ACTA UNIVERSITATIS AGRICULTURAE SUECIAE

VETERINARIA 52

Host Interactions of the Intracellular Bacterium *Coxiella Burnetii*

Internalisation, induction of bacterial proteins and host response upon infection

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Host interactions of the intracellular bacterium *Coxiella burnetii*. Internalisation, induction of bacterial proteins and host response upon infection.

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Akademisk avhandling som för vinnande av veterinärmedicine doktorsexamen kommer att offentligen försvaras i Ettans föreläsningssal, Klinikcentrum, SLU, Uppsala, måndagen den 7 juni 1999, kl 13.15.

Abstract

Q fever is a zoonosis caused by the obligate intracellular bacterium *Coxiella burnetii*. The aim of this thesis was to study internalisation of *C. burnetii*, the induction of bacterial proteins upon infection, and the initial host response to *C. burnetii* infection. A comparison of host response after infection with virulent and avirulent *C. burnetii* was made.

C. burnetii infects a variety of cells, including macrophages. Previously, *C. burnetii* has been reported to enter host cells passively via phagocytosis. To further examine the mechanism of *C. burnetii* internalisation, macrophage monolayers were treated with well known inhibitors of endocytosis. The results indicate that *C. burnetii* may utilise both pinocytosis and phagocytosis for internalisation.

C. burnetii proliferates within the acidic phagolysosome of eukaryotic cells. To study proteins induced during phagolysosome-like conditions bacteria were incubated in an acidic *in vitro* system. One of these proteins was identified as an Hsp70 homologue. The *C. burnetii* Hsp71-kDa protein is one of the predominant proteins induced early in acid activated bacteria.

In intracellular infection, macrophages have been shown to secrete cytokines that are capable of modulating the cellular immune response. To study early host response *in vitro*, macrophages were infected with virulent and avirulent *C. burnetii*. We found that virulence of the bacteria, seems to be correlated to an enhancement of IL-1α production during the initial hours of infection.

To further characterize the host response an *in vivo* model in mice was used. Mice infected with virulent *C. burnetii* appeared depressed and grossly splenomegaly was observed. In lungs of these mice histological lesions appeared earlier and did not resolve as soon as in infection with avirulent bacteria. Typical changes in lung consisted of interstitial inflammatory reactions, with infiltration of mononuclear cells. In liver multifocal granulomas were observed, the frequency of granulomas was considerably higher in mice infected by virulent *C. burnetii*.

Cytokine mRNAs were analysed in lung. An obvious induction of IL-1β mRNA was observed in lungs of mice infected with virulent *C. burnetii* during the acute phase of infection. The results from the *in vitro* and *in vivo* infection model indicates that the innate immune response in virulent *C. burnetii* infection is correlated to an early induction of IL-1.

Keywords: Q fever, *Coxiella burnetii,* internalisation, endocytosis, heat shock proteins, Hsp70 homologue, cytokines, macrophage, LPS, IL-1, mouse infection model.

Distribution:
Swedish University of Agricultural Sciences
Department of Pathology
S-750 07 Uppsala, Sweden

Uppsala 1999
ISSN 1401-6257
ISBN 91-576-5425-5

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Acta Universitatis Agriculturae Sueciae
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ISSN 1401-6257
ISBN 91-576-5425-5

Tryck: SLU Service/Repro, Uppsala 1999

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Tujulin, E. 1999. *Host interactions of the intracellular bacterium* Coxiella burnetii. *Internalisation, induction of bacterial proteins and host response upon infection.* Doctor´s dissertation.

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Contents

Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Tujulin, E., Macellaro, A., Lilliehöök, B., and Norlander, L. 1998. Effect of endocytosis inhibitors on *Coxiella burnetii* interaction with host cells. Acta. Virol. 42:125-131.

II. Macellaro, A., Tujulin, E., Hjalmarsson, K., and Norlander, L. 1998. Identification of a 71-kDa surface-associated Hsp70 homologue in *Coxiella burnetii*. Infect. Immun. 66:5882-5888.

III. Tujulin, E., Lilliehöök, B., Macellaro M., Sjöstedt, A., and Norlander, L. 1999. Early cytokine induction in P388D1 macrophages infected by virulent or avirulent *Coxiella burnetii*. Vet. Immunol. Immunopathol. 68:xx-xx. (Accepted for publication).

IV. Tujulin, E., Macellaro, A., Jönsson, L., Sjöstedt, A., and Norlander, L. Pathologic changes and induction of proinflammatory cytokines after intranasal infection of mice with *Coxiella burnetii*. (Submitted).

Introduction

History

In 1935, Derrick investigated an outbreak of fever with unknown etiology observed among abattoir workers in Brisbane. He named the disease Q (for query) fever. Burnet and Freeman could isolate an organism, which was characterized as a *Rickettsia* (*Rickettsia burneti*) using samples received from Derrick. Independently and almost simultaneously Davis and Cox isolated a filter-passing rickettsia-like agent (*Rickettsia diaporica*) from a tick near Nine Mile Creek, Montana. As laboratory workers became infected and showed clinical symptoms of Q fever it was shown that the filter-passing agent *Rickettsia diaporica* was identical to *Rickettsia burnetii* (Babudieri, 1959, Fiset and Woodward, 1982). In 1948 Philip proposed a new genus, *Coxiella*, as the agent of Q fever markedly differs from other *Rickettsia* (Philip, 1948).

Classification and characteristics of *C. burnetii*

C. burnetii is the sole species of its genus and belongs to the family *Rickettsiaceae*. More recently, molecular biology techniques including 16S rRNA gene sequence analysis have placed *C. burnetii, Wolbachia persica* and *Legionella pneumophila* in the gamma subdivision of Proteobacteria. whereas *Rickettsia, Ehrlichia* and *Bartonella* spp. belongs to the alpha group (Weisburg *et al*, 1989, Stein *et al*, 1993).

C. burnetii is an obligate intracellular bacterium that infects a variety of cells, including macrophages (Baca and Paretsky, 1983). *In vitro, C. burnetii* can establish persistent infection in several cell lines (Baca *et al*, 1981). Replication occurs only in the acidic phagolysosomes of eucaryotic host cells (Burton *et al*, 1978, Hackstadt and Williams, 1981). Intracellular multiplication of *C. burnetii* is prevented if the phagolysosomal pH is manipulated by lysosomtropic amines (Hackstadt and Williams, 1981). *In vitro* biochemical studies have shown that if *C. burnetii* is maintained in media containing oxidizable substrate, like glutamate, the substrate is transported and metabolized on condition that the pH is in range 2.0-5.5 (Hackstadt and Williams, 1981). This phenomenon is termed acid activation, and results in DNA and protein bioynthesis by *C. burnetii* in the absence of host cells (Chen *et al*, 1990, Redd and Thompson, 1995, Zuerner and Thompson, 1983).

The shape of *C. burnetii* is coccobacillary and the cell wall is similar to that of gram-negative bacteria. Characteristic for *C. burnetii* is lipopolysaccharide (LPS) phase variation. A similar phenomenon with smooth to rough LPS variation is observed in gram-negative enteric bacteria. *C. burnetii* phase I bacteria are isolated from infected humans and animals, and are characterized by smooth-type LPS and high virulence. Transition to *C. burnetii* phase II, expressing rough LPS and reduced virulence, occurs after repeated passages in cell cultures and

embryonated eggs (Baca and Paretsky, 1983, Kazar *et al*, 1974, Moos and Hackstadt, 1987). The endotoxic activity of *Coxiella* LPS is poor. In comparison with *E. coli* and *S. typhimurium* LPSs, *C. burnetii* LPS is 100- to 1000-fold less endotoxic when assayed in galactosamine-sensitized mice (Amano *et al*, 1987).

C. burnetii is a slow growing bacteria with a doubling time of 5-7 hours (Afseth and Mallavia, 1997). Propagation of *C. burnetii* occurs through a typical bacterial growth cycle involving transverse binary fission. Furthermore, examination of the ultrastructure of *C. burnetii* has revealed the existance of sporogenic differentiation *in vitro* (McCaul and Williams, 1981). Sporelike formations have also been observed in infected cardiac valves (McCaul *et al*, 1994). Morphologically two distinct variants are observed, a small cell variant (SCV) and a large cell variant (LCV). The SCV is metabolically dormant and able to survive extracellularly. In contrast, the LCV has lost the resistant properties and exhibits higher metabolic activity. Sporogenic differentiation, with development of dense bodies in one pole of the cell, occurs in this cell type. The developmental cycle of *C. burnetii,* in the phagolysosome, leads to formation of a heterogenous mixture of pleomorphic cell types (Figure 1).

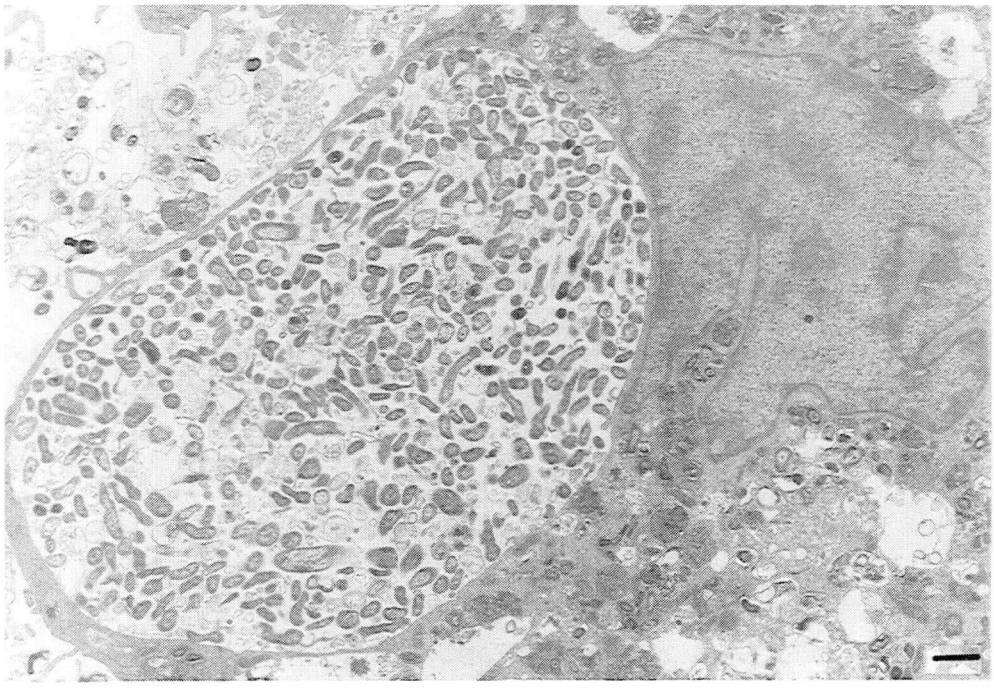

Figure 1. Electron micrograph of a BGM cell heavily infected with *C. burnetii* phase I, strain Nine mile after 72 hours (Bar 1μm).

C. burnetii is unusually resistant to physical and chemical agents (Williams, 1991). The stability in the environment is probably due to the SCV and the ability to spore-formation (McCaul and Williams, 1981, Hackstadt and Williams, 1981).

The size of the *C. burnetii* chromosome has been determined from pulsed-field gel electrophoresis (PFGE) to be 2103 kb (Willems *et al*, 1998). The existence of cryptic plasmids has been shown in *C. burnetii* (Samuel *et al*, 1983, Thiele *et al*, 1994). Studies on 16S rRNA has demonstrated that *C. burnetii* isolates have high degree of DNA homology and are closely related (Stein *et al*, 1993). The use of restriction fragment length polymorphism (RFLP) has revealed restriction groups that may be considered as different strains. The different RFLP patterns correspond to the geographical origin of the isolate, no correlation to virulence has been detected (Jäger *et al*, 1998).

C. burnetii infection in man

Human infection with *C. burnetii* can be either subclinical, acute or chronic. Asymptomatic Q fever infection is common, since the number of serologically positive persons is much higher than the incidence rate of the disease (Aitken *et al*, 1987, Marrie and Raoult, 1997). In acute Q fever the symptoms resembles of influenza (fever, headache and myalgia), which can become complicated with pneumonia and/or granulomatous hepatitis. Fever usually lasts for one to two weeks (Aitken *et al*, 1987). Therefore Q fever is often misdiagnosed as common cold or influenza. The incubation time for acute Q fever ranges from two to four weeks.

C. burnetii has been isolated from placenta and breast milk from mothers who have had an acute Q fever infection a few years before delivery. This indicates that resting *C. burnetii* undergoes reactivation during pregnancy. Some of the mothers gave birth to healthy children, while others suffered miscarriages and delivery of abnormal children (Sawyer *et al*, 1987, Téllez *et al*, 1998).

Chronic Q fever, is most often characterized by endocarditis, and may develop years after an acute infection. In contrast to acute Q fever, chronic Q fever is often fatal. Rather than the infecting strain, immunosuppression or underlying heart disease are the most common risk factors for Q fever endocarditis (Koster *et al*, 1985, Mege *et al*, 1997)

If acute Q fever is diagnosed antibiotic treatment is recommended, but the disease usually resolves also without treatment. Tetracycline compounds, especially doxycycline and also quinolone compounds are currently used to treat Q fever (Marrie and Raoult, 1997). In chronic Q fever treatment with a combination of several antibiotics lasting from one to several years is necessary because the risk of recurrence (Aitken *et al*, 1987, Sawyer *et al*, 1987).

C. burnetii infection in animals

C. burnetii can infect a large number of animal species including several domestic animals. In animals *C. burnetii* does not usually cause respiratory disease as in humans. Instead bacteria localizes in the female reproductive tract, and massive proliferation of bacteria occurs in placenta and mammary glands (Lang, 1990). Domestic animals rarely become systemically ill, therefore the term coxiellosis is preferred to Q fever, when it comes to *C. burnetii* infection in animals. Infection with *C. burnetii* can be subclinical, but is also associated with late abortions, stillbirth and delivery of weak offspring. Furthermore, herds with cattle infected with *C. burnetii* suffer from retention of the afterbirth, metritis, and infertility (Aitken, 1989, Lang, 1990).

Gross lesions in sheep and goat are primarily confined to the intercotyledonary areas of the placenta, which is thickened and leathery and covered by copious inflammatory exudate. Histologically a diffuse acute suppurative placentitis is observed mainly in the intercotyledonary areas (Jubb *et al*, 1985). Lesions in aborted foetuses have not been reported (Raju *et al*, 1988).

There appears to be differences in coxiellosis of cattle, sheep and goats. In contrast to sheep both cattle and goats may become chronically infected, and lactating animals may show prolonged excretion of *C. burnetii* into milk (Lang, 1990). Goats and sheep are predisposed to abortions during coxiellosis epizootics, whereas abortions in cattle herds are more sporadic (Rady *et al*, 1985, Stanford *et al*, 1994). In sheep abortion rates from 10-60 % has been reported (Zeman *et al*, 1989). The abortion rate in goats is usually 5-50 %, but may reach to more than 90 % (Miller *et al*, 1986).

There are several reports describing coxiellosis in parturient cats (Langley *et al*, 1988, Marrie *et al*, 1988, Pinsky *et al*, 1991). In a study from Canada, seven of ten cats with *C. burnetii* infection had one or more stillborn kittens, the rate of stillborn kittens is normally around 10 % (Marrie *et al*, 1988).

There are also two reports from Canada concerning contagion from dogs. In both reports all the members of the families became ill with Q fever after the family dog had given birth to pups. One of the dogs was probably infected by rabbits she had caught during her pregnancy. All of her four pups died soon after birth (Buhariwalla *et al*, 1996). The other dog was fed deer liver a week before delivery. One of her pups was stillborn and two of the pups died within the first week of life. *C. burnetii* was isolated from this dogs uterus (Laughlin *et al*, 1991).

Experimental intranasal Q fever infection in mice

The susceptibility for Q fever infection varies between mice strains. Exposure to a high infectious dose may result in asymptomatic infection in mice which are resistant. In contrast mice, which are intermediately sensitive, show signs of

disease, and mortality is observed in sensitive mice to the same infectious dose (Scott *et al*, 1987).

The route of infection and the size of the inoculum seems to determine the predominant manifestation of Q fever infection in mice. Pathological changes in lungs are more pronounced after intranasal infection, whereas greater changes in liver are observed after intraperitoneal infection (Marrie *et al*, 1996).

There are several publications concerning experimental intranasal or aerosol infection of mice with *C. burnetii* (Hall *et al*, 1981, Khavkin and Tabibzadeh, 1988, Marrie *et al*, 1996, Perrin and Bengtson, 1942). Symptoms of disease like lethargy, ruffled fur, and also deaths have been recorded (Marrie *et al*, 1996). Gross lesions consisting of enlargement of spleen have been reported 7-14 days post-infection (Hall *et al*, 1981). Intranasal Q fever infection can cause pneumonia and hepatitis in mice. Typical lesions in lungs of Balb/c mice infected intranasally with *C. burnetii* 10 days post-infection are predominantly interstitial alveolitis (Marrie *et al*, 1996). The changes consists of interstitial infiltrates of mononuclear cells in the walls of airways, and there is proliferation of bronchiolar epithelium and desquamation of cells. At this time point changes in liver consists of focal mononuclear cell aggregates, in some of these aggregates necrotic hepatocytes are observed (Marrie *et al*, 1996). Q fever infection is a self-limiting disease. In experiments performed by Hall *et al* (1981) aerosol infected mice contained Q fever infection within 3 weeks.

Epidemiology

The host range is unusually broad, since *C. burnetii* has been identified in arthropods, reptiles, fish, birds, rodents, marsupials and livestock (Aitken, 1989, Baca and Paretsky, 1983). *C. burnetii* is highly infectious by the respiratory as well as the parenteral route. In wild vertebrates transmission can occur by ticks (tick-bite and/or contamination of the bite wound by tick feces). In domestic animals contamination of the environment by infectious birthfluids, placenta, milk, urine and feces is of considerable epidemiological importance, since it may allow inhalation of the organism (Ormsbee, 1965). In herds of cattle, sheep and goats, tick-independent cycles of infection develop, especially when large number of animals are held in a restricted area during calving or lambing. The organism remains viable for months in tick feces or dust due to the sporulation process (Ormsbee, 1965). Q fever is a zoonosis and one inhaled bacteria is capable of causing Q fever in man (Tigertt *et al*, 1961). Humans may also become infected after ingestion of contaminated dairy products, but this route seems less frequent (Aitken, 1989, Marrie and Raoult, 1997).

Geographical distribution

C. burnetii is worldwide in distribution and exists on all five continents. Q fever is observed mainly in countries where enough attention is paid to its importance.

The epidemiological situation in Europe varies. Sporadic cases of Q fever have been observed, but some large outbreaks have also occurred. A large outbreak occurred in Switzerland in 1983, when flocks of sheep were herded from alpine pastures. Approximately 400 human cases of Q fever occurred in inhabitants along the route (Dupuis *et al*, 1987). In Slovakia another Q fever outbreak affecting 113 persons was reported in 1993. The source of infection was imported goats in which abortions had occured (Kovácová *et al*, 1998). In the Netherlands 28 human Q fever cases were reported in 1985. A serological survey performed in the Netherlands revealed that 21,4 % of 1160 dairy cows and 3,5 % of 3603 sheep were seropositive. In contrast, of 219 dogs and 26 cats tested none was found positive (Houwers and Richardus, 1987).

In Germany 54 human cases of Q fever were reported in 1984. A continuous increase in reported cases of cattle has been observed between 1972-1985 in Germany. Several seroepidemiological studies show that approximately 8 % of a large number of cows tested were positive. Interestingly the prevalence of antibodies against *C. burnetii* increases in herds with infertility and abortion problems, 40 % of 1193 serum samples tested were positive (Krauss *et al*, 1987).

The prevalence of acute Q fever in France is 50 per 100 000 inhabitants (Mege *et al*, 1997). Of more than 300 analyzed cases in France, hepatitis was a more common presentation than pneumonia (Dupont, 1992). Chronic Q fever is also more frequently reported from France than other countries (Brouqui *et al*, 1993).

For a long time, Sweden was thought to be free of Q fever. In 1990 *C. burnetii* was isolated for the first time from a Swedish source (Åkesson *et al*, 1991). Isolation was successful from two sheep placentas originating from two different herds located on the island of Gotland. These sheep were healthy with normal lambnings. Seroepidemiological studies in humans suggest that Q fever is endemic in Sweden (table 1)(Macellaro *et al*, 1993). In Sweden, sporadic cases of imported or suspected domestic Q fever have been previously reported (Kindmark *et al*, 1985, Kindmark *et al*, 1986). Recently, a domestic case of Q fever has been diagnosed. A man became ill with severe influenza-like symptoms after he had emptied an old barn containing mouldy hay (Rustscheff *et al*, 1999). He was later shown to have high antibody titres against *C. burnetii*. Samples of the hay was collected and *C. burnetii* was successfully isolated from these samples.

Table 1. Prevalence of individuals seropositive to *C. burnetii* (Macellaro *et al*, 1993).

Sheep farmers	42/147	28,5 %
Veterinarians	17/132	12,9 %
Draftees	5/89	5,6 %
Hospital employees	4/56	7,1 %

In North America Q fever is highly prevalent in Maritime Canada. Q fever accounted for 20 % of all cases of pneumonia admitted to hospitals in Nova Scotia in 1983 (Marrie *et al*, 1985a). The highest risk for acquisition of pneumonia caused by *C. burnetii* in this area is exposure to parturient cats or skinning of wild hares (Marrie *et al*, 1986, Marrie *et al*, 1988). In seroepidemiological studies in the same area it has been shown that 24 % of 216 cats and 49 % of 730 snowshoe hares (*Lepus americanus*) had antibodies to *C. burnetii* (Marrie *et al*, 1985b, Marrie *et al*, 1993). A seroepidemiological study performed in 1980 in California on domestic animals showed highest prevalence of antibodies against *C. burnetii* in dogs (53 % of 1040 dogs), whereas 32 % of 28 cattle, 26 % of 121 horses and 9 % of 80 cats had antibodies against *C. burnetii* (Willeberg *et al*, 1980). In earlier studies in California more than 80% of 1052 dairy cows were seropositive, and 23 % of 840 cows tested were shedding *C. burnetii* in the milk (Biberstein *et al*, 1974). *C. burnetii* infection is widely distributed throughout the State of California since wildlife serotested revealed 63 % prevalence of serum antibodies against *C. burnetii* among coyotes and foxes and 26 % prevalence among bobcats (Riemann *et al*, 1978). In a survey conducted by the Centers for Disease Control between 1948 and 1977, 67 % of 1164 human Q fever cases reported in the US were from California (D'Angelo *et al*, 1979).

Entry into and proliferation within host cells

Bacteria can reach the intracellular compartment by mechanisms requiring active participation by bacteria and host, others rely on host cellular functions, endocytosis, for uptake (Moulder, 1985). Endocytic mechanisms, phagocytosis and pinocytosis, occur at a high rate and are used by eucaryotic cells to internalise various molecules into the cell (Goldstein *et al*, 1979). Phagocytosis is an efficient process for internalisation of a variety of microbes, and some of the pinocytic pathways have also been described as ways of internalisation of bacterial species. Four distinct pinocytic pathways have been described, clathrin-dependent pinocytosis (coated pits), non-coated pinocytic vesicles, macropinosomes and caveolae (Lamaze and Schmids, 1995, Watts and Marsh, 1992). The caveolar vesicles, which are approximately 95 nm, are too small for bacterial transport (Anderson, 1993).

Previously, *C. burnetii* has been shown to enter host cells passively via phagocytosis. Moreover, *C. burnetii* phase II has been shown to infect cells more readily than phase I bacteria (Baca *et al*, 1993). In a recent report Meconi *et al* (1998) show that virulent *C. burnetii* stimulates morphological changes in THP-1 monocytes. These changes are similar to the initiation of macropinocytosis by *Salmonella typhimurium*. *S. typhimurium* stimulates large portions of the macrophage surface, and the bacteria are internalised when the membrane ruffles close to form intracellular vesicles (Alpuche-Aranda *et al*, 1994). In *C. burnetii*, contact between the cell and the bacterium induces membrane protrusions and

polarized projections. The morphological changes cover an extensive region of the surface of THP-1 monocytes. F-actin and myosin colocalize in the cell protrusions, indicating that tension of actin-myosin filaments may be responsible for the morphological changes stimulated by *C. burnetii* (Meconi *et al*, 1998).

Members of the obligate intracellular species *Chlamydiaceae* show many similarities to *C. burnetii*. Both the *Chlamydiaceae* species and *C. burnetii* have an infectious, metabolically inactive extracellular form and a metabolically active intracellular form. Furthermore, they utilise a passive mode of entry, i.e. they rely on host cellular functions for their internalisation (Baca *et al*, 1993, Moulder, 1991). The *Chlamydia* species gain entrance to their host cells by phagocytosis and at least two pinocytic modes of entry (Hodinka and Wyrick, 1986, Hodinka *et al*, 1988). One of the entry mechanisms is mediated by coated pits, whereas another occurs through inducible clathrin-independent pinosomes. Similarly, *C. burnetii* should derive advantage from multiple entrance mechanisms into host cells.

Immunological aspects

In intracellular infection, activated macrophages have important immuno-regulatory functions in development of protective immunity. Proinflammatory cytokines like IL-1, TNF-α and IL-12, which are secreted by infected macrophages, are capable of modulating the cellular immune response.

IL-1 (IL-1α and IL-1β) and TNF-α are known to induce a wide variety of genes (Dinarello, 1994, Liles and Voorhis, 1995). By inducing chemokines and upregulating cell adhesion molecules, IL-1 and TNF-α facilitates the emigration of inflammatory cells to the site of infection (Dinarello, 1994, Furth *et al*, 1994). IL-1 has an enhancing role in IL-2 receptor expression and induction of IL-2 synthesis by T-cells, which results in T-cell proliferation (Mizel, 1987, Lowenthal *et al*, 1986). Moreover, TNF-α plays a role in granuloma formation and their generation of bactericidal properties (Kindler *et al*, 1989). IL-1Ra, also released by macrophages, is a specific receptor antagonist and inhibits the biological activity of IL-1α and IL-1β (Liles and Van Voorhis, 1995). The mechanisms for differential release of IL-1 and its inhibitor is probably of major importance (Roberts *et al*, 1986). IL-12 enhances differentiation of T-cells into protective T helper 1 (Th1) cells (Trinchieri, 1994, Locksley, 1993, Hendrzak and Brunda, 1995).

In Q fever infection, macrophages play a central role, since *C. burnetii* inhabits their acidic phagolysomes. By avoiding their microbicidal activities, *C. burnetii* is able to maintain infection. *C. burnetii* possesses superoxide dismutase and catalase, which can limit the microbicidal effects of reactive oxygen intermediates (Akporiaye and Baca, 1983). Clearence of infection with intracellular bacteria like *C. burnetii* is dependent of cellmediated immunity. It has been shown that infection of athymic mice (lacking T-cells) with *C. burnetii*

phase I causes a progressive nonlethal disease. In contrast, their normal euthymic littermates cleared the infection (Hall *et al*, 1981).

Since paper III was accepted this spring, several reports concerning cytokine involvement in host response to *C. burnetii* infection has been published. IFN-γ, known to activate antimicrobial activity in macrophages, promotes killing of *C. burnetii* (Dellacasagrande *et al*, 1999, Turco *et al*, 1984). Furthermore, IFN-γ induces cell death by apoptosis of monocytes infected with *C. burnetii*. Both events are mediated in part by TNF-α (Dellacasagrande *et al*, 1999). In another report, also published this spring, blood from patients with acute Q fever were analysed and the levels of TNF-α and IL-1Ra were significantly increased, whereas IL-1β levels were low (Capo *et al*, 1999).

C. burnetii infection and vaccination usually induces long-lasting protection against further disease. However, persistance of *C. burnetii* in recovered individuals may lead to chronic or recurrent disease (Aitken *et al*, 1987). Onset of chronic Q fever is often dependent of immunological status of the host (Mege *et al*, 1997). T cell-mediated immunity is depressed in Q fever endocarditis. Lymphocytes from patients with Q fever endocarditis fail to proliferate *in vitro* in response to *Coxiella* antigen. The unresponsiveness of lymphocytes is antigen-specific, since *Candida* can stimulate lymphocyte proliferation. The lack of granulomas in chronic Q fever is probably due to the inefficient response to *C. burnetii* (Koster *et al*, 1985). Monocytes from patients with recently diagnosed Q fever endocarditis release high amount of TNF-α and IL-1β (Capo *et al*, 1996). The levels of TNF-α are markedly enhanced in patients with endocarditis, when compared to patients with acute infection (Capo *et al*, 1999). TNF-α and IL-1β upregulate adhesion molecules in circulating monocytes and endothelial cells and could therefore be involved in the development of endocarditis (Capo *et al*, 1996).

Aims of the study

- To investigate mechanisms for internalisation of *C. burnetii* into the host cell.
- To study early proteins induced in acid-activated *C. burnetii.*
- To study host response to infection with *C. burnetii* in an *in vitro* and *in vivo* model system.
- To compare host responses after infection with virulent and avirulent *C. burnetii.*

Methodological considerations

Detailed description of materials and methods used in the different studies can be found in respective papers.

Cells and bacterial growth

C. burnetii Nine Mile phase I and II were grown in the cell line BGM. Confluent cell layers were infected with bacteria and incubated at 37 °C. Fresh media was added after 20-24 h. *C. burnetii* were collected from the media of actively growing cultures after 7-8 days by a differential centrifugation method. Prior to the experiments the bacterial suspensions were passed through an injection needle, to disintegrate aggregates.

The number of bacteria was initially estimated by A_{565} measurements, and the concentration was verified by plaque counting as described in paper I. Bacteria were heat-killed by incubation in PBS at 65°C for 1 hour (Ransom and Huebner, 1951, Williams, 1991).

The mouse macrophage-like cell line P388D1 was cultured for studies of uptake and for the *in vitro* infection and activation model. Two days prior to infection P388D1 cells were scraped off, washed and seeded in cell culture flasks to accomplish equal and suitable cell density. The medium was shifted 2 hours prior to infection. Bacteria (multiplicity of infection, 100:1) were added to P388D1 cells and the flasks were incubated at 37°C. For LPS stimulation purified *C. burnetii* LPS or commercial *E. coli* LPS were used.

In vitro acid activation and labeling of *C. burnetii*

A modification of an *in vitro* acid activation buffer system was used for labelling proteins in the bacteria (Thompson *et al.*, 1990). Extracellular *C. burnetii* were isolated as described above and resuspended in acidic buffers. Radioactive L-[^{35}S]-methionine was added, and the mixture was incubated for indicated time.

To fractionate bacteria according to their size, some of the labelled bacteria were applied on a sucrose gradient (25-60 %) (Wachter *et al.*, 1975) and centrifuged for 40 hours. The absorbance, A_{280}, and counts per minute (c. p. m.) were measured in each fraction (1 ml). This showed that the labeled bacteria consists of a heterologous population of small as well as large cells.

Inhibition experiments

There are a number of inhibitory substances available for studies of uptake processes in eukaryotic cells. In the studies for uptake we used the following substances; colchicine, methylamine, and amiloride. Colchicine has an influence on endocytosis by binding to and inducing a local unfolding of the growing ends of microtubuli (Sackett and Varma, 1993). Methylamine inhibits the membrane

enzyme transglutaminase, which catalyses cross-linking of proteins and is involved in receptor-mediated endocytosis (Davies *et al*, 1980, Kaplan and Keogh, 1981, Lorand *et al*, 1976). Amiloride is a substance that blocks Na^+/H^+ exchange and interferes with a number of receptors and formation of pinosomes, but not coated pits (Hewlett *et al*, 1994, West *et al*, 1989). An image of the cell viability after treatment with inhibitors was obtained by the trypan blue exclusion test and the MTT test (Bagge Hansen *et al*, 1989; Philips, 1973).

Cell-layers were pre-incubated with the inhibitors methylamine, colchicine or amiloride, and labelled bacteria were added with a multiple of infection of 100. After incubation at 37 °C the non-bound bacteria were removed, the cell layers were washed, and the cells were scraped off into PBS. Total cell-associated bacteria were determined by liquid scintillation counting of one third of this mixture. The remaining cells were lysed by intensive shaking for 3 minutes and centrifuged to remove cell debris. The bacterial content of the supernatants (intracellular fraction) was determined by liquid scintillation counting and expressed as c. p. m. This was assumed to give a rough estimation of the amount of internalised bacteria.

Bacterial cell fractionation

Bacterial cells were fractionated using a modification of the Osborne method. Cytoplasmic and membrane proteins were subjected to a density gradient centrifugation. The gradient was fractionated from the bottom and A_{280} of each of the fractions was measured (Osborn *et al*, 1972).

Protein and LPS preparation and separation

Samples for protein separation were withdrawn at various time intervals after a shift to the acidic buffer and/or increased temperature and the bacteria were harvested by centrifugation. The pellets were dissolved in sample buffer, sonicated and boiled. LPS was extracted from *C. burnetii* phase I and phase II according to Amano *et al* (1987) with some modifications

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a modified Laemmli buffer system (Laemmli, 1970). The protein samples were separated on a uniform 12.5% polyacrylamide gel and LPS samples were separated on a 10-17.5 % polyacrylamide gel. The PAG was stained in Comassie brilliant blue R (proteins) and silver-stained (LPS) as described by Tsai and Frasch (1982).

In the two-dimensional gel electrophoresis, the first dimension electrophoresis was performed by isoelectric focusing of acid-activated *C. burnetii* proteins, and the second dimension electrophoresis was performed as described above.

Immunoblotting and autoradiography of separated proteins

Autoradiography was performed by exposure of Enhance®-treated, dried polyacrylamide gels to Hyperfilm-MP. For Western blotting proteins were transferred from PAG to NC membranes by the use of a semi-dry electroblotter. Anti-71-kDa protein specific antiserum was raised by immunizing rabbits with protein, which had been prepared by electroelution from PAG slices. Anti-71-kDa protein antibodies were purified from the polyclonal serum by immunoadsorption using NC membrane spots containing the 71-kDa protein.

Cloning and DNA sequencing

Preparation of chromosomal DNA was performed by a modification of the procedure described by Vodkin *et al* (1986). Random cutting of the *C. burnetii* chromosomal DNA was generated by digestion of total DNA with *Eco*RI and fragments between 2 and 9 kb were selected. The purified fragments were ligated to Lambda ZAP II vector. Positive clones were detected with antibodies directed to the 71-kDa protein. The nucleotide sequence was determined by the dideoxynucleotide chain-termination method. The software package GCG was used to assemble and analyze the sequences, and homologies to other proteins or genes were analyzed using the FASTA and Gap methods of the GCG Wisconsin Sequence Analysis Package™, version 8.0, and the GenBank or SWISS Protein databases.

Cellular localisation of the *C. burnetii* 71-kDa protein

Bacteria were isolated from continuously infected cultures and fixed in formaldehyde. The samples were frozen in liquid N_2 and cryosectioned. The sections were mounted on formvar-coated nickel grids. The grids were first incubated with anti-71-kDa protein antibodies and then collodial gold conjugated goat anti-rabbit IgG antibodies. The immunolabeled sections were examined in an electron microscope.

Cytokine immunoassay

The amount of IL-1α, IL-12 and TNF-α protein in the cell culture supernatant, was determined by ELISA. Statistical analysis was done with ANOVA: One factor test. A p value less than 0.05 was considered significant.

Infection of mice

Balb/cJ mice, which are intermediately sensitive to *C. burnetii* infection, were used for the *in vivo* infection model. Approximately 10 weeks old female mice were divided into three groups, each consisted of three mice. One group received approximately 1 x 10^7 viable *C. burnetii* phase I in 10 µl isotonic-saline intranasally. The second group was infected intranasally with the same number of *C. burnetii* phase II. The third group served as control animals and received isotonic-saline by the same route. After 3, 5, 7 and 14 days mice were euthanised with CO_2.

Presence of *C. burnetii* in organs

RNA was isolated from lung, liver and spleen from three mice after homogenization of the tissues. Complementary DNA was synthesized from RNA as described in paper IV. To normalize for variation in amount of tissue β2-microglobulin, which is constitutively expressed, was used as an internal standard to the bacterial marker *C. burnetii* 16S rDNA. PCRs using primers for β2-microglobulin (Ehlers *et al*, 1992) and *C. burnetii* 16S rDNA were run as described in paper IV to analyse the presence of bacteria in organs. Heat killed *C. burnetii* were used as a positive control. PCR products were analyzed by electrophoresis of the reaction mixture on 2 % agarose gels, which were stained with ethidium bromide.

Histopathology

Paraffin sections of formalin fixed tissue specimens were stained with haematoxylin and eosin (HE) and Verhoeff´s elastic stain. Additional stains were the reticulin stain according to Gordon and Sweet, van Gieson´s method for connective tissue and Martius scarlet blue (MSB). To evaluate the number of granulomas in liver, the same lobe of mice infected 7 or 14 days with virulent or avirulent *C. burnetii* were analysed. From each mouse a total of 40 histological slides were examined, 4 histological slides at 4 μm distance from each other were chosen every 20 μm. Granulomas on each slide were calculated at magnification x 63.

Detection of cytokine mRNA in lung

To detect and quantitate cytokine mRNA expression in lung RiboQuant™ Multi-probe RNase Protection Assay System was used according to the manufacturer. The RPA-assay allows simultaneous detection of several cytokine mRNAs in a single sample. The following cytokine mRNAs were screened: mIL-12p35, mIL-12p40, mIL-10, mIL-1α, mIL-1β, mIL-1Ra, mMIF, mIL-6, mIFN-γ, mTNFβ, mLTβ, mTNF-α. Results from the RPA assay are based on mRNA samples from two mice at each time point. Our experience of the RPA assay in this model system is that the method is not very sensitive. Therefore, a negative result with the RPA assay does not exclude the presence of low amounts of cytokine mRNA. Furthermore the RPA assay was very time-consuming.

Results and discussion

Internalisation of *C. burnetii* into the host cell

C. burnetii is capable of infecting a number of cells, including macrophages (Baca and Paretsky, 1983). In several reports, entry of *C. burnetii* into host cells has been shown to occur by phagocytosis (Brezina and Kazár, 1965, Baca *et al*, 1993, Burton *et al*, 1971, Kazár *et al*, 1975, Kishimoto and Walker, 1976, Wisseman *et al*, 1967). In study I internalisation of *C. burnetii* into the host cell was studied.

Macrophage monolayers (P388D1) were preincubated with well-known inhibitors of endocytosis. To study internalisation, the cell cultures were then infected with *C. burnetii*. Phase II organisms were used in these experiments since they are taken up more readily. The bacteria were obtained from culture supernatants and were labelled *in vitro* with [^{35}S]-methionine. The presence of small as well as large cell sizes of labelled *C. burnetii* was verified by separating the bacteria on a sucrose gradient according to Wachter *et al* (1975). The intracellular fraction of labelled *C. burnetii* was measured three hours post-infection. The inhibitors colchicine, methylamine and amiloride all caused an obvious dose-dependent decrease of radiolabelled *C. burnetii* from the intracellular fraction.

Colchicine affected cell morphology and caused a dose-dependent reduction in mitochondrial enzyme activity. Therefore the reduced internalisation of *C. burnetii* after colchicine treatment could be caused by an unspecific toxic effect on cells, rather than specific reduction of uptake. In contrast, the negative effect of methylamine and amiloride on cell morphology was very weak.

Incubation of macrophages with methylamine has been shown to inhibit pinocytosis at low concentrations and phagocytosis at high concentrations in a dose-dependent manner (Teshigawara *et al*, 1985, Kaplan and Keogh, 1981). Since methylamine caused a reduction of the intracellular fraction of *C. burnetii* both at low (5-10 mM) and high concentrations (20 mM), methylamine seems to block both pinocytic and phagocytic internalisation of *C. burnetii*.

Amiloride is known to inhibit clathrin-independent pinocytosis (West *et al*, 1989). The dose-dependent reduction of the intracellular fraction of *C. burnetii* by amiloride indicates that clathrin is not involved in internalisation of *C. burnetii*.

The passive mode of entry was verified by using heat-killed bacteria. The three inhibitors caused a dose-dependent reduction of intracellular non-viable *C. burnetii*. Thus, the inhibitors caused a similar reduction of intracellular bacteria after infection with either viable or non-viable *C. burnetii*.

The results from our experiments suggest that *C. burnetii,* in analogy to the *Chlamydiaceae* species, may utilise both pinocytosis and phagocytosis for internalisation. The variable size of *C. burnetii* cells offers suitable particle sizes for both pinocytic (small particles) and phagocytic (large particles) modes of uptake. Furthermore, the use of several endocytic mechanisms in a passive mode for internalisation, ensures an efficient proliferation of *C. burnetii* and may explain the broad host cell range of this bacteria. In addition to macrophages, also non-phagocytic cells have been shown to be inhabited by *C. burnetii.* Cells like pneumocytes and fibroblasts in the lungs become infected after intranasal *C. burnetii* infection of mice (Khavkin and Tabibzadeh, 1988).

Recently an interesting report in the subject has been published. In this report virulent *C. burnetii* is shown to induce extensive morphological changes on surface membranes of monocytes (Meconi *et al,* 1998). Similar morphological changes are induced on macrophages by *S. typhimurium,* that utilise macropinocytosis for uptake (Alpuche-Aranda *et al,* 1994). This observation, as well as the results presented in study I, supports that several endocytic mechanisms are involved in internalisation of *C. burnetii.*

Identification of a *C. burnetii* 71-kDa Hsp70 homologue

C. burnetii, incubated in an acidic *in vitro* system, are very active in protein biosynthesis, thus providing a convenient method for studying proteins induced during phagolysosome-like conditions (Thompson *et al,* 1990, Zuerner and Thompson, 1983). Study II was focused on the proteins which are initially produced by acid activated *C. burnetii.*

Protein synthesis was followed by [^{35}S]-methionine labeling of bacteria incubated in an acidic buffer and by separation of proteins on polyacrylamide gels. Autoradiographs of dried gels revealed labeled proteins with apparent molecular weights of approximately 60 000 and 80 000 within one hour of incubation. After 4 hours, [^{35}S]-methionine was incorporated in about 35 proteins. The antigens separated on polyacrylamide gels were transferred to NC membranes for Western blotting. Using anti-GroEL antiserum the 60-kDa protein induced in our experiments was shown to be a previously described 62-kDa *C. burnetii* major protein belonging to the Hsp60 family. Heat shock proteins are among the most highly conserved genetic elements known and have basic functions in folding, assembly and membrane translocation of proteins (Langer and Neupert, 1991, Kaufmann, 1990). Expression of heat shock proteins might result from the stress the bacteria encounter during the infectious process (Buchmeier and Heffron, 1990). Since in the laboratory, elevated temperature is widely used to induce these proteins, they have been termed heat shock proteins. Two well characterized families of heat shock proteins comprise Hsp60 and Hsp70 groups (corresponding to *E. coli* GroEL and DnaK proteins). An immunoblotting using

an anti-DnaK antiserum revealed that the "early" protein migrating as an 80-kDa protein is a Hsp70 homologue.

To further characterize the Hsp70 homologue a gene library was constructed using the Lambda ZAPII vector and *Eco*RI*-digested *C. burnetii* DNA. Positive clones in the *C. burnetii* gene library were identified by using antibodies directed to the purified Hsp70 homologue. Sequence analysis of one of the clones showed a single open reading frame with a size of 1 968 bp. The deduced amino acid sequence contains 656 residues. This corresponds to a protein with a molecular weight of 70 800 and therefore the gene will be referred to as the *C. burnetii* hsp71 gene hereafter. The presence of initiation and stop codons together with the results from the alignment with hsp70 sequences from other bacteria indicated that the clone contained the entire gene. When the clone with the *C. burnetii* hsp71 gene was expressed in *E. coli,* analysis by SDS-PAGE revealed a protein migrating as an 80-kDa protein.

A Shine Delgarno sequence was located at position 57. A -10 promoter sequence identical with the consensus heat shock σ^{32} promoter sequence was identified. Alignement of the postulated -35 promoter region with the consensus sequence and promoter regions of DnaK genes from other bacteria indicates that an *Eco*RI site is located in the -35 region, which is assumed to be located at the border of the cloned fragment. The spacing between the -35 and -10 regions in the *C. burnetii* hsp71 gene is 12 base pairs.

The similarity of the amino acid sequence of the *C. burnetii* Hsp71, compared to Hsp70s of *Legionella pneumophila, Francisella tularensis* and *Escherichia coli* was 86%, 83% and 80%, respectively. The comparison revealed extensive homologies in the N-terminal and central parts of the sequence.

Members of the Hsp70 family are generally considered to be cytoplasmic proteins in most procaryotes, but membrane association and secretion from the cell has been reported (Garduño *et al*, 1998, Zügel and Kaufmann, 1999). To elucidate the cellular localization of the *C. burnetii* Hsp71 protein, immunogold staining of ultrathin sections of *C. burnetii* was performed. Primary antibodies were directed to the 71-kDa protein and the secondary antibodies were conjugated to colloidal gold. Counting of gold particles revealed that 53% of gold particles were located in the cytoplasm and 47% were associated with the cell wall. Furthermore, Western blotting of membrane proteins prepared by the Osborne procedure was performed. The anti-DnaK antibodies recognized a single band in the membrane fractions as well as a faint band in the cytoplasmic fraction. These results support a cell wall-association in addition to the localization in the cytoplasmic compartment for the *C. burnetii* 71-kDa Hsp70 homologue. The *C. burnetii* Hsp60 homologue is also reported to be membrane associated (Vodkin *et al*, 1988).

When *C. burnetii* reaches the phagolysosomal milieu synthesis and accumulation of proteins is induced. The functions of *C. burnetii* proteins, induced early in infection under conditions that closely reflects the phagolysosomal environment, are likely to be of interest for understanding intracellular survival and growth. In this report we show that the *C. burnetii* Hsp60 and Hsp70 homologues are among the first proteins to be induced in acid activated *C. burnetii*, furthermore they are both predominant.

An increase in heat shock protein synthesis has been associated with a role in the developmental cycle of various organisms e.g. *Chlamydia* and *Leishmania* (Lathigra *et al*, 1991, Lundemose *et al*, 1990). Early production of DnaK and GroEL-like stress proteins suggests that they are of importance in transformation of *Chlamydiaceae* elementary bodies to reticulate bodies (Lundemose *et al*, 1990). The course of events for the developmental cycle of *C. burnetii* are not known in similar detail as for *Chlamydia,* and whether heat shock proteins are of importance for the developmental cycle of *C. burnetii* is unknown.

Heat shock proteins have been suggested to be virulence determinants, and they are reported to be major targets of both humoral and cell-mediated immune responses (Kaufmann, 1990, Lathigra *et al*, 1991). Bacterial Hsp has been shown to directly induce cytokine mRNA as well as cytokine secretion (Retzlaff *et al*, 1994, Marcatili *et al*, 1997). In other experiments we have observed that the *C. burnetii* Hsp71 is one of the immunodominant proteins identified by Western blottings, when using sera from naturally infected or vaccinated persons. It would be of great interest to find out whether the *C. burnetii* Hsp71 protein is important in pathogenesis of Q fever infection.

Cytokine induction in P388D1 macrophages infected by *C. burnetii*

C. burnetii infects primarily via inhalation and has been shown to infect alveolar macrophages (Khavkin and Tabibzadeh, 1988). In the lung, alveolar macrophages are strategically situated at the air-tissue interface in the alveoli and alveolar ducts and are among the first cells to encounter inhaled organisms. In paper III, to study host response, the initial cytokine secretion induced by *C. burnetii* was analysed after an *in vitro* infection of the macrophage-like cell line P388D1.

To determine secretion of IL-1α, TNF-α and IL-12 protein from P388D1 cells infected with *C. burnetii*, cell culture supernatants were analysed with ELISA kits. Induction of IL-1α after infection with virulent *C. burnetii* was first observed at 3 hours post-infection, and at 6 and 20 hours of infection further accumulation of significant amounts of IL-1α was observed when compared to non-infected cells. A comparison was made between cytokine induction in cell cultures infected with virulent and avirulent *C. burnetii.* At 3 and 6 hours of infection with avirulent *C. burnetii* the level of IL-1α was below the detection

limit. At 20 hours of infection IL-1α secretion was detected in culture supernatants, but the levels were low and did not significantly differ from non-infected cells.

TNF-α secretion was observed as early as 3 hours post-infection both in culture supernatants from cells infected with virulent and avirulent *C. burnetii* and there was no significant differences between the two variants. At 6 and 20 hours of infection further accumulation of TNF-α was observed in medium of cells infected with *C. burnetii* phase I or II. At 6 hours significantly more TNF-α was secreted in *C. burnetii* phase I-infected cell cultures, whereas *C. burnetii* phase II induced significantly larger amounts of TNF-α at 20 hours of infection. Thus, no uniform trend is obvious for early TNF-α production after infection with virulent or avirulent *C. burnetii*. No IL-12 production was detected in cell culture supernatants.

The difference in the early IL-1α response in macrophages infected with virulent and avirulent *C. burnetii* raised the question of LPS involvement. LPS is the only component known to differ between virulent and avirulent *C. burnetii*. *C. burnetii* phase I and II LPS was isolated and used to stimulate P388D1 cells. Cells exposed to 0.1 μg/ml of each of the two LPS variants induced comparable low levels of IL-1α after 2 and 14 hours of stimulation. A hundred-fold increase of each *C. burnetii* LPS variant (10 μg/ml) caused a moderate increase of IL-1α production in culture supernatants, but still no difference in induction of IL-1α was observed between LPS from *C. burnetii* phase I or phase II. In contrast, 0.1 μg/ml of *E. coli* LPS, which is a known stimulator of IL-1α, was a hundred times more efficient than LPS from either variant of *C. burnetii*.

The limited stimulatory effect by LPS isolated from *C. burnetii* is perhaps due to the unusual chemical composition (Amano *et al*, 1987, Moos and Hackstadt, 1987). Structurally the LPS of *C. burnetii* resembles LPS of *F. tularensis*, which could not stimulate IL-1α production in human monocytes (Sandström *et al*, 1992). The lower secretion of IL-1α after infection with intact phase II bacteria may indirectly be related to LPS composition. The phase I smooth LPS, with an extended carbohydrate structure, sterically blocks surface antigens. This results in differential accessibility of outer membrane proteins (Hackstadt, 1988).

The differential induction of IL-1α could be related to virulence as has been shown in other species. Infection with virulent *Legionella pneumophila* induced higher levels of IL-1 in macrophage cultures than avirulent bacteria (Klein *et al*, 1992). In *Listeria* strains e.g. hemolytic activity has been correlated to ability to induce IL-1α mRNA (Demuth *et al*, 1996, Xiong *et al*, 1994). The induction of cytokines may, futhermore, depend on the various steps of invasion, such as binding, internalization, replication and intracellular localization. The lower IL-1α induction by avirulent *C. burnetii* could be due to some of these steps. There are no reports concerning differences in intracellular replication or survival in

P388D1 cells, and both *C. burnetii* phase I and phase II proliferate within and establish persistent infection of P388D1 cells (Baca *et al*, 1981). In contrast, *C. burnetii* phase II seems to infect various cells more quickly (Baca and Paretsky, 1983). The initial bacterial burden, therefore, does not seem to be correlated to the IL-1α induction of P388D1 cells. In the *in vitro* infection model with *C. burnetii*, virulence of the bacteria, seems to be correlated to an augmentation of IL-1α production in the initial hours of infection.

Pathologic changes and induction of cytokines after intranasal infection of mice with *C. burnetii*

In study IV, we wanted to further characterize the host response and therefore an *in vivo* infection model in mice was used. To simulate natural infection mice were infected intranasally with virulent and avirulent *C. burnetii*, and after 3, 5, 7 and 14 days they were euthanised. The more pronounced infection caused by the virulent *C. burnetii* was reflected by depression of mice, combined with an enlargement of the spleen during the second week of infection. In contrast, mice exposed to the avirulent *C. burnetii* showed no signs of disease, and the spleen weight did not significantly differ from uninfected animals.

Histological changes in the lungs were observed as early as 3 days after infection with virulent *C. burnetii*. Scattered inflammatory foci were characterized by intra-alveolar and bronchiolar exudate composed of oedema fluid, neutrophils and some erythrocytes. However, at day 7 pneumonic foci with predominantly interstitial inflammatory reactions were found both in lungs of mice infected with virulent and avirulent *C. burnetii*. The alveolar walls were diffusely infiltrated with lymphocytes, plasma cells and macrophages. The inflammatory reaction in mice with avirulent infection was nearly resolved 14 days post-infection. In contrast, the interstitial reaction of mice infected with virulent *C. burnetii* was even more pronounced at day 14 than at day 7 post-infection. The dominant feature consisted of macrophage infiltration in alveoli and alveolar walls.

The pathological changes observed in the lungs were correlated to the presence of bacteria by amplifying the *C. burnetii* specific 16S rDNA sequence. The lack of PCR product after avirulent infection indicated that the presence of bacteria is limited. Still mice infected with avirulent bacteria showed obvious pathological changes in lungs 7 days post infection. The inflammatory reaction in the lung could either be induced by the avirulent bacteria present at the time of infection. Alternatively, we are unable to detect the presence of low numbers of bacteria. In contrast, in virulent infection *C. burnetii* 16S mRNA was demonstrated in lung both at 3 and 5 days of infection, which is in agreement with the early inflammatory response observed in lungs of these mice.

Dissemination of virulent *C. burnetii* to liver and spleen was demonstrated by the presence of bacterial mRNA as early as 3 days post infection. Also later at 5 days of infection *C. burnetii* 16S mRNA was present in both organs. On the contrary,

in mice infected with avirulent *C. burnetii,* no bacterial mRNA was detected early in infection neither in liver nor in spleen. However, at 5 days post infection a weak signal was observed. This indicates that the avirulent bacteria transiently infect these organs.

In liver granulomas were observed at 7 and 14 days post-infection, both in mice infected with virulent and avirulent *C. burnetii.* The multifocal granulomas consisted of macrophages and lymphocytes with a few polymorphonuclear leukocytes. The frequency of granulomas in liver specimens was in general considerably higher in mice infected with the virulent variant. This is in agreement with the previously demonstrated bacterial load of virulent *C. burnetii* in liver. Similarly, the less numerous granulomas in the livers of mice infected with avirulent bacteria is a result of the transient infection caused by these bacteria.

The pathological changes observed in the mice infection model are in agreement with earlier publications concerning intranasal or aerosol Q fever infection in mice (Hall *et al,* 1981, Khavkin and Tabibzadeh, 1988, Marrie *et al,* 1996, Perrin and Bengtson, 1942). In conclusion, intranasal infection of mice with either *C. burnetii* phase I or phase II caused a systemic infection. However, several differences were evident when comparing the inflammatory responses after infection with the two variants. Mice infected with virulent *C. burnetii* appeared depressed. Histological lesions in lungs appeared earlier and did not resolve as soon as in mice infected with avirulent *C. burnetii.* Histological changes in livers were more prominent and splenomegaly was observed only in mice infected with virulent *C. burnetii.*

The obvious differences observed in infection of mice with the two *C. burnetii* variants made the question of possible involvement of immune signal substances exciting. The induction of cytokine mRNAs was studied in lung, which is the primary infectious focus, at 3, 5 and 7 days of infection. The RPA assay was used to enable study of several cytokine mRNAs simultaneously, and the predominant cytokines were monitored. In the lung, virulent *C. burnetii* induced an obvious increase of IL-1β mRNA 5 days post-infection. A slight induction of IL-1α, IL-1Ra and TNF-α mRNA was also observed at this time point. No cytokine mRNA was detected at 3 or 7 days and avirulent *C. burnetii* did not induce cytokine mRNAs in the lung at any of the time points tested.

The extensive involvement of macrophages and lymphocytes in the lung as well as in the liver granulomas suggests that the infectious process caused by *C. burnetii* is controlled by acquired cell-mediated immunity. Activation of T lymphocytes and subsequent activation of macrophages for increased intracellular killing, is mediated by cytokines and cell-cell interactions via membrane bound receptors. In our mouse model, the clear induction of IL-1β mRNA, together with the low induction of IL-1α mRNA, detected only in the

lungs of mice infected with virulent *C. burnetii*, indicates that induction of IL-1 may be reflected by a more severe and prolonged inflammatory response. A response which is necessary to clear infection in these mice. IL-1 has been shown to be important for recruitment of T-cells and their activation (Sibille and Reynolds, 1990). A slight induction of IL-1Ra mRNA was also observed. IL-1Ra is known to block IL-1 activity and will therefore regulate the level of IL-1 activity (Arend *et al*, 1998). Expression of IL-1Ra has been shown to occur also in tuberculosis, a disease which is also characterized by granulomatous lesions (Arend *et al*, 1998).

Furthermore, analysis with the fairly insensitive RPA assay, revealed a low induction of TNF-α mRNA. TNF-α is required for macrophage accumulation and differentiation (Kindler *et al*, 1990, Sibille and Reynolds, 1990). In human patients with diagnosed acute Q fever, analysis of cytokines in plasma revealed induction of TNF-α and IL-1Ra, but not IL-1β (Capo *et al*, 1999). However, a distinction should be made between systemic blood cytokine levels and local levels in organs, they might not be similar (Kindler *et al*, 1989). Despite that the RPA assay allowed screening of several cytokine mRNAs not many were detected. Perhaps mRNAs produced in low levels could not be detected due the insensitivity of the assay.

Not only mononuclear cells, but also polymorhonuclear phagocytes (PMN) have been shown to be essential for elimination of intracellular bacteria. PMN exert their bactericidal activity by producing oxygen radicals and releasing granular enzymes (Sibille and Reynolds, 1990). In an *in vivo* infection model in mice, neutrophils have been shown to restrict growth of the facultative intracellular *F. tularensis* early in infection. So as to prevent an overwhelming number of bacteria, until a specific immune response has developed (Sjöstedt *et al*, 1994). Besides, neutrophil elastase has been shown to be important in combating gram negative bacterial infection in mice (Belaaouaj *et al*, 1998). Polymorhonuclear phagocytes stimulated with LPS have also been shown to secrete TNF-α, IL-1β and IL-1Ra (Cassatella, 1995). These cells secrete smaller quantities of cytokines per cell, when compared to mononuclear cells. But they could influence the development of the immune response since they are among the first cells to migrate into tissues with an inflammatory reaction and they are present early in *C. burnetii* infection.

To further elucidate the immune response during *C. burnetii* infection it is important to find out whether cytokine mRNA levels are reflected in cytokine protein expression. In addition, it would be of interest to look closer at the T-cell response. Cytokines produced at an early stage of infection by macrophages and natural killer (NK) cells can polarize the subsequent T-cell response from a precursor Th0 cell, to a Th1 or Th2 cell response. Predominance of a Th1-like response provides better cell-mediated immunity, whereas a development towards a Th2-like response is important for an efficient B-cell development and

antibody production (humoral immunity). The two functional subsets of T lymphocytes are characterized by the cytokines that they secrete. Th1 cytokines include IFN-γ, IL-2 and TNF-β, whereas Th2 cytokines includes IL-4, IL-5, IL-6, IL-10 and IL-13 (Lucey *et al*, 1996). The obvious differences observed in infection with virulent and avirulent *C. burnetii* may largely be due to differently polarized T lymphocytes. In agreement with other intracellular infections Q fever could be expected to be controlled by a protective Th1-mediated response.

Conclusions

The obligate intracellular bacterium *Coxiella burnetii* is capable of infecting a variety of cells of different origins. Typical for *C. burnetii* is the existence of an intracellular developmental cycle, which leads to formation of a mixture of pleomorphic cell types. The utilization of different pathways for uptake should enable an efficient uptake of small as well as large cell variants into the host cell. *C. burnetii* has previously been reported to reach the intravacuolar compartment of host cells passively by phagocytosis. To further study internalisation of *C. burnetii,* well characterized inhibitors of endocytosis were used. The results indicate that internalisation of *C. burnetii* phase II is mediated by pinocytosis as well as phagocytosis. The use of various endocytic pathways could also explain how *C. burnetii* ensures an efficient uptake in a wide range of host cells. The small cell variant, being the infectious form with ability to survive extracellularly, could be expected to utilize pinocytosis, in addition to phagocytosis, due to its small size. The existence of alternative endocytic pathways are supported by a recent report, where *C. burnetii* phase I is shown to induce intense cell protrusions in monocytes. Similar morphological changes has been observed in murine macrophages exposed to *S. typhimurium,* followed by macropinocytosis of the bacteria.

C. burnetii proliferates only within the phagolysosome of eukaryotic cells. The small genome of *C. burnetii* is activated by the acidic pH of the phagolysosome, and as we and others have shown a limited number of proteins are induced. In this unique niche, the scarce metabolism is sufficient for proliferation of the bacteria. Bacteria incubated in an acidic *in vitro* system, are very active in protein biosynthesis, thus providing a convenient method for studying proteins induced during phagolysosome-like conditions. We have identified one of the predominant proteins, synthesized early in acid activated *C. burnetii* as a Hsp70 homologue. Previously, one of these proteins, a 62-kDa protein has been demonstrated to be a member of the Hsp60 family. The early and strong induction of the *C. burnetii* Hsps may indicate a role for the proteins in natural infections. Induction of heat shock proteins by other intracellular pathogens has been shown to be pertinent for the disease process and important for survival in the host.

Hsps are typical cytosolic proteins, which functions as molecular chaperones, nevertheless surface expression is reported by numerous studies. A dual localization of the *C. burnetii* Hsp71-kDa is suggested, since presence of the protein was demonstrated in association with the cell wall as well as in the cytoplasm. Surface exposure could explain our previous observation that the *C. burnetii* Hsp71 is one of the immunodominant proteins, recognized by sera from naturally infected or vaccinated persons. This finding is in agreement with other reports where heat shock proteins have been shown to be major targets for

antibodies in many bacterial infections (Kaufmann, 1990, Zügel and Kaufmann, 1999).

In intracellular infection macrophages play a central role, since the activated macrophages are capable of killing intracellular bacteria. Furthermore, by secreting proinflammatory cytokines, like IL-1 (IL-1α and IL-1β) and TNF-α, infected macrophages, are capable of modulating the cellular immune response. Both IL-1 and TNF-α favours accumulation of inflammatory cells at the site of infection. IL-1Ra, also secreted by macrophages, is a specific receptor antagonist and inhibits the biological activity of IL-1.

C. burnetii infects primarily via inhalation and has been shown to infect alveolar macrophages. Early in infection *C. burnetii* is able to survive and proliferate within the macrophages, until the cell-mediated immune response is fully developed. To study the early immune signals in *C. burnetii* infection, an *in vitro* infection model was used. The macrophage-like cell line P388D1 was infected with virulent and avirulent *C. burnetii,* and cytokine secretion into cell culture supernatants was determined by ELISA. Significant secretion of the cytokine IL-1α by macrophages was correlated to infection with virulent *C. burnetii* in the initial hours of infection. TNF-α was secreted by macrophages within the first hours of infection both into culture supernatants from cells infected with virulent and avirulent *C. burnetii.* The only known difference between virulent and avirulent *C. burnetii* is LPS. LPS was therefore isolated from the two variants and used to stimulate P388D1 cells. The two LPS variants induced comparable low levels of IL-1α. The limited stimulatory effect by LPS isolated from *C. burnetii* is perhaps due to the poor endotoxic activity of *Coxiella* LPS. Thus LPS is not responsible for the difference in IL-1α induction. However, LPS could be involved indirectly, as the smooth LPS of phase I bacteria with its extended carbohydrate structures may block surface antigens and this results in differential exposure of surface proteins of phase I and phase II bacteria.

To further characterize the host response an *in vivo* infection model in mice was used. Intranasal infection with virulent or avirulent *C. burnetii* was microscopically characterized by accumulation of mononuclear cells (macrophages and lymphocytes) and granulomas in internal organs. But, the inflammatory response in mice infected with avirulent *C. burnetii* was milder and resolved sooner. Infiltration of mononuclear cells in the lung was not as pronounced, and the liver granulomas were less frequent in avirulent infection. Furthermore, only mice infected with the virulent variant appeared depressed and grossly enlargement of the spleen was observed in these mice. Thus, there are clear differences in the mouse model after intranasal infection with virulent and avirulent *C. burnetii.* The avirulent infection is transient, whereas the virulent bacteria establishes a more protracted infection.

The obvious differences in infection caused by the two variants of *C. burnetii* opened the question of possible involvement of cytokines. A screening of cytokines in lung during the first week of infection by *C. burnetii* was performed. Cytokine mRNAs could not be detected in lungs after avirulent infection, indicating a limited induction of cytokines. In contrast, an obvious induction of IL-1β mRNA was observed in the lung in the acute phase of infection in mice infected with virulent *C. burnetii.* Furthermore, low levels of IL-1α, IL-1Ra and TNF-α mRNAs were detected in these mice.

Clearance of *C. burnetii* infection is dependent of cell-mediated immunity. The significant induction, by only the virulent variant, of IL-1α in the *in vitro* model and IL-1β mRNA in the mouse model indicates that IL-1 may be crucial for the development of an early inflammatory response. Thus, the number of bacteria is limited until a specific immune response has developed capable of eliminating the bacteria. IL-1 has been shown to be important in cell-mediated immunity as it may induce recruitment of T-cells and their activation. The low induction of IL-1Ra may be involved in regulating the level of IL-1 activity. TNF-α, a key cytokine in cell-mediated defense against intracellular bacteria, seems to be involved also in *C. burnetii* infection. TNF-α probably enhances the differentiation of macrophages and plays a role in granuloma formation observed in mice infected with *C. burnetii.* The initial cytokine response induced in *C. burnetii* infection affects the later activation of T-cells, so important in cell-mediated immunity. Similar to other intracellular infections, one could expect that development of protective Th1 cells are necessary for successful elimination of virulent *C. burnetii.*

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Acknowledgements

I wish to express my gratitude to the following persons:

Lena Norlander, Division of Microbiology, Defence Research Establishment, DRE (Försvarets Forskningsanstalt, FOA), my main supervisor, for enthusiastically introducing me to the fascinating world of *Coxiella burnetii*. For all support and encouragement given during the years of my doctoral studies. For all the time you have taken for fruitful discussions and for the constructive critisism of my work.

Lennart Jönsson, head of the Department of Pathology and my supervisor, for being so positive and for supporting me in different practical matters during the doctoral studies. For helping me with the pathological parts of my work.

Anna Macellaro, Division of Microbiology, FOA, with your wast knowledge in almost every method I have used in this study, you have taken time to show me the crucial steps of many of the methods and this has been invaluable help in all laboratory work. For all the help to cultivate bacteria and cells. For always being helpful and having time for my questions, and for being a good friend.

Thorsten Johansson and Helena Lindgren, Division of Microbiology, FOA, for technical assistance.

Bo Lilliehöök and Anders Sjöstedt[1], Division of Microbiology, FOA, co-authors, for fruitful collaboration.

Per Wikström, Division of Microbiology, FOA, for a positive attitude and for all the computer support.

Majken Karlsson, secretary at the Division of Microbiology, FOA, for always being cheerful and for her assistance in different practical matters.

Carin Stenlund, librarian, FOA, for rapidly supplying me with publications needed during the work.

Leonore Johansson, Division of Biomedicine, FOA, for technical assistance.

Ulla Hammarström and Tapio Nikkilä, Department of Pathology, for technical assistance with histological slides and electron microscopic pictures.

Anders Bucht, Division of Microbiology, FOA, and Åke Åkesson, Department of Infectious Diseases, Kalmar Hospital, for critical reading of manuscripts.

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I want to thank all other friends, colleagues and personnel at FOA and at the Department of Pathology for creating a good atmosphere to work in.

Heidi and Bella, my horses, for supplying my brains with fresh air, beautiful scenery and for keeping me fit during the years of my doctoral studies.

Axel my joyful little baby boy, for making my life happy every day.

I also want to thank all baby-sitters; my parents, Riitta and Gustav, Staffans parents, Margit and Harald, and our dear neighbour Mrs Eivor Grenholm.

Staffan, Axels father, for encouraging support in every way, for taking care of the household duties and for keeping up my spirits.

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ISSN 1401-6257
ISBN 91-576-5425-5