells infected for 3 hrs with the labelled rickettsiae. This fraction was obtained from the supernatant of carefully washed cells which have been lysed by shaking and centrifuged. Loosely bound rickettsiae were lost in the washing steps and thus the rickettsiae retained from the supernatant were mainly intracellular.

The results show that amiloride, colchicine and methylamine substantially reduce the amount of radiolabelled *C. burnetii* obtained from the intracellular fraction of lysed cells. The reduction was dose-dependent and the effect was obvious also at low concentrations of the inhibitors. The general negative effects of amiloride and methylamine on uninfected cells was weak. Thus, the reduction of the rickettsiae in the intracellular fraction could be due to a specific inhibition of uptake of the rickettsiae by the host cell.

In contrast to amiloride and methylamine, colchicine seemed to have a more general negative effect on the cells. It caused a strong dose-dependent reduction of mitochondrial enzyme activity and there was an obvious effect on the cell morphology also at low concentrations of the inhibitor. Thus, the inhibitory effect of colchicine on internalisation of *C. burnetii* in this system might be due to a more general reduction of the cell activity and might not be associated to inhibition of uptake processes *per se*.

Incubation of macrophages with methylamine has been demonstrated to cause a dose-dependent inhibition of pinocytosis as well as phagocytosis (Teshigawara *et al.*, 1985; Kaplan and Keogh, 1981). However, pinocytosis was more sensitive to low concentrations of methylamine than phagocytosis; the concentration of methylamine which inhibited pinocytosis (2 mmol/l) was one-tenth of that inhibiting phagocytosis (20 mmol/l). The results presented in this paper show a marked effect of methylamine on the intracellular fraction at lower (5 – 10 mmol/l) as well as and higher concentrations (20 mmol/l and more). This implies a methylamine block of pinocytic as well as phagocytic internalisation of *C. burnetii*.

Amiloride is reported to interfere with the clathrin-independent pinocytosis but not the formation of clathrin-dependent pinosomes (West *et al.*, 1989). The intracellular fraction of *C. burnetii* is efficiently reduced in amiloride-treated macrophages and the pathway used by the rickettsiae is consequently considered not to involve clathrin.

In conclusion, the present results indicate that, by analogy to the *Chlamydiaceae* species, *C. burnetii* may utilise several cell uptake pathways of a passive mode of internalisation. Obligate intracellular bacteria like *Chlamydiaceae* and *C. burnetii* should have great benefit of multiple mechanisms of entrance into host cells. The heterogeneous size population of extracellular *C. burnetii* offers suitable particle sizes for both the pinocytic and phagocytic modes of uptake. Furthermore, the utilisation of entrance pathways other than phagocytosis by *C. burnetii* may explain the broad host cell range of this rickettsia.

Acknowledgement. We are grateful to Thorsten Johansson for performing the computer figures and to Karin Hjalmarsson, Gunnar Sandström and Anders Sjöstedt for fruitful discussions and critical reading of the manuscript.

References

Alpuche-Aranda CM, Raccosin EL, Swanson JA, Miller SI (1994): *Salmonella* stimulate macropinocytosis and persist within spacious phagosomes. *J Exp. Med.* **179**, 601–608.

Anderson RGW (1993): Caveolae: Where incoming and outgoing messengers meet. *Proc. Natl. Acad. Sci. USA* **90**, 10909–10913.

Baca OG, Paretsky D (1983): Q-fever and *Coxiella burnetii*: a model for host-parasite interactions. *Microbiol. Rev.* **47**, 127–149.

Baca OG, Klassen DA, Aragon AS (1993): Entry of *Coxiella burnetii* into host cells. *Acta Virol.* **37**, 143–155.

Bagge Hansen M, Nielsen SE, Berg, K. (1989): Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immun. Methods* **119**, 203–210.

Brezina R, Kazár, J. (1965): Study of the antigenic structure of *Coxiella burnetii*. IV. Phagocytosis and opsonization in relation to the phases of *C. burnetii*. *Acta Virol.* **9**, 268–274.

Burton PR, Kordová, N, Paretsky D (1971): Electron microscopic studies of the rickettsia *Coxiella burnetii*: entry, lysosomal response and fate of rickettsial DNA in L-cells. *Can. J. Microbiol.* **17**, 143–158.

Casella JF, Flanagan MD, Lin S (1981): Cytochalasin D inhibits actin polymerization and induces depolymerization of actin filaments formed during platelet shape change. *Nature* **293**, 302–305.

Davies PJA, Davies DR, Levitzki A, Maxfield FR, Milhand PM, Willingham MC, Pastan I (1980): Transglutaminase is essential in receptor-mediated endocytosis of 2-macroglobulin and polypeptide hormones. *Nature* **283**, 162–167.

Francis CL, Ryan TA, Jones BD, Smith SJ, Falkow S (1993): Ruffles induced by Salmonella and other stimuli direct macropinocytosis of bacteria. *Nature* **364**, 639–642.

Goldstein JL, Anderson RGW, Brown MS (1979): Coated pits, coated vesicles, and receptor-mediated endocytosis. *Nature* **279**, 679–685.

Hackstadt T, Williams JC (1981): Biochemical stratagem for obligate parasitism of eucaryotic cells by *Coxiella burnetii*. *Proc. Natl. Acad. Sci. USA* **78**, 3240–3244.

Heinzen RA (1997): Intracellular development of *Coxiella burnetii*. In Anderson B, Friedman H, Bendinelli M (Ed.): *Rickettsial Infection and Immunity*. Plenum Press, New York, pp. 99–129.

Hewlett LJ, Prescott AR, Watts C (1994): The coated pit and macropinocytic pathways serve distinct endosome populations. *J. Cell Biol.* **124**, 680–702.

Hodinka RL, Wyrick PB (1986): Ultrastructural study of mode of entry of *Chlamydia psittaci* into L929 cells. *Infect. Immun.* **54**, 855–863.

Hodinka RL, Davies CH, Choong J, Wyrick PB (1988): Ultrastructural study of endocytosis of *Chlamydia trachomatis* by McCoy cells. *Infect. Immun.* **56**, 1456–1463.

Kaplan J, Keogh EA (1981): Analysis of the effect of amines on inhibition of receptor-mediated and fluid-phase pinocytosis in rabbit alveolar macrophages. *Cell* **24**, 925–932.

Kaplan J, McVey Ward D (1990): Movement of receptors and ligands through the endocytic apparatus in alveolar macrophages. *Am. J. Physiol.* **258**, 263–270.

Kazár, J., Škultetyová E, Brezina R (1975): Phagocytosis of *Coxiella burnetii* by macrophages. *Acta Virol.* **19**, 426–431.

Kishimoto RA, Walker JS (1976): Interaction between *Coxiella burnetii* and guinea pig peritoneal macrophages. *Infect. Immun.* **14**, 416–421.

Lamaze C, Schmid SL (1995). The emergence of clathrin-independent pinocytic pathways. *Curr Opin. Cell Biol.* **7**, 573–580.

Lorand L, Weissman LB, Epel DL, Lorand JB (1976): Role of the intrinsic transglutaminase in the Ca^{2+}-mediated crosslinking of erythrocyte proteins. *Proc. Natl. Acad Sci. USA* **73**, 4479–4481.

Moulder JW (1985): Comparative biology of intracellular parasitism. *Microbiol. Rev.* **49**, 298–337.

Moulder JW (1991): Interaction of Chlamydiae and host cells *in vitro*. *Microbiol. Rev.* **55**, 143–190.

Ofek I, Rest RF, Sharon N (1992): Nonopsonic phagocytosis of microorganisms. *Am. Soc. Microbiol. News* **58**, 429–435.

Philips HJ (1973): Dye exclusion tests for cell viability. In Kruse PF Jr., Patterson MK (Ed.): *Tissue Culture Methods and Applications*. Academic Press, New York, pp. 406–408.

Pratten MK, Lloyd JB (1986): Pinocytosis and phagocytosis: the effect of size of particulate substrate on its mode of capture by rat peritoneal macrophages cultured *in vitro*. *Biochim. Biophys. Acta* **881**, 307–313.

Ransom SE, Huebner RJ (1951): Studies on the resistance of *Coxiella burnetii* to physical and chemical agents. *Am. J. Hyg.* **53**, 110–119.

Reynolds DJ, Pearce JH (1990): Characterization of the cytochalasin D-resistent (Pinocytic) mechanisms of endocytosis utilized by Chlamydiae. *Infect. Immun.* **58**, 3208–3216.

Sackett DL, Varma JK (1993): Molecular mechanism of colchicine action: induced local unfolding of ß-tubulin. *Biochem.* **32**, 13560–13565.

Schneider W (1989): Titration of *Coxiella burnetii* in Buffalo Green Monkey (BGM) cells cultures. *Zbl. Bakt* **271**, 77–84.

Teshigawara K, Kannagi R, Nora N, Masuda T (1985): Possible involvement of transglutaminase in endocytosis and antigen presentation. *Microbiol. Immunol.* **29**, 737–750.

Thompson HA, Bolt CR, Hoover T, Williams JC (1990): Induction of heat shock proteins in *Coxiella burnetii*. *Ann. NY Acad. Sci.* **590**, 127–135.

Waag DM, Williams JC, Peacock MG, Roult D (1991): Methods of isolation, amplification and purification of *Coxiella burnetii*. In Williams JC, Thompson HA (Ed): *Q-Fever: The Biology of Coxiella burnetii*. CRC Press, Boca Raton, pp. 73 – 115.

Wachter RF, Briggs GP, Gangemi JD, Pedersen CE Jr (1975): Changes in buoyant density relationsships of two cell types of *Coxiella burnetii* phase I. *Infect. Immun.* **12**, 433–436.

Watts C, Marsh M (1992): Endocytosis: what goes in and how? *J. Cell. Sci.* **103**, 1–8.

West MA, Bretscher MS, Watts C (1989): Distinct endocytic pathways in epidermal growth factor-stimulated human carcinoma A431 cells. *J. Cell Biol.* **109**, 2731–2739.

Williams JC (1991): Infectivity, virulence, and pathogenecity of *Coxiella burnetii* for various hosts. In Williams JC, Thompson HA (Ed): *Q-Fever: The Biology of Coxiella burnetii*. CRC Press, Boca Raton, pp. 22–71.

Wisseman CL Jr, Fiset P, Ormsbee RA (1967): Interaction of Rickettsiae and phagocytic host cells. V. Phagocytosis and opsonic interaction of phase 1 and phase 2 *Coxiella burnetii* with normal and immune human leucocytes and antibodies. *J. Immunol.* **99**, 669–674.