Long-term effects of amino acids on chlamydia infections

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La chance ne sourit qu'aux esprits bien préparés

Louis Pasteur (1822-1895)

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Zusammenfassung der Arbeit:

Diese Arbeit untersucht die Effekte von erhöhten Aminosäurekonzentrationen auf chlamydiale Infektionen *in vitro* und *in vivo*.

Im ersten Teil der Arbeit wurden Zellen mit *Chlamydia trachomatis* bzw. *Chlamydophila pneumoniae* infiziert, und hohe Konzentrationen der proteinogenen Aminosäuren zum Wachstumsmedium gegeben. Von allen getesteten Substanzen inhibierten Leucin, Isoleucin, Methionin und Phenylalanin am stärksten das chlamydiale Wachstum. Ein deutlicher Unterschied in der Inhibierung wurde für Methionin beobachtet, das die Vermehrung von Retikularkörperchen bei *C. trachomatis* inhibiert, jedoch nicht bei *C. pneumoniae*.

Der zweite Teil der Arbeit untersucht die Wirkung der am stärksten inhibierenden Aminosäuren auf *C. trachomatis*. Zuerst wurde ausgeschlossen, dass die Hemmung des chlamydialen Wachstums durch negative Effekte auf die Wirtszellen hervorgerufen wurde. Dann wurde gezeigt, dass die Bakterien nicht vollständig aus der Kultur eliminiert wurden, sondern sich eine kleine Bakterienpopulation ausbildete, die zwar nicht infektiös, aber metabolisch aktiv war. Die Infektivität ließ sich durch Wiederherstellung der normalen Nährstoffkonzentration reaktivieren, weshalb schlussgefolgert wurde, dass die Aminosäuren in den Chlamydien Persistenz induzieren. Dies scheint jedoch eine neue Art von Persistenz zu sein, da das Transkriptionsmuster von den bisher bekannten Persistenzmodellen abwich.

Im dritten Teil der Arbeit wurde die Hemmung der Chlamydien *in vivo* untersucht. Zuerst wurde im Langzeitversuch getestet, ob die Aminosäuren negative Auswirkungen auf gesunde Mäuse hatten. Dies war für Leucin und Isoleucin nicht der Fall, weshalb Leucin bei Mäusen angewandt wurde, welche mit *C. pneumoniae* infiziert waren. Diese Behandlung führte zu einer deutlichen Verringerung der Bakterienzahl in den Lungen der Tiere.

Zusammen mit anderen Ergebnissen unserer Gruppe schlussfolgern wir, dass vor allem die verzweigtkettigen Aminosäuren Leucin und Isoleucin durch Kompetition an einem chlamydialen Aminosäuretransporter zu Valinmangel führten. Obwohl dadurch eine persistente Bakterienpopulation entstand, könnte diese Behandlung ein Potential für die Behandlung von chlamydialen Infektionen haben.

Abstract:

This thesis deals with the effects that elevated amino acid concentrations have on chlamydial infections *in vitro* and *in vivo*.

In the first part, cell cultures were infected with *Chlamydia trachomatis* or *Chlamydophila pneumoniae* and then grown in medium containing elevated concentrations of the proteinogenic amino acids. Of all substances tested, it was shown that leucine, isoleucine, methionine and phenylalanine completely inhibited chlamydial growth. A significant difference in degree of inhibition was observed by methionine, which inhibits replication of reticulate bodies in *C. trachomatis*, but not in *C. pneumoniae*.

The second part of this thesis tries to determine the fate of the inhibited bacteria. The most inhibitory amino acids which were determined in part one were used. First, negative side effects that elevated concentrations of these amino acids might have on the host cells were excluded. Regarding the bacteria, it was shown that the organisms are not completely eradicated from the cultures, but that a small population of viable, metabolically active but non-infectious bacteria remains. The abolished infectivity can be reactivated upon removal of the excess amino acids. It was therefore concluded that the amino acid treatments induce persistence in chlamydiae. However, this seems to be a novel form of persistence, as the transcriptional pattern of these bacteria was different from the persistence models known.

The third part of this thesis deals with the administration of amino acids to mice in the drinking water and the effect on an *in vivo* infection with *C. pneumoniae*. First, it was verified that long term administration of the inhibitory amino acids leucine and isoleucine has no negative influence on uninfected mice. Then it was shown that leucine given to infected mice significantly reduces the chlamydial load in the lungs of the animals after two weeks.

Together with other data from our group, we conclude that elevated concentrations of branched chain amino acids eradicate chlamydiae by an antagonistic mechanism. Even though a small persistent population is induced, a potential for treating chlamydial infections could be shown.

Figures and tables

Abbreviations

AOA	aminooxyacetate
APS	ammonium persulfate
CGM	cell growth medium
COPD	chronic obstructive pulmonary disease
Cpn	*Chlamydophila pneumoniae*
Ctr	*Chlamydia trachomatis*
DIF	direct immunofluorescence
d p.i.	days post infection
EB	elementary body
EDTA	ethylenediaminetetraacetic acid
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HRP	horseradish peroxidase
IDO	indoleamin 2,3-dioxigenase
IFNγ	interferon gamma
IFU	inclusion forming unit
IM	infection medium
iNOS	inducible nitrogen oxide synthase
LD50	lethal dose, 50%
LPS	lipopolysaccharide
MM	maintenance medium
MOI	multiplicity of infection
MTOC	microtubule organizing center
NP-40	nonidet P-40
PFA	paraformaldehyde
p.i.	post infection
PVDF	polyscreen polyvinylidene fluoride
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RB	reticulate body
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEM	transmission electron microscopy
TEMED	tetramethylethylenediamine
Tris	trishydroxymethylaminomethane

1. Introduction

1.1 Chlamydiae

Members of the order *Chlamydiales* are obligate intracellular bacteria, which infect a wide range of host species (Peeling and Brunham, 1996). They exhibit a two-stage developmental cycle and are disseminated by aerosol or by contact, requiring no alternate vector.

1.1.1 Phylogenesis

After Halberstaedter and von Prowazek's discovery of chlamydiae in 1907, they were first considered to be viruses until being recognized as gram negative bacteria (Page, 1966) and placed into the order *Chlamydiales* (Schachter and Caldwell, 1980). Based on rRNA sequence analysis, this order was regrouped in 1999 (Everett *et al.*, 1999;Schachter and Caldwell, 1980) as outlined in figure 1.1.

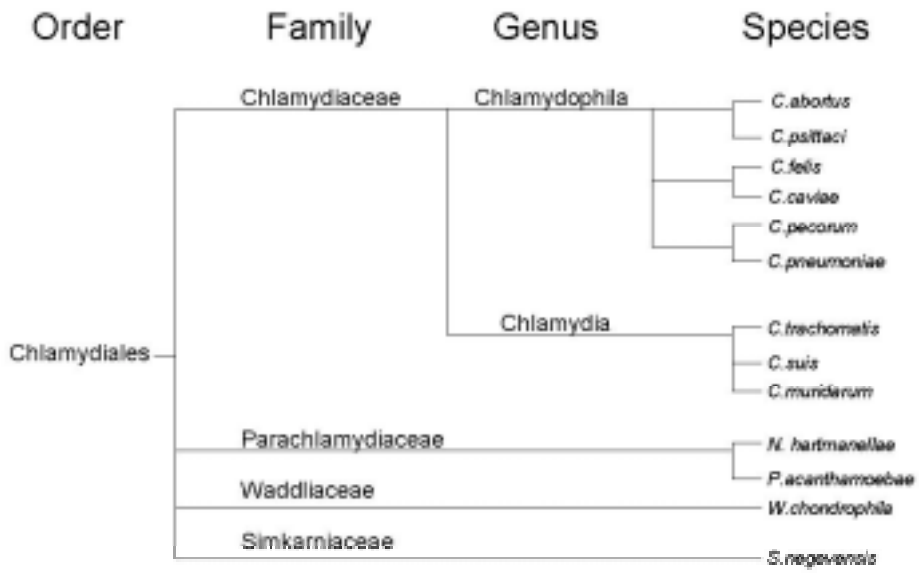


Figure 3.1 – Taxonomy of the order *Chlamydiales* groups the nine chlamydial species

Full-length 23S rRNA genes were used for construction. Lengths of lines do not represent actual phylogenetic distances. The order Chlamydiales contains four families; the family chlamydiaceae contains two genera: *Chlamydophila* (consisting of six species) and *Chlamydia* (consisting of three species). Adopted from (Bush and Everett, 2001).

Chlamydia (C.) trachomatis and ***Chlamydophila (C.) pneumoniae*** are the two major human pathogens. All other chlamydial strains mostly infect animals, but can sometimes cause zoonoses.

1.1.2 *Chlamydia trachomatis*

The species can be divided into two human biovars (biovariants, characterized by their biochemical and physiological properties): "Trachoma" and "Lymphogranuloma venereum" (LGV). These biovars contain different serovars (serovariants, characterized by different antigens). The trachoma biovar, containing serovars A-K, infects primarily mucosal epithelial cells. The LGV biovar, containing serovars L1-L3, can invade lymphatic tissue.

The ocular serovars A, B and C cause trachoma. This chronic follicular conjunctivitis, transmitted by flies and smear infections, causes the infected peoples' eye-lashes to turn inwards, thereby scarring the cornea. Over the course of years, this leads to a gradual decrease of visual capacity. Trachoma is the worlds leading cause of preventable blindness, the World Health Organization (WHO) estimates that 84 million people mostly in developing countries are affected by trachoma, 8 million of which are visually impaired. Since 1997, the WHO program "GET 2020" (Global Elimination of Trachoma) is promoting the "SAFE" strategy (lid Surgery, Antibiotic treatment, Facial cleanliness and Environmental changes) to fight this disease.

In Western countries, serovars D to K, which infect the urogenital tract, are considered the most common cause of sexually transmitted diseases – the WHO estimates that chlamydiae are responsible for over 90 million new infections per year (Gerbase *et al.*, 1998;Schachter, 1999). Ascending chlamydial infections of the female upper genital tract, known as pelvic inflammatory diseases, can cause salpingitis. This can lead to fibrosis and scarring of the fallopian tubes and subsequently to ectopic pregnancy or tubal infertility (Cates, Jr. and Wasserheit, 1991). Following vertical transmission through an infected birth canal, neonatal conjunctivitis (Schachter *et al.*, 1986) as well as pneumonia (Beem and Saxon, 1977) can occur. In men, the urethral infection is mostly asymptomatic, therefore facilitating further spread of the pathogen (Schachter, 1999;Thylefors *et al.*, 1995;Thylefors, 1995). It is also considered possible that this infection leads to male infertility (Ness *et al.*, 1997).

Serovars L1, L2 and L3, the so called "LGV serovars", are also transmitted sexually, but in contrast to serovars D to K, can cross the epithelial barrier. Systemic transmission can lead to an invasion of the lymph nodes (Hossain, 1989;Raulston, 1995;Schachter and Caldwell, 1980)

causing the systemic lymphogranuloma venereum (LGV). The disease consists of three stages: In the first stage, a self-limiting painless genital ulcer occurs at the contact site after about one week, which heals in a few days and is rarely recognized by women and by only one third of infected men. In stage two, after 10 to 30 days, the infection has spread to the lymph nodes. In men, depending on the site of infection, it causes lymphangitis, proctitis or inflammation of the colonic mucosa associated with diarrhea and abdominal cramps. In women, cervicitis, perimetritis or salpingitis may occur. In both sexes, fever, decreased appetite and malaise may occur. The lymph nodes enlarge (then called buboes) and become painful, while their overlaying skin is thinning. Symptoms of phase three involve necrosis of the lymph nodes, resulting in abscesses and fibrosis as well as lymphatic obstruction and mostly permanent edema.

Acute *Chlamydia trachomatis* infections can relatively easy be treated with lipophilic antibiotics that can pass through the membranes to reach the susceptible reticulate bodies (RBs). Common drugs used include macrolides (erythromycin and azithromycin), quinolones (ofloxacin and lefofloxacin) and cyclins (doxycycline or other tetracyclines), as well as amoxicillin for pregnant women.

1.1.3 *Chlamydophila pneumoniae*

Chlamydophila pneumoniae (which also used to be called *Chlamydia pneumoniae*) is emerging as an important cause of pneumonia and other pulmonary diseases. Airborne respiratory infections with *C. pneumoniae* occur in the majority of people – with increasing age, epidemiologic prevalence reaches up to 80% (Grayston *et al.*, 1990). In 90% of the cases, the infection is asymptomatic. If symptoms develop, they can range from a mild cold to more severe sequelae: It is estimated that *C. pneumoniae* causes 10% of community-acquired pneumonia and 5% of bronchitis and sinusitis cases (Kuo *et al.*, 1995). If unresolved, respiratory infections may contribute to chronic inflammatory lung diseases, such as asthma (Hahn *et al.*, 1991), chronic obstructive pulmonary disease (COPD) (Blasi *et al.*, 1993) and sarcoidosis (Kuo *et al.*, 1995). *C. pneumoniae*, which can also disseminate from the site of the initial infection (Moazed *et al.*, 1998) has been associated with chronic diseases such as atherosclerosis (Byrne and Kalayoglu, 1999;Kuo *et al.*, 1993;Kuo *et al.*, 1995;Saikku *et al.*, 1988), and potentially lung cancer (Laurila, A.L. *et al.*, 1997).

1.1.4 Pathogenesis of non-human chlamydial strains

Chlamydophila abortus strains are endemic among ruminants and efficiently colonize the placenta. *C. abortus* is primarily associated with cases of abortion and weak neonates. Sporadic zoonotic abortion due to *C. abortus* infection has been confirmed by genetic analysis of isolates from women who work with sheep (McKinlay *et al.*, 1985). ***Chlamydophila psittaci*** infects birds, where it is often systemic. Infections can be unapparent, severe, acute or chronic with intermittent shedding. Most organs become infected, e.g. the conjunctiva, the respiratory system and the gastrointestinal tract. The bacteria can also be passed in the eggs. *C. psittaci* is known to cause sporadic zoonotic disease in humans, other mammals and tortoises (Vanrompay *et al.*, 1995). ***Chlamydophila felis*** is endemic among house cats, causing inflammation of the conjunctiva, rhinitis and the respiratory system. It can be recovered from the stomach and reproductive tract. Only very few zoonotic infections of humans with *C. felis* have been reported (Browning, 2004). ***Chlamydophila caviae*** can be recovered from the conjunctiva of guinea-pigs suffering from ocular inflammation and eye discharge. *C. caviae* infects primarily the mucosal epithelium and is not invasive (Ahmad *et al.*, 1977). ***Chlamydophila pecorum*** has been isolated from cattle, sheep, goats, koalas and swine. It has been associated with reproductive diseases, infertility and urinary tract infections, as well as with abortion, conjunctivitis, encephalomyelitis, enteritis, pneumonia and polyarthritis (Kaltenboeck *et al.*, 1993).

Chlamydia suis has been isolated from swine, where it causes conjunctivitis, enteritis, pneumonia, and a high incidence of apparently asymptomatic infections. Antibody cross reactions with *C. trachomatis* due to nearly identical epitopes in the *ompA* gene product may lead to errors in serotyping (Everett and Ward, 2003). Two strains of ***Chlamydia muridarum***, MoPn and SFPD have been isolated from mice and hamsters. MoPn infection may be asymptomatic or produce pneumonia in mice. SFPD is an enteric isolate and was obtained concurrently with a causative agent of proliferative ileitis (Fox *et al.*, 1993;Zhang *et al.*, 1993).

1.1.5 Lifecycle (Developmental cycle)

The intracellular developmental cycle of chlamydiae involves alternation between two morphologically distinct forms: The infectious, spore-like, inert elementary body (EB) and the metabolically active multiplying reticulate body (RB). A scheme of the lifecycle is depicted in figure 1.2.

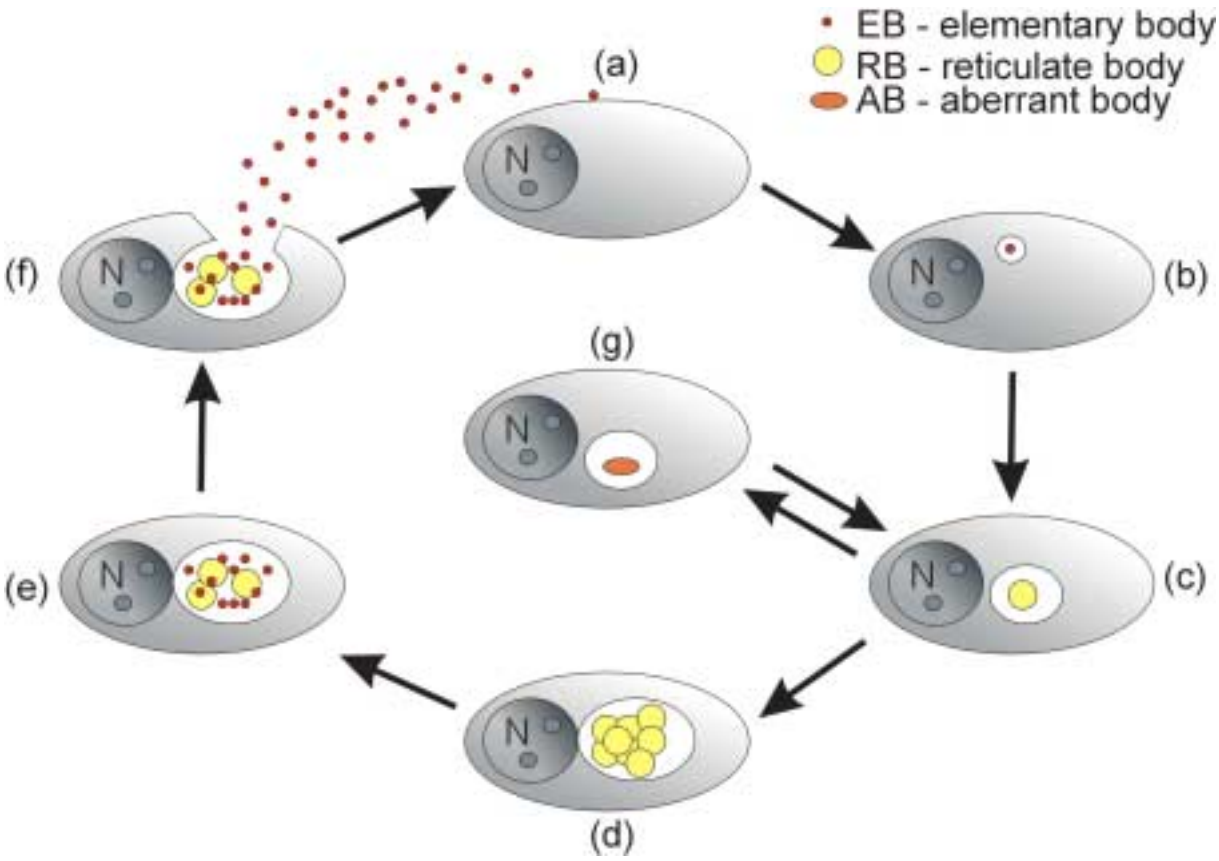


Figure 1.4 – Scheme of chlamydial life cycle shows intracellular development of chlamydiae

a) The chlamydial EB attaches to a host cell and enters. b) The EB gets transported to the perinuclear region. c) The EB differentiates into an RB. d) The RB multiplies. e) The RBs differentiate back to EBs. f) The cell is lysed, and new cells are infected. g) Under certain conditions, the bacteria go into a persistent state and form ABs. N = nucleus. For further details see text.

a) Attachment and entry.

The attachment of the negatively charged EB to the host cell surface is thought to be electrostatically mediated by the glucosaminoglycans heparin and heparan-sulfate which are present on the host's cell wall (Watt, 1980). More specific interactions with so far unidentified host-receptors are thought to occur (Dautry-Varsat, 2005). On the chlamydial side, several surface proteins such as Hsp70 or PmpD seem to be involved (Raulston 1993, Wehrl et al., 2004). After cell adhesion, the chlamydiae are either endocytosed or phagocytosed – both clathrin-dependent and –independent mechanisms are proposed (Dautry-Varsat, 2005). During uptake, the chlamydial protein Tarp (translocated actin-recruiting phosphoprotein) is injected into the host cell, most probably via a type-II secretion system leading to a remodelling of the actin cytoskeleton (Clifton et al., 2004). Also, important signal transduction pathways are modulated, such as small GTPases (Rho, Rac, Cdc42, Arf6), PI3-kinases or MEK/ERK (Dautry-Varsat, 2005).

The host-derived endosome is termed an "inclusion", and its membrane gets considerably modified. This way, the host cell does not recognize it as part of the degradative pathway. Fusion with lysosomal compartments is avoided which permits the microbial invader to escape destruction. The inclusion is devoid of known early or late endosomal markers such as transferrin/transferrin receptor or mannose-6-phosphate receptor, and it also lacks the lysosomal markers LAMP1, LAMP2, cathepsin D and the vacuolar H^+-ATPase (Cirillo *et al.*, 1998).

b) Migration to perinuclear area

Within two hours, bacterial gene expression is initiated and the inclusion actively migrates to the Microtubule Organizing Center (MTOC) (Clausen *et al.*, 1997). Close physical association with the Golgi apparatus and recycling endosomes seems to be important for creating a nutrient-rich environment for replication. The chlamydial inclusion intercepts the exocytic host cell membrane transport from the Golgi apparatus, thereby capturing mostly sphingolipids, phospholipids and cholesterol (Hackstadt et al., 1995, Carabeo et al., 2003) (Scidmore *et al.*, 1996). Other nutrients such as amino acids (Hatch *et al.*, 1975; Karayiannis and Hobson, 1981), Nucleotides (McClarty et al, 1993) or iron (Al Younes *et al*., 2001) need to be recruited from the host cell as well.

c) and d) EB to RB transition, inclusion development and bacterial replication

Between 6 and 10 hours after the infection, the EB (diameter 200-300nm) starts to transform into the RB (diameter >1000nm), the metabolically active form, which divides by binary fission. In parallel, the inclusion increases in size.

In addition to the re-routing of endosomal traffic, intracellular chlamydiae also disturb other cellular functions: N-cadherin dependent cell-cell junctions are disrupted and apoptosis is inhibited (Greene *et al.*, 2004;Rajalingam *et al.*, 2001). The MHC Class I and Class II response is also disturbed through the secretion of a protease which cleaves transcription factors of MHC antigens (Heuer *et al.*, 2003;Zhong *et al.*, 2001). Export of the mostly unknown factors is facilitated by a Type-III-secretion system (Kalman *et al.*, 1999).

e) and f) RB to EB transition and cell lysis

After 8 to 12 rounds of multiplication, the RBs start to asynchronously transform back into infectious EBs. The host cells that are now completely filled with bacteria are disrupted either by lysis (Abdelrahman and Belland, 2005) or via a novel form of exocytosis (Beatty, 2007) and the released EBs infect neighboring cells (Moulder, 1991). Under optimal growth conditions in cell culture, the cycle is completed within 44 to 72 h, depending on the chlamydial strain and on cell culture conditions. *In vivo*, where nutrients are scarce, this process can take considerably longer (Harper et al, 2000).

g) Development of persistent forms

As an interruption of the classic lifecycle, persistent infections can occur, which are induced by external stimuli. During persistence, RBs do not redifferentiate into EBs, but develop into so called ABs (aberrant bodies) with significantly modified morphology (de la Maza *et al.*, 1987). The bacteria stay metabolically active but are unable to establish a productive infection. By this means, chlamydiae are inaccessible to the host's immune system and can survive for a long period of time. If the stimulus is removed, the bacteria are reactivated and continue to undergo the regular, acute developmental cycle.

1.1.6 Persistence

As mentioned above, the chlamydial biphasic infection may deviate *in vivo* and *in vitro* towards persistence, in which chlamydiae fail to complete their development from RBs into EBs, and remain non-culturable. The persistent chlamydial forms, the ABs, are morphologically aberrant (Shatkin *et al.*, 1985), form small inclusions, do not lyse the host-cells, but retain metabolic activity. Their metabolism and gene expression differ considerably from RBs (Molestina, 2002; Belland 2003). Protein synthesis in general is decreasing and less Mump (major outer membrane protein), OmcB and LPS (lipopolysaccharide) are produced. On the other hand, the amount of Hsp60, one of the most important chlamydial antigens, stays constant or is even increasing.

In general, chlamydial persistence can be defined as a state in which the bacteria are **viable** but **non-infective** and **non-proliferating**. Persistent bacteria can be **reactivated** to return to their normal infectious lifecycle, when the persistence stimulus is removed.

1.1.6.1 Chlamydial persistence *in vitro*

In vitro, a persistent infection can be induced by the addition of antibiotics, by the simulation of inflammatory conditions through addition of IFN-γ or by deprivation of essential nutrients, such as iron or amino acids.

Antibiotics which lead to persistence are penicillin, ofloxacin and ciprofloxacin (Dreses-Werringloer *et al.*, 2000;Johnson and Hobson, 1977;Segreti *et al.*, 1992). Penicillin-treated bacteria which consist of enlarged ABs, the so-called "penicillin forms", resume normal growth after penicillin removal. This phenomenon could explain the often observed antibiotic treatment failures of chlamydial infections (Mpiga and Ravaoarinoro, 2006).

In human cells, persistence through the addition of the cytokine **IFN-γ** (Byrne *et al.*, 1986;Summersgill *et al.*, 1995) is caused by the induction of indoleamin 2,3-dioxigenase (**IDO**) which cleaves L-tryptophane, thereby reducing the intracellular concentration of the free amino acid. IFN-γ also upregulates the **tryptophane-tRNA synthetase**, thereby further depleting the amino-acid pool (Cheshire and Baldwin, Jr., 1997;Flohr *et al.*, 1992;Takikawa *et al.*, 1988). An excess of tryptophane in the growth medium reverses the effects and allows the bacteria to resume the acute lifecycle. Genital serovars of *C. trachomatis* possess a tryptophane-synthetase which can convert indole – produced by other genital bacteria – into

tryptophane. This way, these strains circumvent the IFNγ-induced persistence (Caldwell *et al.*, 2003;Shaw *et al.*, 2000a).

Iron-depletion by addition of the chelating compound deferoxamine mesylate (DAM) also leads to persistence, mostly because a number of chlamydial enzymes require iron for their activities. One of them, rib nucleotide reductase (Iliffe-Lee and McClarty, 1999;Roshick *et al.*, 2000), can be partially or totally inhibited, affecting DNA synthesis. The iron-depletion effect can be reversed by the addition of iron-loaded transferrin (Al Younes, 2001;Hogan *et al.*, 2004;Kutlin *et al.*, 2001;Wolf *et al.*, 2000). *In vivo*, the induction of iron deficiency via an increased expression of iron-binding proteins such as transferrin, ferritin or lactoferrin is an efficient strategy against intracellular pathogens (Schaible and Kaufmann, 2004)

Limiting availability of certain **nutrients** (amino acids or glucose) also leads to growth arrest of *C. trachomatis* and *C. psittaci* and to the development of persistent particles (Coles *et al.*, 1993;Harper *et al.*, 2000;Hatch, 1975;Morgan, 1955). As chlamydiae are intracellular, the nutrient concentrations within the cell are crucial for their development. For example, if amino acid concentrations are reduced by only 25%, persistent chlamydial forms are induced.

1.1.6.2 Chlamydial persistence *in vivo*

In vivo, chlamydiae can become persistent as a reaction to the **immune system** or after **antibiotic treatment**. This chronic or persistent state is much more difficult to eradicate and necessitates long-term treatment which quite often does not totally clear the infection.

The **immune system** fights chlamydial infections via an intense cell mediated immunity (CMI). This CMI is important in protecting against mucosal infections, and is mediated predominantly by $CD4^+$ type 1 lymphocytes and IFN-γ (Beatty *et al.*, 1994;Caldwell *et al.*, 2003;Kim and DeMars, 2001). A reactivation of infections that have become persistent following the CMI is thought to be possible and has been described as a result of immune suppression (Malinverni *et al.*, 1995;Yang *et al.*, 1983).

The induction of persistence can also occur due to **antibiotic treatment** of chlamydiae and has been described *in vivo*. The persistent forms are shown to be refractory even to prolonged courses of treatment with antibiotics (Hooton *et al.*, 1990;Roblin and Hammerschlag, 1998;van der Willigen *et al.*, 1988;Xu *et al.*, 2000).

A growing body of evidence suggests a significance of chlamydial persistence in the pathogenesis of certain human diseases. Persistent infections have been implicated in many chronic diseases like trachoma, infertility (*C. trachomatis*) and chronic pulmonary infections (*C. pneumoniae*). Persistent chlamydial infections have also been associated with diseases previously thought to be of non-infectious origin, including pelvic inflammatory disease, arthritis, asthma, and atherosclerosis (Dean *et al.*, 2000;Gieffers *et al.*, 2001;Hammerschlag *et al.*, 1992;Hogan *et al.*, 2004;Mahony and Coombes, 2001;Nanagara *et al.*, 1995;Schachter, 1999;Stamm, 2001).

1.1.6.3 Antigenic properties of persistent chlamydiae

Important genes in chlamydial pathogenicity and persistence are *groEL*, *omp1*, and *omcB*. These genes belong to three different temporal classes of chlamydial genes (Shaw *et al.*, 2000b): ***groEL***, encoding the heat shock protein Hsp60, begins to be expressed 2h after infection, and is thought to be a key immunopathologic modulator (Morrison *et al.*, 1989b;Morrison *et al.*, 1989a). ***omp1***, encoding the outer membrane protein Momp, is a mid-cycle gene expressed between 6 and 12h p.i., playing a role in protective humoral immunity (Su *et al.*, 1990;Zhang *et al.*, 1987). ***omcB***, encoding the 60 kDa cysteine-rich outer membrane protein, is a late gene, whose expression can be detected by 20h after infection. It is therefore thought to be involved in the final stage of the developmental cycle (Hatch *et al.*, 1986). The gene encodes a protein thought to form a supramolecular lattice in the periplasm, suggesting a role in the stability of developing bacteria.

Gene and protein expression profiles of persistent chlamydiae differ from those of acute infections: It has been shown *in vitro* that upon induction of persistence the amount of bacterial proteins decreases, especially Momp, Omc-B, whereas the amount of the chlamydial heat-shock protein Hsp60, one of the most important chlamydial antigens, stays constant or is increased (Beatty *et al.*, 1993;Mehta *et al.*, 1998).

The correlation to diseases has also been elucidated on a molecular level. An excess production of the immunopathological Hsp60 compared with a decrease in expression of the immuno-protective Momp or other chlamydial proteins could contribute to hypersensitivity or autoimmunity (Gerard *et al.*, 1998;Morrison *et al.*, 1989a). The reason for the pathogenic and tissue-destroying infections could therefore be the immunological host reaction, namely the

continuous secretion of inflammatory cytokines causing chlamydiae to go into persistence (Knight *et al.*, 1995).

1.1.7 Genetics

The genomes of various chlamydial strains (*C. trachomatis, C. pneumoniae*, *C. muridarum* and *C. psittaci*) have recently been sequenced (Read *et al.*, 2000;Read *et al.*, 2003;Stephens *et al.*, 1998). Both strains contain a most likely functional but incomplete glycolytic pathway with a linked tricarboxylic acid cycle. They also possess type-III secretion genes as well as serine/threonine protein kinases. However, the *C. trachomatis* genome contains around 1 megabase with a coding potential of 894 proteins of which approximately 28% have no orthologs in the available protein sequence databases. Genome comparison between the different strains revealed that *C. trachomatis* contains about 70 genes without homologues in *C. pneumoniae* and that *C. pneumoniae* has about 200 genes not present in *C. trachomatis* (Kalman *et al.*, 1999). The most obvious difference is the lack of a tryptophan biosynthesis operon (*trpA*, *trpB*, *trpC* and *trpR*) in *C. pneumoniae*.

This amount of genomic information and the intra-species differences provide considerable possibilities to determine gene functions related to the pathogenic lifestyle. Unfortunately, because chlamydiae are currently not amenable to genetic manipulation, experiments prove to be rather difficult.

1.2 Amino acids

Amino acids are classified by the properties of the side chains attached to their α-carbon atom: Hydrophobic, hydrophilic and amino acids that have both properties (figure 1.3)

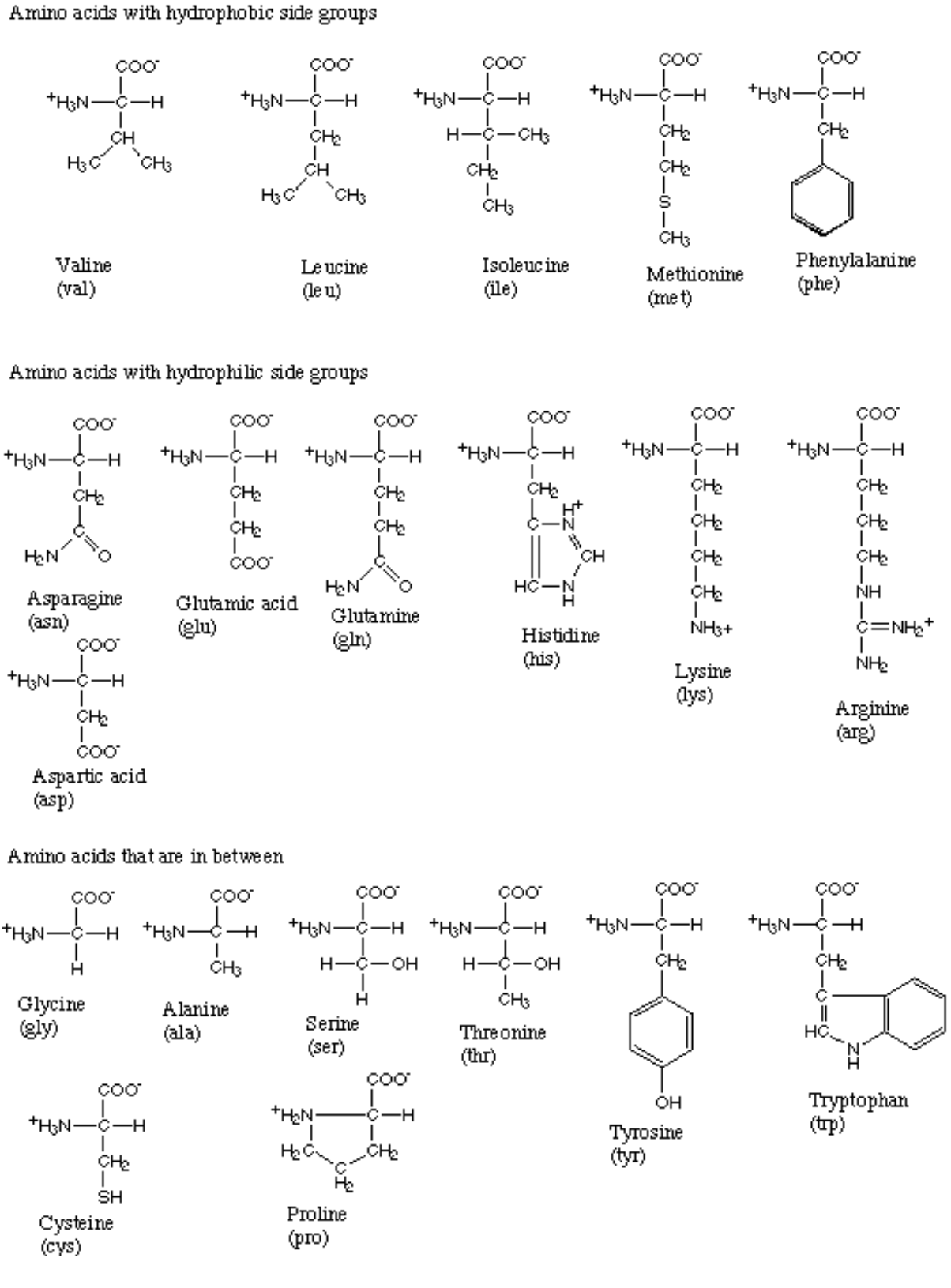


Figure 1.3 –The proteinogenic amino acids differ in the structure of their side groups

Source: Massachusetts Institute of Technology Biology Hypertextbook, http://web.mit.edu/esgbio/www/7001main.html

1.2.1 Amino acid metabolism

Proteins ingested in the diet are cleaved into amino acids or small peptides that can be absorbed by the intestine and transported in the blood. Amino acids are then used for protein biosynthesis in the cells of the body. Normal blood concentrations of the 20 proteinogenic amino acids are listed in Table 1.1. Other sources of amino acids are the targeted degradation of unneeded cellular proteins via **ubiquitination** or the unspecific degradation via **autophagy**. Amino acid concentrations in different tissues vary considerably and are subject to changes depending on the physiological state of the organism (Brosnan, 2003).

Concentrations [mM] of L-amino acids.					
Alanine (Ala, A)	0,333	Arginine (Arg, R)	0,080	Asparagine (Asn, N)	0,041
Aspartic acid (Asp, D)	0,003	Cysteine (Cys, C)	0,052	Glutamic Acid (Glu, E)	0,024
Glutamine (Gln, Q)*	0,586	Glycine (Gly, G)	0,230	**Histidine (His, H)***	0,082
Isoleucine (Ile, I)*	0,062	**Leucine (Leu, L)***	0,123	**Lysine (Lys, K)**	0,188
Methionine (Met, M)	0,025	**Phenylalanine (Phe, F)***	0,057	Proline (Pro, P)	0,168
Serine (Ser, S)	0,114	**Threonine (Thr, T)**	0,140	**Tryptophan (Trp, W)**	0,070
Tyrosine (Tyr, Y)	0,059	**Valine (Val, V)***	0,233		

Table 1.1 – Blood plasma concentrations of proteinogenic amino acids range from 3 to 586 µM

Adopted from (Cynober, 2003). Essential amino acids in bold. Arg is essential only for children. Amino acids essential for chlamydiae (see section 1.2.2)

In case of nutritional deficiencies, humans can synthesize the 11 nonessential amino acids from metabolic intermediates of the TCA cycle (**glutamate, glutamine**, **asparagine**, **proline** and **arginine** from α-ketoglutarate, **aspartate** from oxaloacetate**)**, of the glycolysis (**alanine** from pyruvate, **serine**, **glycine**, **cysteine** from 3-phosphoglycerate) or from essential amino acids which must be supplied in the diet (**tyrosine** is synthesized from phenylalanine).

Oversupply of amino acids is quite common and shows no negative effects. Especially the branched chain amino acids (BCAA) **Leu**, **Ile** and **Val** are used as nutritional supplements by athletes to increase muscle size and improve performance. Long-term administration of those amino acids for up to 90 days has positive effects, mainly on muscle integrity and hematopoiesis (Ohtani *et al.*, 2006). In case of oversupply, amino acids not used for protein genesis are catabolized to metabolic fuel, as the body does not have a store of free amino acids. In the liver or in the intestine, which can immediately provide its nitrogenous products to the liver via the hepatic portal vein, the α-amino group is first removed by transaminases and then converted by multiple steps in the urea cycle from ammonia into urea which is secreted via the urine. The remaining branched-chain α-ketoacids are then subjected to oxidative decarboxylation by the α-ketoacid dehydrogenase complex (BCKDC, Harris, Joshi,

Jeoung, 2004), converted into intermediates of the TCA cycle and then into glucose or degraded to CO_2 to deliver energy. The keto acid derived from leucine (α-ketoisocaproate) inhibits BDK (BCKDC kinase) thereby dephosphorylating and thus activating the BCKDC. A defect in BCKDC causes maple syrup urine disease – an excess buildup of **Leu**, **Ile** and **Val**, which can lead to brain damage and progressive nervous system degeneration.
In other cells that use amino acids for the generation of fuel, the nitrogen is transferred onto pyruvate to form alanine or onto glutamate to form glutamine which is released in the blood and transported to the liver. This way, amino acid catabolism occurs in a manner that does not elevate blood ammonia.

Uptake and release of amino acids from the blood into the cell and vice versa is mediated by a number of classes of transporters that differ in specificity, mechanism, regulation and localization (Hyde, 2003). The intracellular concentration of amino acids is generally higher than outside of the cell which is the result of indirect active transport. The driving force is the gradient of Na^+, K^+ and H^+. Also, some exchangers use the gradient of one type of amino acids for the transport of others.
In general, the transport systems are highly redundant. No amino acid has only one transporter and the majority of them are specific for various amino acids, which are most of the time structurally related. For instance, systems L and $B^{0,+}$ transport mainly hydrophilic amino acids such as **Leu**, **Ile**, **Met**, **Val** and **Phe**.

1.2.2 Amino acids and chlamydiae

Because of the obligatory intracellular lifestyle of Chlamydiae and its inability to synthesize a number of metabolic substrates, which are required for growth and multiplication, this pathogen appears to acquire a number of substances from host pools (Grieshaber *et al.*, 2002;Hatch, 1975;Kalman *et al.*, 1999;McClarty, 1994;Tipples and McClarty, 1993). As already mentioned, the chlamydial inclusion intercepts the exocytic host cell membrane transport from the Golgi apparatus, thereby capturing mostly sphingomyelin and cholesterol (Scidmore *et al.*, 1996). In addition, metabolites needed due to the incomplete TCA-cycle as well as lipids and NTPs are acquired via transporters in the modified inclusion membrane (Hackstadt *et al.*, 1995;Kalman *et al.*, 1999;Taraska *et al.*, 1996). *C. trachomatis* and *C. pneumoniae* encode the branched chain amino acid transporter *brnQ*, as well as single amino acid transporters (e.g. *glnPQ*, *braB* and *aaaT* as well as genes for the di- and oligopeptide ATP binding cassette (ABC) transporters (*dpp* and *opp* operons). Also, the

Arginin/Ornithin-Antiporter *arcD*, the Tyrosin/Tryptophan-transporter *tyrP_1* and *tyrP_2* can be found. Homologies to *alsT* (Na^+/Alanine Symporter) and *gltP* (Na^+/H^+-Dicarboxylat-symporter) are found in the sequenced chlamydial genomes

Since chlamydiae are dependent on the host's amino acid pool, perturbations in the levels of these precursors inside host cells potentially affect chlamydial growth. Indeed, depletion of single amino acids or restriction of the amino acid content in the host-cell growth medium causes noticeable perturbation of chlamydial growth (Allan et al., 1985;Allan and Pearce, 1983a;Coles et al., 1993;Harper et al., 2000;Karayiannis and Hobson, 1981;Kuo and Grayston, 1990). Essential for most chlamydial strains are the branched chain amino acids **Leu**, **Ile** and **Val** as well as **His**, **Gln** and **Phe** (Allan and Pearce, 1983b)

Only few other genes involved in amino acid biosynthesis are found in the chlamydial genome (Stephens et al, 1998). Although aminotransferases (AspC and TyrB) and a serin-hydroxymethyltransferase (GlyA) responsible for the last step of **Asp**, **Phe**, **Tyr** and **Gly** synthesis are present, the preceding steps are not. It is therefore likely that chlamydiae are dependent on the host for all amino acids, either directly or indirectly through precursors.

2. Materials & Methods

2.1 Materials

2.1.1 Bacteria and cell lines

Chlamydia trachomatis L2	Lymphatic isolate (ATCC VR-902B)
Chlamydophila pneumoniae CWL029	Respiratory isolate (ATCC VR-1310)
HEp-2	Human epidermoid laryngeal epithelium (ATCC CCL23)

2.1.2 Cell media and solutions

RPMI 1640

Concentrations [mM] of L-amino acids					
Glycine (Gly)	0.13	Arginine (Arg)	1.15	Asparagine (Asn)	0.38
Aspartic acid (Asp)	0.15	Cystine (Cys)	0.08	Glutamic Acid (Glu)	0.14
Glutamine (Gln)	2.05	Histidine (His)	0.10	Hydroxyproline (H-Pro)	0.15
Isoleucine (Ile)	0.38	Leucine (Leu)	0.38	Lysine (Lys)	0.27
Methionine (Met)	0.10	Phenylalanine (Phe)	0.09	Proline (Pro)	0.17
Serine (Ser)	0.28	Threonine (Thr)	0.17	Tryptophan (Trp)	0.03
Tyrosine (Tyr)	0.11	Valine (Val)	0.17	Alanine (Ala)	none

Cell growth medium (CGM)
RPMI 1640
FCS (heat inactivated) 10%
Gentamycin (10µg/ml)

Infection medium (IM)
RPMI 1640
FCS (heat inactivated) 5%

Maintenance medium (MM)
IM
Cycloheximide (1µg/ml)
Gentamycin (10µg/ml)

2.1.3 Chemicals and consumables (alphabetically)

Acrylamide / Bisacrylamide	Roth
Agarose	Seakem, Biozym
Ammonium Acetate	Ambion
AOA	Sigma-Aldrich
APS	Roth
β-Mercaptoethanol	Roth
Bromophenol blue	Sigma
BSA	Biomol
Cell culture flasks (150 and 75cm^2)	TPP
Cell culture plates (6-, 12-, 96 well plates)	TPP
Cell strainer	BD Biosciences
Chloroform	Merck KGaA
Coverslips	Roth
Cycloheximide	Sigma-Aldrich
DNAse I	Qiagen
DTT	Sigma
EDTA	Roth
Test tubes (1,5ml)	Sarstedt
Ethanol	Merck KGaA
Ethidium bromide	Sigma-Aldrich
Centrifuge tubes (50ml)	Sarstedt
Centrifuge tubes (15ml)	Sarstedt
FCS	Biochrome
Gentamycine	Gibco
Glass beads (3mm)	Roth
Glycerol	Roth
Glycogen, 5µg/µl	Ambion
HCl	Gibco-BRL, Paisley, Scotland
Injection needle	Braun
Isopropanol	Merck KGaA
Ketavet	Pharmacia & Upjohn, Ch.-B.: 13731
Methanol	Merck KGaA
Microscope slides	Roth
Mitotracker orange	Molecular Probes
Mowiol	Sigma-Aldrich
NP-40	Fluka
PBS (sterile)	Gibco
Percoll (30% v/v)	Pharmacia
Pfa	Fluka
Phenol-chloroform-isoamyl alcohol	Roth
Polybed	Polysciences
PVDF transfer membranes	Perkin Elmer
Random primer (nonamers)	GE Healthcare
Rompun	Bayer, PZN-1320422
RPMI 1640	Gibco
Saccharose	Roth
SDS	Biomol
Sodium acetate, pH 5,2	Ambion

Syringe	Transject
TEMED	Roth
Tris-base	Applichem
Trypsin-EDTA	Gibco
Triton X-100	Calbiochem
Tween20 (0,5%)	Merck KGaA
Vancomycin	Gibco
Water (RNAse-free)	Gibco
all other chemicals and amino acids	Roth

2.1.4 Antibodies

Primary antibodies for immunofluorescence (IF) and immunoblotting (IB)

Target	Source	Species	dilution
Hsp60	Alexis, Grünberg, Germany, 804-072-R100 A5789	mouse	IB: 1:3000 IF: 1:500
MOMP	Department of Pathology, University of Washington, USA	mouse	IB: 1:3000
LPS	Milan Analytica AG	rabbit	IF: 1:60
Chlamydial LPS (DAKO IMAGEN)	Dako, Hamburg, Germany (now Oxoid)	FITC-labeled antibody (DIF)	IF: 1:1
Tubulin	Sigma T-9026 Lot 093K4880	mouse	IB: 1:5000

Secondary antibodies for immunofluorescence (IF) and immunoblotting (IB)

target species	Source	Species	dilution
rabbit, mouse	Amersham Biosciences, Freiburg, Germany	HRP coupled	1:3000 (WB)
Mouse	Jackson Immuno Research	IgG, CY3-coupled	IF: 1:100
Mouse	Jackson Immuno Research	IgG, CY2-coupled	IF: 1:100
Rabbit		IgG, green	

2.1.5 Buffers and solutions (alphabetically)

Blocking buffer (Western blot)
TBS-T
3 % dry milk

Mowiol
2,4 g Mowiol 4-88
6 g Glycerin
6 ml H_20
well overnight
12 ml 0,2 M Tris pH 8,5
stir and heat to 60°C, centrifuge at 4000 min^{-1}, aliquot

PBS
0,2 g KCl
0,2 g KH_2PO_4
8 g NaCl
2,16 g Na_2HPO_4
ad 1 l H_20

Permeabilization medium
BSA (0,2 %)
Triton X-100 (0,2 %) in PBS

PFA (4 %)
8 g PFA
20 ml 10x PBS
8 g Saccharose
ad 200 ml H_20; pH 7,4

RIPA-buffer
20 mM Tris/HCl; pH 7,5
150 mM NaCl
0,5 % NP-40
0,5 % Triton X-100
0,5 mM EDTA
1 mM DTT

SDS-electrophoresis buffer (10x)
1,92 M Glycine
250 mM Tris/HCl
1 % SDS

SDS loading buffer (4x, reducing)
125 mM Tris/HCl pH 6,8
6 % SDS
40 % Glycerol
10 % β-Mercaptoethanol
2-10 mg Bromophenolblue

SDS loading gel buffer (4x)
6,05 g Tris/HCl
0,4 g SDS
ad 100 ml H_20
adjust to pH 6,8 with HCl

SDS loading gel (4%)
1,4 ml loading gel buffer
1,75 ml 30% Acrylamide / 2% Bisacrylamide
1,2 ml H_2O
50 µl 10% APS
7 µl TEMED

SDS separating gel buffer (4x)
91 g Tris/HCl
2,0 g SDS
ad 500 ml H_2O
adjust pH to 8,8 (with HCl)

SDS separating gel (10%)
2,6 ml separating gel buffer
3,4 ml 30% acrylamide / 2% bisacrylamide
3,9 ml H_2O
50 µl 10% APS
5,5 µl TEMED

SPG buffer
75 g Sucrose
0.52 g KH_2PO_4
1,22 g Na_2HPO_4
0,72 g L-glutamic acid
fill up to 1 l with aqua dest.
adjust pH to 7.4
steril filter and store at 4° C

Stripping buffer
62,5 mM Tris/ HCl pH 6,7
100 mM β-Mercaptoethanol
2 % SDS

TBE buffer
89,15 mM Tris/HCl
88,95 mM Boric acid
2 mM EDTA

TBS-buffer
20 mM Tris/HCl
140 mM NaCl
pH 7,5
for TBS-T, add 0,05 % Tween 20

Wet blot transfer buffer
6 g Tris
28,8 g Glycine
2 g SDS
20 % Methanol
ad 2 l with H_20

2.1.6 Kits

Immunodetection kit	Enhanced chemiluminescent Western Lightning™ Chemiluminescent Reagent Plus, Perkin Elmer
LDH	Roche
OneStep RT-PCR kit	Qiagen
Omniscript Transcriptase	Qiagen
QuantiTect® SYBER® Green	Applied Biosystems
TRIzol Reagent	Invitrogen
WST-1	Colorimetric assay, Roche

2.1.7 Primers

All primers were purchased from Biotez, Berlin.

RT-PCR primers

16S rRNA (forward) 5'-tcg caa tgg acg gaa gtc tg-3'
16S rRNA (reverse) 5'-acg cat ttc acc gct aca cg-3'

groEL (*hsp60*) (forward) 5'-tcg cgt tgg agc tgc aac ag-3'
groEL (reverse) 5'-tgc gcc agg cat tgc tgg ag-3'

omp1 (*momp*) (forward) 5'-gtg ggg aat cct gct gaa cc-3'
omp1 (reverse) 5'-tag gct tgg cac cca ttt gg-3'

omcB (forward) 5'-agt gat cca gcg aca act cc-3'
omcB (reverse) 5'-aca agc gca tac tgt tgc ag-3'

gapdh (forward) 5'-atc atc cct gcc tct act gg-3'
gapdh (reverse) 5'-tgg gtg tcg ctg ttg aag tc-3'.

real-time PCR primers

16S rRNA (forward) 5'-tcg caa tgg acg gaa gtc tg-3'
16S rRNA (reverse) 5'-acg cat ttc acc gct aca cg-3'

gapdh (forward) 5'-ggt atc gtg gaa gga ctc atg ac-3'
gapdh (reverse) 5'-atg cca gtg agc ttc ccg ttc ag-3'

2.1.8 Equipment

Bioanalyzer	Agilent 2100
Chemiluminescence reader	LAS3000 Fuji Image Reader
Cooling centrifuge	Sorvall Du Pont
Digital camera	Morada, SIS
Electrophoresis equipment	BioRad
ELISA-Photometer	Molecular Devices, USA
Epifluorescence microscope	Nikon
Incubators	Heraeus und Thermo Life Science
Laser scanning microscope (confocal)	Leica (Kr-Ar Laser), TSC-5
Roller	Assistant RM5
Sequence detection system	ABI PRISM 7000, Applied Biosystems
Shaker	IKA MS1
Sonicator	Bandelin Sonorex
Table top centrifuge	Eppendorf
Thermocycler	Perkin Elmer
TEM	Leo 906E
UV-workbench	MWG-Biotech
Wet blot system	BioRad

2.1.9 Software

Adobe Photoshop
CorelDraw
Image Reader LAS3000
Microsoft Office
Mozilla
Reference Manager 11
SDS7000
SigmaPlot 2002
Visicapture
Windows XP

2.2 Methods

2.2.1 Cell culture

Chlamydiae can only propagate in living cells. In this work, the immortalized cell line HEp-2 was used as a host, grown aseptically in CGM at 37°C in 5 % CO_2, either in 75 cm^2 culture flasks, six-, twelve-, or 96- well plates. The cells were tested monthly for mycoplasma contamination by PCR.

Passaging of cells
Before passaging, the cells were washed with warm sterile PBS. They were then incubated with 1ml (per 75 cm^2) Trypsin/EDTA at 37°C for about 10min, until they detached. The trypsin was inactivated by adding CGM, and the cells were divided into several culture flasks depending on the desired dilution. About 3 days after a dilution of 1:4, the cells had reached confluency and had again to be passaged.

2.2.2 Chlamydiae

***C. trachomatis*-infection of cells**
HEp-2 cells (about 80 % confluency) were infected with an MOI as indicated (in this work, an MOI of 0,5 represented a stock dilution of 1:1000). After removing the CGM, the cells were incubated with 500 µl (per six-well) of diluted chlamydial stock in IM for 2h at 35°C in 5% CO_2. The inoculum was then replaced with new IM.

***C. pneumoniae*-infection of cells**
HEp-2 cells (about 80% confluency) were infected with an MOI as indicated (in this work, an MOI of 0,5 represented a stock dilution of 1:3000). After removing the CGM, the cells were incubated with 1ml (per six-well) of diluted chlamydial stock in IM. Cells were centrifuged at 920 g for 1h at 35° and then incubated for another hour. The medium was removed and replaced with new IM.

Preparation of chlamydial stocks
For the preparation of *C. trachomatis / C. pneumoniae* stocks, the bacteria were propagated in HEp-2 cells grown in 150cm^2 / 75cm^2 cell culture flasks with 24ml / 12ml MM. 48h / 72h after infection, the cells were detached using 3mm glass beads, combined in 50ml falcon tubes and ruptured by vortexing for 4 minutes with glass beads. The lysates were centrifuged at 1000g for 10min at 4°C to sediment nuclei and cell debris. The supernatants were removed and centrifuged again at 48.000g for 60min at 4°C to pellet the chlamydiae. The harvested bacteria were resuspended in 15ml SPG buffer using a 21-22 gauge injection needle. 50µl aliquots were prepared, stored at 4°C for 30min, frozen at -75°C and freshly thawed for each experiment.

Assessment of infectivity titer
To determine chlamydial infectivity in *C. trachomatis* (*C. pneumoniae*) infected cells, the cells were harvested at 48h (72h) with glassbeads from two 6-wells, transferred into 15ml falcon tubes, lysed by vortexing with 4ml glassbeads and serially diluted 1:10, 1:100, 1:1000, 1:10.000, 1:100.000, $1:10^6$ (1:5, 1:25, 1:125, 1:625, 1:3125, 1:15625) in IM.

To determine chlamydial infectivity of the stock, serial dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 (1:5, 1:25, 1:125, 1:625, 1:3125, 1:15625) of thawed EB-stocks were prepared in 100 µl (500 µl) of IM.

HEp-2 cell monolayers grown on coverslips in 24 well (12 well) plates were infected with the dilutions and grown in MM. 40h (48h) after infection, developed chlamydial inclusions were detected using immunofluorescent staining. For each dilution, inclusions in 10 microscopic fields at 400x magnification were enumerated using an epifluorescence microscope, and the average number was calculated as inclusion-forming units (I.F.U.) per milliliter.

Treatment of chlamydiae infected cells with amino acids
At the times indicated, medium was replaced with amino acids dissolved at 10mM in IM (or at concentrations indicated). The pH value of amino acid containing IM was adjusted to 7.28 (pH of pure RPMI 1640) by addition of 1,0 N HCl. During long term treatment, the media were replaced every other day.

2.2.3 Microscopy

Immunostaining
Infected cells grown on coverslips were washed once with cold PBS and incubated with Pfa for 20min, followed by 30min incubation with permeabilization medium. After washing with PBS, they were incubated for 1h with 20µl of primary antibody diluted in 0.2% BSA in PBS for one hour. Three times five minutes washing with PBS were followed by incubation with 60µl of secondary antibody diluted in 0.2% BSA in PBS for 1 hour, followed by three times five minutes washing with PBS and rinsing with deionized water. The coverslips were then dried and mounted on microscope slides using mowiol.

Direct immunofluorescent microscopy
Infected cells grown on coverslips were washed once with cold PBS and fixed / permeabilized with ice-cold methanol for 10 min. 10µl of DAKO Imagen kit antibody were loaded onto the coverslip and placed for 20 min in the incubator at 37°C. After washing with PBS, the coverslips were dried and mounted on microscope slides using mowiol. The DAKO Imagen kit contains polyclonal FITC-labeled rabbit antibodies against chlamydial LPS and *Evan´s Blue* for counterstaining the cytoplasm. For the confocal analysis, a Leica SP *Laser-scanning* microscope with 63×, 40× or 20×-objective was used. FITC was excited at 488 nm, *Evan´s Blue* at 543 nm.

Transmission electron microscopy
For fine structural analysis, cells were fixed with 2,5% glutaraldehyde, postfixed with 1 % osmiumtetroxide, contrasted with uranylacetate and tannic acid, dehydrated and embedded in

Polybed. After polymerization, specimens were cut at 60 nm and contrasted with lead citrate. Specimens were analyzed in a transmission electron microscope using a Morada digital camera.

Immuno-EM
For immunodetection, cells were fixed with 4% PFA and embedded in a mixture of 2 5% sucrose / 10 % PVA. Ultrathin sections were cut at –105°C, blocked, reacted with primary antibodies followed by secondary antibodies coupled to 6 or 12 nm gold particles. Specimens were analyzed in a Leo 906E transmission electron microscope.

Determination of cytotoxicity and host cell viability, proliferation
Metabolic activity and proliferation of host cells were determined using WST-1, a water-soluble tetrazolium salt (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzen-disulfonat). The rate of WST-1 cleavage by mitochondrial dehydrogenases into a formazan-dye (absorption maximum: 450 nm) correlates with the number of viable cells in the culture. HEp-2 cells were seeded in 96-well plates and continuously supplemented with IM plus individual amino acids. Control cells were incubated with IM only. Media were changed each second day for 15 days. The WST-1 test was carried out following the manufacturer's instructions.

Long-term cytotoxic effects of incubation with amino acids on host cells were evaluated by the **LDH test.** This colorimetric assay, used for the quantification of cell death and cell lysis, is based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant. Cells were seeded with medium containing the respective amino acids for 13 days. The medium was replaced every other day. On day 15, the LDH release into the medium was measured following the manufacturer's protocol. AOA, an aminotransferase inhibitor, was used as a negative control.

To further assess the viability of treated cells, the **Mitotracker assay**, using mitochondrial membrane potential-sensitive dye was performed. Cells were plated in culture flasks and provided with amino acids. Thirteen days post-treatment, cells were trypsinized, seeded onto coverslips in 12-well plates and incubated for another two days in the presence of excess amino acids. Cells were then stained with Mitotracker dye and examined.

2.2.4 Proteins

Gel electrophoresis and immunoblotting
Host cells treated as indicated were collected and lysed with RIPA-buffer (30 min on ice followed by 15 min centrifugation at 20.000 g to remove cell debris). Protein samples were subjected to SDS-PAGE and transferred onto PVDF Transfer Membranes using the Wet Blot System over night. To block non-specific binding, blots were blocked for 1h at room temperature on a roller. The blots were then incubated with the appropriate primary antibody in blocking buffer for 1h at room temperature on a roller. Blots were then washed in TBS-T for one hour on a roller, while the medium was replaced three times. After 1 h incubation with the secondary antibody coupled to peroxidase, the blots were washed as above, developed with an immunodetection kit and detected using a chemiluminescence reader.

2.2.5 Nucleic acids

RNA isolation
Total RNA was purified from cells using TRIzol Reagent. 1ml of Trizol-cell lysate from one six-well was sonicated for 5 minutes, extracted with 200µl chloroform and centrifuged at 12.000 g for 10min at 4°C. From the upper aqueous phase, the RNA was precipitated with 0,5 ml isopropanol, 120µl 5M ammonium acetate and 10µl glycogen at -20° for 15 min. After another round of centrifugation, the RNA pellet was washed with 75% ethanol, dried and DNAse treated for 1h at 37°C. To remove proteins, the RNA (resolubilized in 100 µl water) was extracted with 180ul phenol-chloroform-isoamyl alcohol, separated by centrifugation for 10 min at 4°C at 12.000 g and precipitated with 0.1 volume of 2M sodium acetate and two volumes of absolute ethanol by placing it at –20°C overnight or at –80°C for 30min. The pellet obtained by centrifugation for 15 min at room temperature at 12.000g was desalted by washing with 70% ethanol. After air drying, the RNA was solubilized in 20µl RNAse free water. The quality of purified RNA was determined using a bioanalyzer.

Each preparation of purified RNA was confirmed to be DNA-free by PCR targeting the host *gapdh* or the bacterial genes in the absence of reverse transcription. Positive control used in the assay was DNA prepared from harvested bacteria.

One-step reverse transcription
For cDNA preparation and its amplification, a **OneStep RT-PCR** kit was employed according to the manufacturer's instructions using 100 ng of total RNA per reaction. The thermocycler protocol was as follows:

30 min @ 50°
15 min @ 95°
30x 1 min @ 94°
1 min @ 55°
1 min @ 72°
10 min @ 72°

Reverse transcription
Simple reverse transcription was performed with **Omniscript Transcriptase** and random primers according to the manufacturer's protocol.

Agarose gel electrophoresis
Amplified cDNA was loaded on a 2% agarose gel (in TBE-buffer) and separated by applying a voltage of 100V. Bands were visualized by adding ethidium bromide to the agarose.

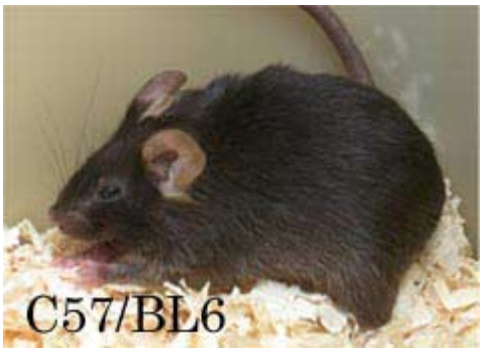
Real-time PCR
cDNA was analyzed using the SYBR Green kit on a sequence detection system with the appropriate primers. The following temperature profile was used:
15 min @ 95°
40x 20sec @ 95°
40sec @ 60°C

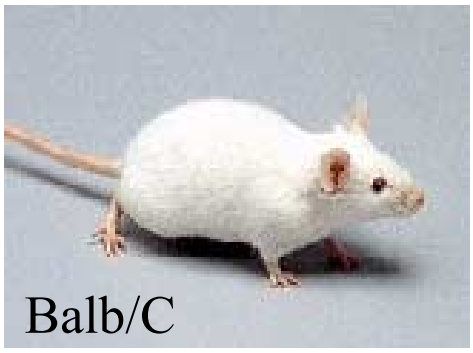
Data were analyzed using the SDS7000 software by calculating the ratio of 16S to GAPDH in each sample. The ratio of the sample "Leucine, 1d treatment" was arbitrarily set to one, and the other ratios were calculated accordingly.

2.2.6 *In vivo* experiments

The establishment of an animal model was based on publications that infected the mice either intranasally (Kaukoranta-Tolvanen *et al.*, 1993;Tormakangas *et al.*, 2004a;Yang *et al.*, 1995), intravenously (Yang *et al.*, 1995) or subcutaneously (Yang *et al.*, 1995).

The animal experiments were performed under the authorization number Reg0288/03 (Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit, Berlin). Six weeks old female C57/BL6-mice were used for the infectivity assays, following the literature (Tormakangas *et al.*, 2004b). For the amino acid susceptibility assays, Balb/C-mice were used.





Weight of animals as well as water consumption were recorded daily.

Anaesthetization
Animals were anaesthetized by subcutaneous administration of 50 µl Rompun, Ketavet, PBS (1:1:3).

***C. pneumoniae* infection:**
$1x10^7$ IFUs of *C. pneumoniae* (20 µl of stock containing $5x10^8$ IFU/ml) were placed under the nostrils of each anaesthetized animal until completely inhaled.

Preparation of amino acid supplemented drinking water
The maximum amount of amino acids was dissolved in drinking water. The concentrations (mmol/l) obtained were **Leu**: 166, **Ile**: 269, **Met**: 226, **Phe**: 179, **Ser**: 476.

Sacrifice and dissection
Animals were anaesthetized and decapitated. Blood was collected directly from the aorta in an eppendorf tube. Lungs were collected through the opened thorax.

Lung infectivity
The lungs were squeezed through a cell strainer and homogenized by vortexing in 1ml SPG with glass beads in a 50ml falcon tube. Homogenized lungs were centrifuged for 5 minutes at 500g. Supernatant was sevenfold serially diluted 1:10 in IM, and 24well plates with HEp-2 cells grown on coverslips were infected with 250µl of dilutions. After centrifugation and incubation, lung debris was washed away with MM containing vancomycin (20µl/ml, to avoid contamination with gram positive bacteria) and cells were incubated with MM for another 2 days, until coverslips were immunostained and inclusions were counted.

Blood serum amino acid concentration
The determination of amino acid concentrations was performed by Jeanette Klein (Neugeborenenscreening, Charité, Berlin, Germany). Collected blood was placed at 4° C overnight and spun at 20.000 g for 20 minutes. Serum was removed and amino acids extracted with TCA. 200 µl of the extract were mixed with 20µl internal standard (nor-Leu, 2.2µmol/ml) and 200 µl sample dilution buffer. After filtration (VectaSpin Micro Centrifuge Filter, Laborservice Onken) 100µl were injected into the HPLC. Quantification was achieved by one point calibration using a standard mixture of amino acids obtained from Pickering Laboratories (Mountain View, California, USA). Amino acids were determined by ion-exchange chromatography using the Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, UK or. Laborservice Onken, Gründau/Breitenborn, Germany) with lithium hydroxide / lithium acetate chemistry and post-column ninhydrine reaction. All buffers were obtained as solutions ready to use from Laborservice Onken. Analytes were detected photometrically at 570 nm (primary amino acids) and 440 nm (proline and hydroxyproline). The standard program for physiological amino acids was applied except for Val, Leu, and Ile. They were determined by a short program originally designed for therapeutic monitoring of branched chain amino acids.

2.2.7 Statistics

To determine statistically significant differences, the Student's t test was performed. The difference was considered significant when P was <0,05. For *in vivo* experiments, the logarithm of the measured values was taken to ensure a Gaussian distribution. Outliers were removed according to the standard 4σ-Method.

3. Results

3.1 Overview of amino acid influence on chlamydiae

3.1.1 Introduction

Previous studies in our institute that investigated the correlation between chlamydiae and autophagy found that supplementation with certain amino acids had an inhibitory effect on chlamydiae (Al Younes *et al.*, 2004). However, not all amino acids were tested, and only the strain *C. trachomatis* was used. Therefore, this part of the thesis tries to determine the effect of elevated concentrations of all proteinogenic amino acids on both *C. trachomatis* and *C. pneumoniae*.

All L-amino acids present in the growth medium (CGM) were included in this study, at a concentration of 10mM (~1000x serum level), except for **Cys** (not used due to its high toxicity), **Lys** and **Trp** (used at 0.5 and 1 mM, because of their toxicity at higher concentrations). 30 mM **Asn** was also used as previously recommended (Hoyvik *et al.*, 1991;Schaible *et al.*, 1999). The amino acids were grouped into essential and non-essential (for humans) for easier overview of the results.

Incubation with free amino acids for 72h at the concentrations indicated neither destroyed the monolayers nor affected the viability and metabolic activity of host cells, as determined by the WST-1 assay (data not shown). The developmental cycle of *C. trachomatis* L2 / *C. pneumoniae* CWL029 is completed within 44-48 h / 72-96 h. Thus, in the **first** group of experiments, by introducing the amino acids in the **middle of the infection cycle**, i.e. at 19 h / 30 h p.i., the influence on exponentially growing RBs (Grieshaber *et al.*, 2002;Nicholson *et al.*, 2003;Wolf *et al.*, 2000) was determined. In the **second** group of experiments, amino acids were administered at the **beginning of the infection cycle** (2 h p.i.) to study the effect of amino acids on chlamydial EBs.

3.1.2 Influence of essential amino acids on chlamydiae

Most single **essential amino acids** added at the **middle of the infection** had no or only negligible effects on the size of *C. trachomatis* and *C. pneumoniae* inclusions (figure 3.1.1 A, left). Only **Leu, Ile and Phe** delayed their growth. **Met** was less effective in arresting the growth of *C. pneumoniae* inclusions, compared to its considerable effect on

C. trachomatis inclusions. These findings were in accordance with the amino acids' effect on progeny infectivity (figure 3.1.1 A, right), except for **His, Lys and Thr** which showed moderate inhibitory effects.

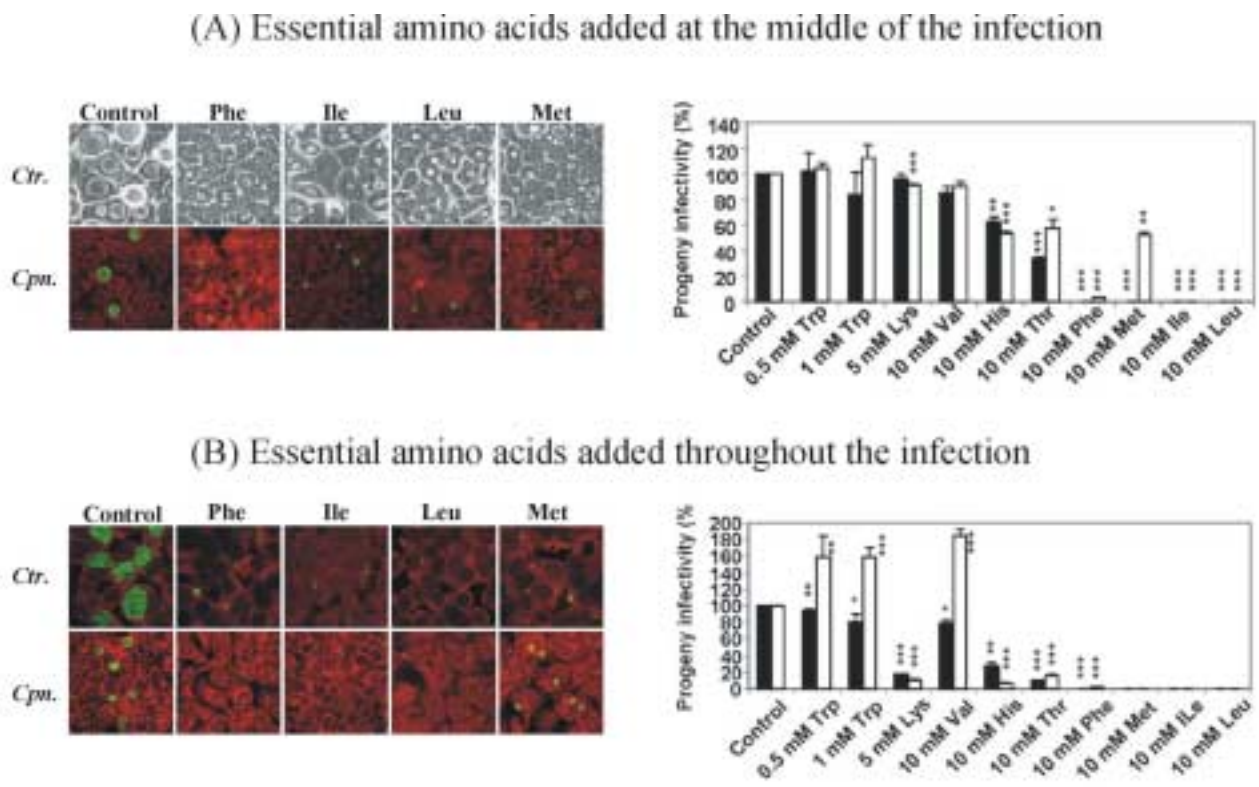


Figure 3.1.1 - Some essential amino acids inhibit inclusion growth and infectivity

Cells were infected at an MOI of 0.5 with either *C. trachomatis* or *C. pneumoniae* and single essential amino acids were added either at the middle of infection (A) or 2h p.i. (B) until the end of the infection period. **Left**: Cells were stained using the DAKO IMAGEN kit and analyzed by confocal microscopy. Micrographs show 80x80µm, Ctr, *C. trachomatis*; Cpn, *C. pneumoniae*. Other amino acids had no effect on inclusion size. **Right**: Relative infectivity titers were determined.
Closed bars: *C. trachomatis*; open bars: *C. pneumoniae*.
Assays for each treatment represent at least two independent experiments. Data presented are means+SDs. The 100% infectivities for the *C. trachomatis* controls represent $1.3x10^8$ (in A) and $2x10^8$ (in B) recoverable i.f.u./ml. The 100% infectivities for the *C. pneumoniae* controls represent $3.4x10^7$ (in A) and $5.2x10^6$ (in B) recoverable i.f.u./ml. *, P <0,05; **, P<0,01; ***, P<0,001.

When **essential amino acids** were added **throughout the infection** (figure 3.1.1 B), **Ile, Leu and Phe** severely inhibited inclusion growth of both species, except for **Met** which had a weaker effect on *C. pneumoniae* inclusion size (left). All these amino acids (including Met) completely abrogated the infectivity of chlamydial progeny. None of the other essential amino acids affected inclusion maturation, only **His, Lys** and **Thr** inhibited infectivity to about 20% (figure 3.1.1 B right). Interestingly, although **Trp and Val** had only weakly suppressed *C. trachomatis* progeny, they lead to an increase of *C. pneumoniae* infectivity.

3.1.3 Influence of non-essential amino acids on chlamydiae

Most **non-essential amino acids** (**Ala, Asn, Asp, Glu, Gln** and **Pro**) did neither adversely affect the size of chlamydial inclusions when introduced at the **middle of infection**, nor did they influence the progeny infectivity. Only **Gly, Hyp** and **Ser** very slightly decreased the inclusion size of both species and (together with **Arg** and **Tyr**) affected progeny infectivity (Fig 3.1.2, left). Like it was the case for **Met**, *C. trachomatis* was more susceptible to **Gly**, **Hyp** and **Ser** treatment than *C. pneumoniae*.

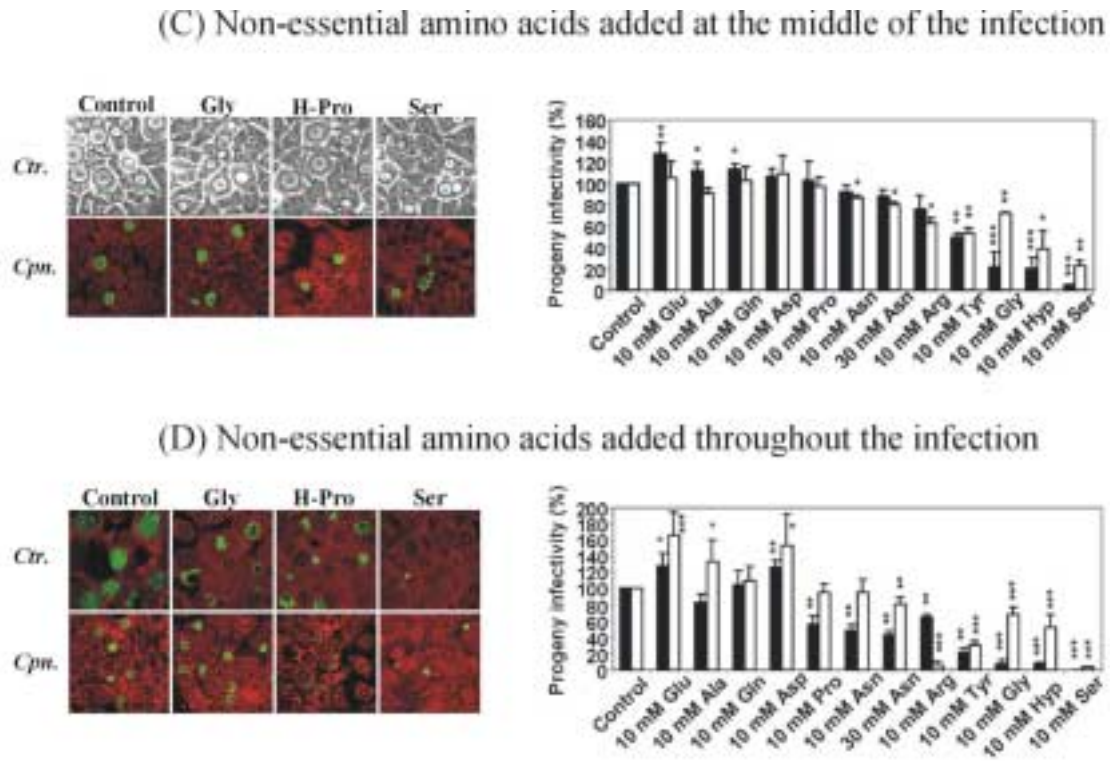


Figure 3.1.2 - Some non-essential amino acids inhibit inclusion growth and infectivity
see figure 3.1.1 for details

Accordingly, the addition of most **non-essential amino acids throughout the infection** had no effect on the development of chlamydial inclusions. Only **Gly, Hyp and Ser** lead to a decrease in size (figure 3.1.2) as well as to an inhibition of progeny infectivity. Their effect and the effect of **Asn and Pro** are more pronounced on *C. trachomatis*. An exception is **Arg** which inhibits *C. pneumoniae* more than *C. trachomatis*. An enhancement of infectivity of both species could be seen with **Glu and Asp**.

The amino acid's effects on chlamydial development (inclusion size and progeny infectivity) are summarized in table 3.1.1.

Effect on inclusion size and progeny infectivity	**Essential AAs (figure 3.1.1)**	**Non-essential AAs (figure 3.1.2)**
Strong	**Ile**, **Leu**, Met, **Phe**	
Weak when added in middle of infection	**His**, Lys, Thr	Gly, Hyp, Ser, Tyr
Weak when added throughout infection		Arg, Asn, Pro
None		Ala, **Gln**
Promoting	Trp, **Val**	Asp, Glu
Differential behavior between *Ctr* and *Cpn*	Met, Trp, **Val**	

Table 3.1.1 –Amino acids can be grouped according to their inhibitory effects on chlamydial development
The different groups are discussed in part 3.1.6. Bold: Amino acids essential for chlamydiae

For further experiments, a focus was set on those amino acids that strongly inhibited chlamydial growth when added both at the beginning and in the middle of the infection, namely **Ile**, **Leu**, **Met** and **Phe**.

3.1.4 Differential activities of inhibitory amino acids

To determine the difference in inhibition effects of **Ile, Leu** and **Met**, their dose dependent effects were determined and are shown in Figure 3.1.3.

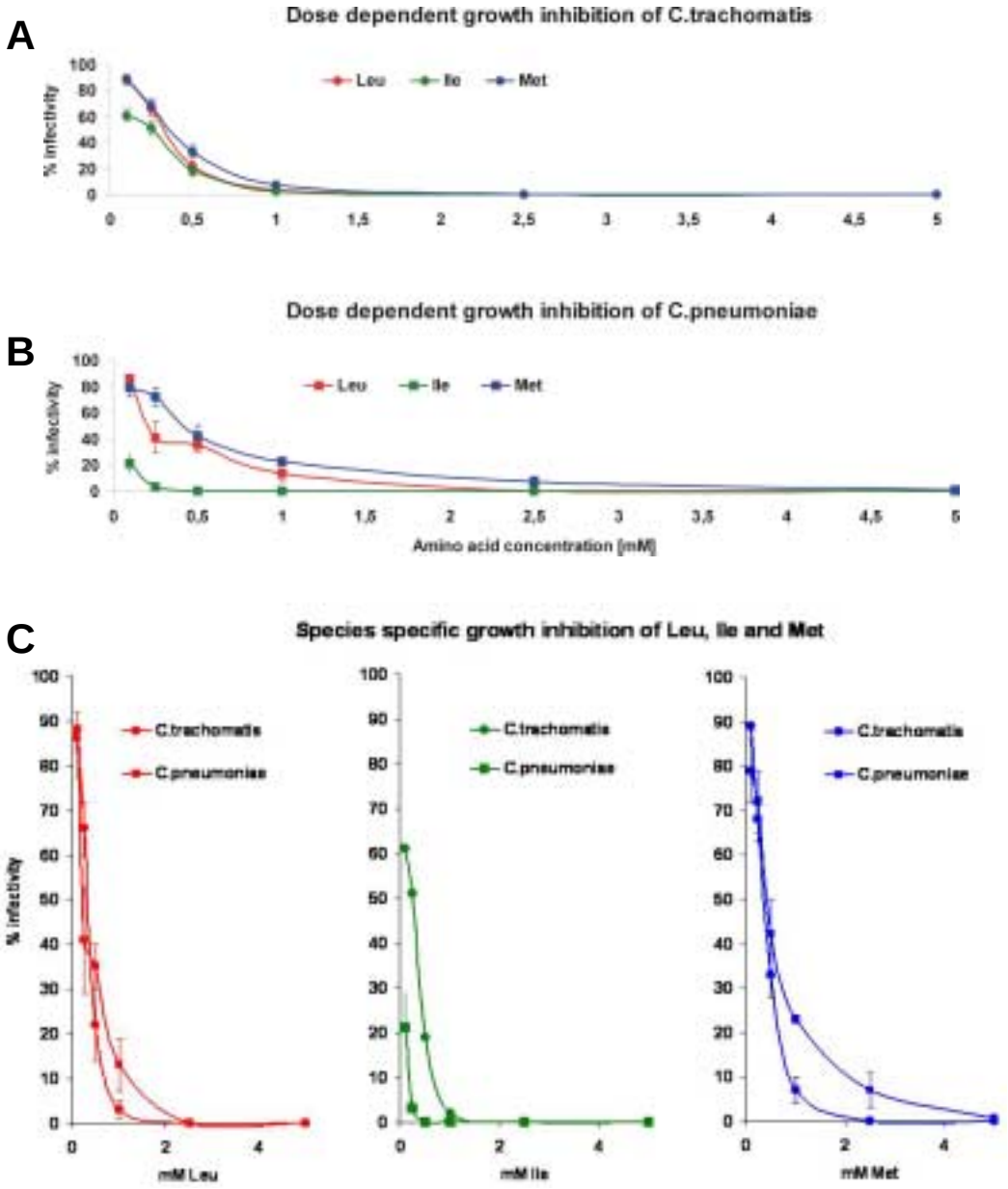


Figure 3.1.3 – Ile, Leu and Met reduce infectious progeny of *C. trachomatis* and *C. pneumoniae* in a dose-dependent fashion

HEp-2 cells were infected at an MOI of 0.5. Incubation with amino acids (concentrations: 0.1, 0.25, 0.5, 1, 2.5 and 5mM) was started 2h p.i. Control cells were infected in the absence of exogenous amino acids. At the end of the infection (44h and 72h p.i. for *C. trachomatis* and *C. pneumoniae*, respectively), progeny infectivity was determined. The data are the average±range of percentage progeny infectivity determined from two independent experiments. The 100% infectivities for the controls of *C. trachomatis* and *C. pneumoniae* represent $1.11x10^8$ and $1.34x10^6$ recoverable i.f.u. ml^{-1}, respectively.
A+ B. Effects of different amino acids on the same strain. At one tenth of the **Leu** and **Met** concentration (0.1mM vs. 1mM), **Ile** shows the same level of inhibition for C. pneumoniae. **C**: Effects of amino acids on different strains. **Leu** and **Met** are more inhibitory on *C. trachomatis*, **Ile** is more inhibitory on *C. pneumoniae*.

The amino acids demonstrated clear differential activities against the two species: **Ile** was more potent in decreasing the generation of infectious *C. pneumoniae* compared to *C. trachomatis*, **Met** and **Leu** were more effective against *C. trachomatis*.

3.1.5 EM analysis of chlamydial inclusions

The lack of infectious *C. pneumoniae* EBs in cultures continuously treated with **Met** was unexpected, since the inclusions were relatively large compared to *C. trachomatis* (figure 3.1.1). Further investigation was therefore performed. Electron microscopy revealed the presence of only the non-infectious RBs of *C. pneumoniae* (figure 3.1.4 D). **Met** appeared not to halt the binary division of *C. pneumoniae*, while it completely inhibited the *C. trachomatis* proliferation (figure 3.1.4 B).

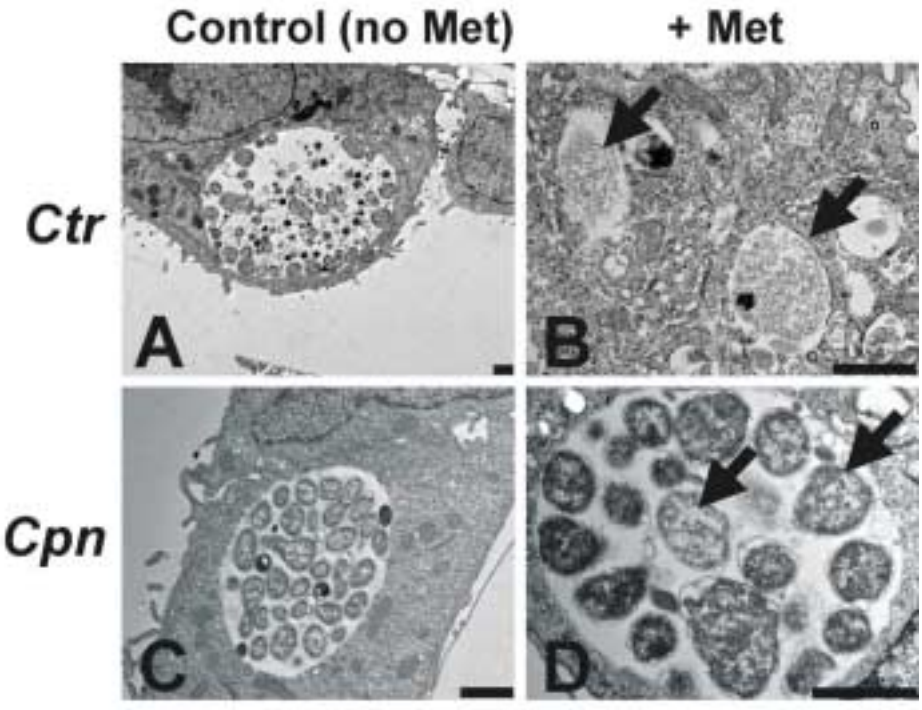


Figure 3.1.4 - Transmission electron micrographs reveals that Met inhibits RB proliferation in *C. trachomatis,* but not in *C. pneumoniae*

Cells were infected with *C. trachomatis* (A and B) or *C. pneumoniae* (C and D) for 2h and then fresh IM either without (A and C) or with (B and D) exogenous 10mM **Met** was added for 48h to the cells. Infections in (A), (C) and (D) were done using an MOI of 0,5. In order to find inclusions in the ultrathin sections, the infection in (B) was carried out using an MOI of 10. Only single *C. trachomatis* RBs are present [arrows in (B)] in cells incubated with excess **Met**, compared to an appreciable number of RBs per *C. pneumoniae* inclusion [arrows in (D)] also treated with **Met**.
Ctr, *C. trachomatis*; Cpn, *C. pneumoniae*. Bars: 1 μm.

3.2 Natural amino acids elicit long-term anti-chlamydial effects

3.2.1 Introduction

In previous experiments (Al Younes *et al.*, 2004 and part 3.2 of this thesis), the amino acids **Leu**, **Ile**, **Met** and **Phe** demonstrated striking adverse effects on chlamydial growth in cells infected for up to 5 days. Single additions of amino acids arrested inclusion maturation and prevented the production of infectious progeny. However, infectious progeny could be recovered, when additives were removed 1 or 2 days after treatment, but only negligible infectivity could be obtained after removal from cultures treated for periods as long as 5 days. Al Younes *et al.* therefore concluded that this inability of additive withdrawal to reverse the suppressive effects could indicate two things: Prolonged amino acid treatments generate **persistent** chlamydiae that fail to grow normally when the growth suppressor is removed or that bacteria are distorted with time and **killed**. To determine, which of the two fates the bacteria meet, the excess **Leu**, **Ile**, **Met** or **Phe** were administered 2h or 30h after infection on *C. trachomatis* L2 development for up to 15 days. For the experiments, *C. trachomatis* was chosen due to its larger inclusions size which made it more easily detectable in electron microscopy.

3.2.2 Assessment of long-term cytotoxicity of amino acid overload

The potentially negative effects of amino acid overload on the metabolic and proliferative activities of the host cells were verified by WST-1, LDH and mitotracker assays (figure 3.2.1). The values of all assays were comparable to those of untreated cells. Overall, supplementation with single amino acids used here affected neither cell viability nor cell activity during the 15 day incubation period.

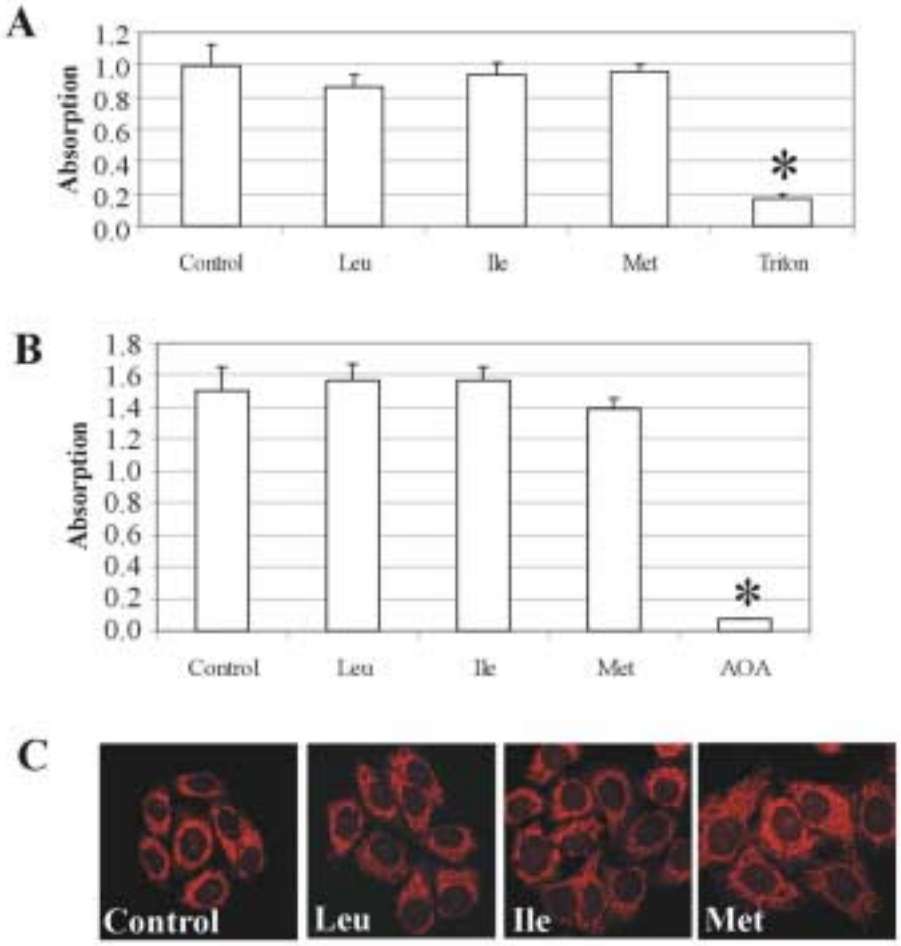


Figure 3.2.1 - Prolonged exposure to Ile, Leu and Met is not harmful to host cells

To test host cell viability and metabolic activity of HEp-2 cells after 15 days of amino acid supplementation, WST-1 (A), LDH (B) and Mitotracker stain (C) assays were performed. For WST-1 and LDH tests (A and B), cells were seeded in 96-well plates and eight replicates were prepared for each treatment. Triton X-100 and 10µM AOA (aminooxyacetate) were used as negative controls in WST-1 and LDH assays, respectively. Statistically significant differences of treated versus untreated (control) cells are indicated by asterisks. Data are presented as means ± SD. (C) Mitotracker dye was retained in mitochondria of amino acid-treated cells similar to untreated cells, indicating that extended incubation with amino acids did not generate loss of mitochondrial membrane potential, thus reflecting viability of exposed host cells. Micrographs show 80x80µm.

3.2.3 Administration of amino acids on an established infection

To explore effects of amino acids on an established *C. trachomatis* infection, HEp-2 cells were infected for 30h and then exposed to 10mM **Leu**, **Ile**, **Met** or **Phe**, the amino acids having the strongest inhibitory effect in previous experiments. Upon *C. trachomatis* infection of untreated cells, monlayers were almost completely destroyed within 4 to 6 days p.i. In contrast, amino acid addition conferred protection from destruction to infected monolayers throughout the experimental period.

3.2.3.1 Immunofluorescence

Immunostaining revealed that amino acid-exposed inclusions increased in size until 3d p.i., but exhibited a decreased staining intensity, which is indicative of a lower number of bacteria (figure 3.2.2). Four days p.i., these large inclusions mostly disappeared and new small inclusions emerged, most probably as a result of a new cycle of infection by released EBs. These new inclusions remained relatively small until the end of the experiments and did not destroy their host cells.

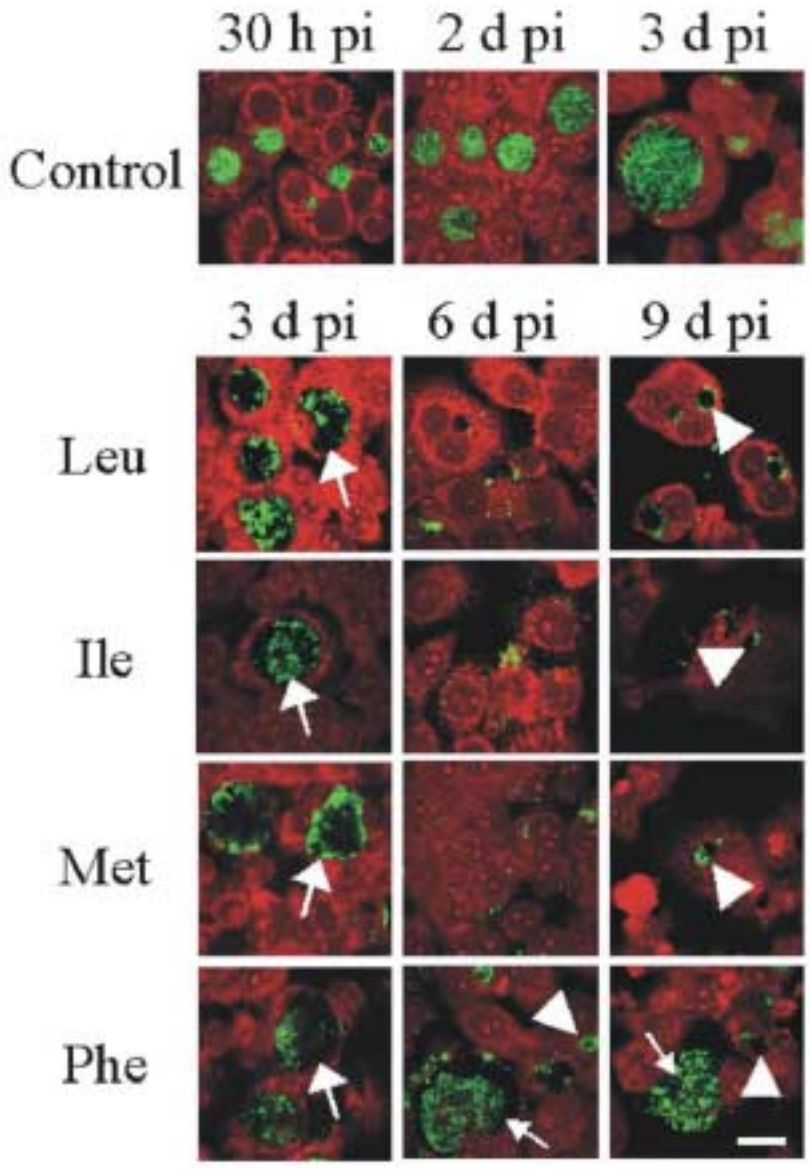


Figure 3.2.2 – Addition of Ile, Leu and Met 30h p.i. reduces growth of secondary inclusions

HEp-2 cells were infected with an MOI of 0.5 for 30 h. Infected cell cultures were then supplemented with 10mM **Leu**, **Ile**, **Met**, or **Phe** for up to 9 days. Chlamydiae (green) were visualized by staining with FITC-labeled anti-Chlamydia antibody and examined by confocal microscopy. After 3d, control cells started to lyse and detach. Bar represents 20µm.

3.2.3.2 Infectivity recovery

The production of infectious bacteria was also reduced drastically compared to the control (figure 3.2.3). Infectivity steadily decreased, and within 7 days p.i., infectious EBs were not detected except in **Phe**-treated culture.

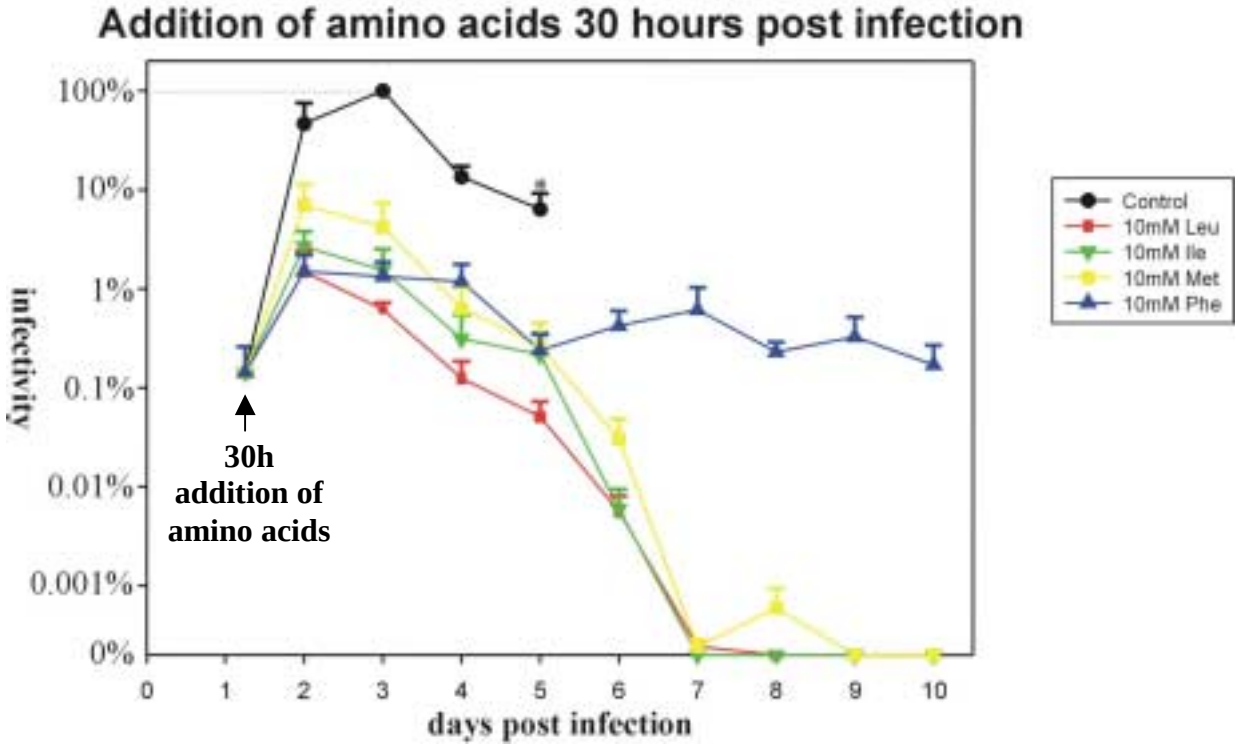


Figure 3.2.3 – Addition of Ile, Leu and Met on an established infection leads to a decrease in progeny infectivity

HEp-2 cells were infected at an MOI of 0.5 for 30h. Infected cell cultures were then supplemented with 10mM Leu, Ile, Met, or Phe for up to 10 days. Treated and untreated infected cells were harvested at indicated time intervals and the progeny infectivity was assessed. The highest number of infectious progeny (control, 3 days p.i.) was set to 100%. The experiment was performed in triplicates. Data presented are the means ± SD. Control HEp-2 cells were completely detached after five days.

3.2.4 Administration of amino acids on an early infection.

The long-term influence of amino acids on the intracellular development of chlamydiae was also analyzed in cells infected for 2 h and then continuously treated for 15 days.

3.2.4.1 Immunofluorescence

Atypical small inclusions during the first 2 days of infection were demonstrated in exposed cells (figure 3.2.4 and figure 3.2.5). Inclusions grown in the presence of **Leu**, **Ile** and **Met** showed a slight increase in the inclusion size, which did not further increase until 15 days p.i. In **Phe**-treated cells, however, the inclusions slightly increased in size throughout the course of the experiment (figure 3.2.4. arrows)

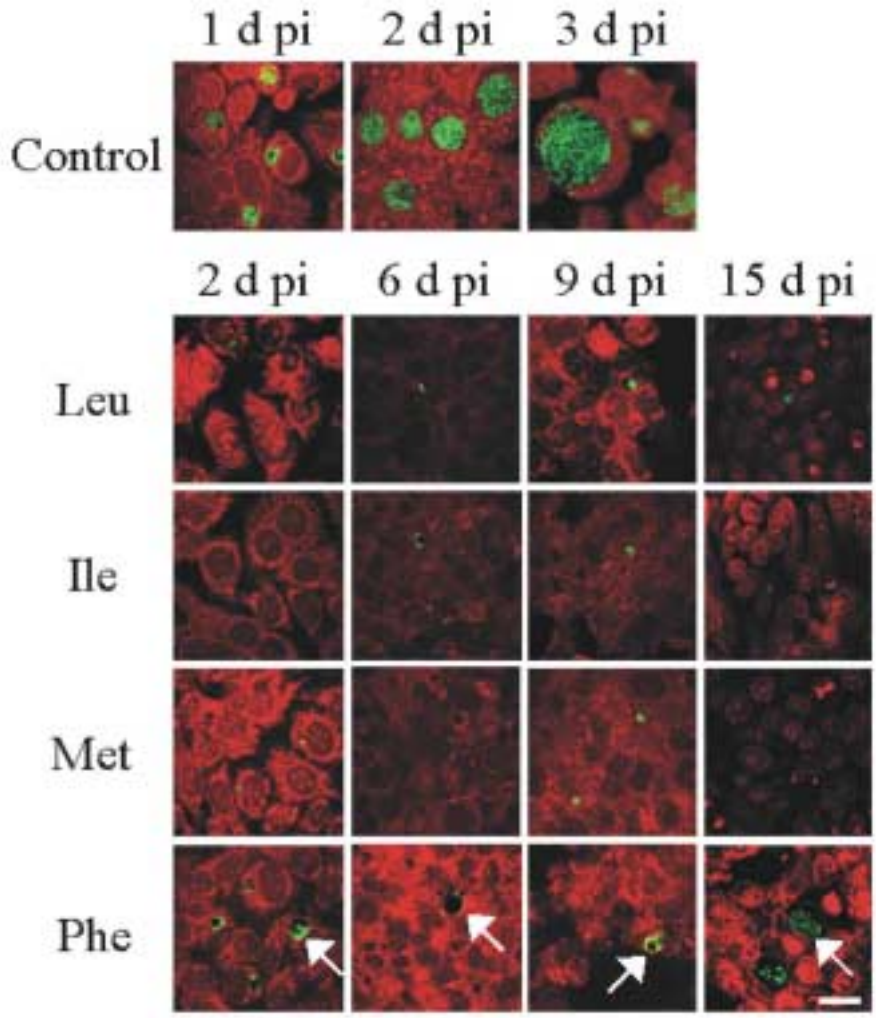


Figure 3.2.4 – Addition of Ile, Leu and Met on a fresh infection inhibits development of chlamydial inclusions

HEp-2 cells were infected at an MOI of 0,5, and amino acids were administered 2h pi. Chlamydiae (green) was visualized by staining with the IMAGEN kit and examined by confocal microscopy. Bar represents 20µm.

To also determine the overall condition of the infected cell monolayers exposed to increased amino acid concentrations, pictures were taken with a light microscope (figure 3.2.5).

3.2.4.2 Condition of infected monolayers after amino acid treatment

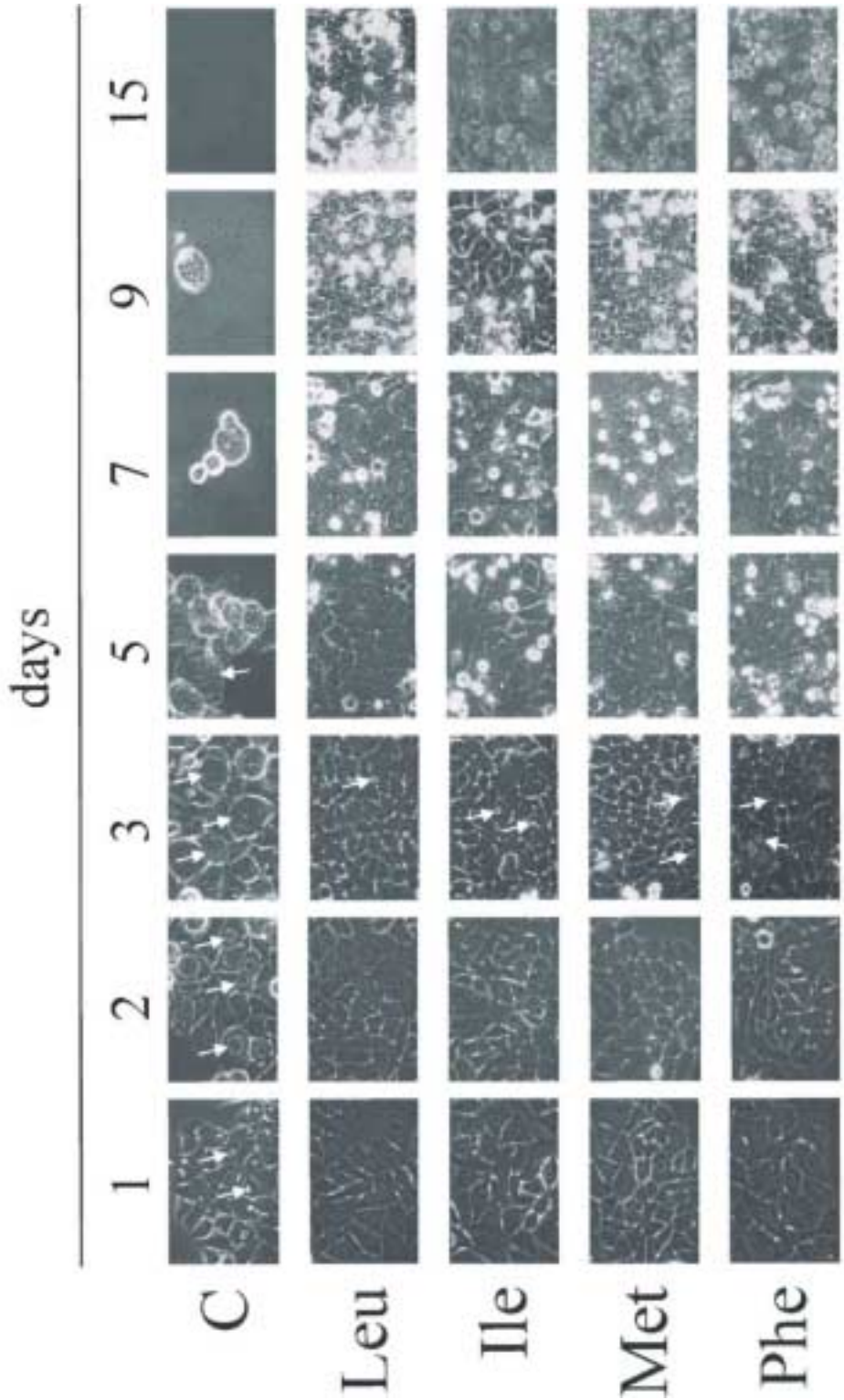


Figure 3.2.5 – Prolonged exposure to 10mM amino acids prevents lysis of monolayer

HEp-2 cells were infected with *C. trachomatis* for 2h at an MOI of 0,5 and incubated with IM containing 10mM of indicated amino acids for 15 days. Medium was changed every other day. In the control infection, inclusions develop (arrows) and cells start to lyse after 3 days. Few cells containing mainly inclusions are left. All treated monolayers stay intact. Inclusions in the treated cells are seen after three days (arrows). At later timepoints the inclusions are hard to detect due to cell overgrowth.

3.2.4.3 Quantification of inclusions

Numbers of inclusions continuously decreased during the whole period of infection in the presence of excess amino acids (figure 3.2.6).

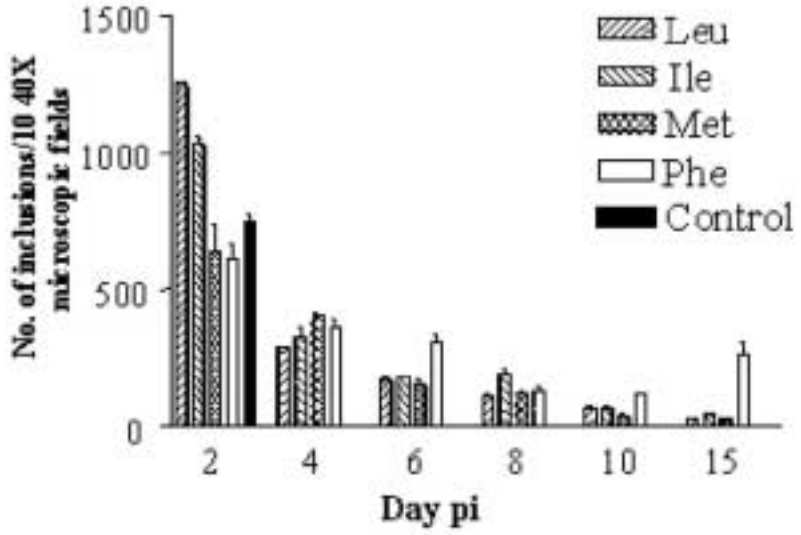


Figure 3.2.6 – Inclusion number decreases upon treatment with Ile, Leu, Met and Phe

HEp-2 cells were infected with *C. trachomatis* at an MOI of 0.5. Incubation with 10mM concentrations of excess single amino acids was started 2h pi until the end of a 15-day period. This experiment was performed with 3 coverslips for each treatment. Data presented are means ± SD.

3.2.4.4 Infectivity recovery

Infectious bacteria were never harvested from cells supplied with **Ile, Leu** or **Met** (Table 3.2.1 A). In contrast, constant production of infectious bacteria, although minimal, was obtained on each day after treatment with **Phe**.

The ability to **recover** the bacterial infectivity in treated cells by additive withdrawal was investigated. Results are shown in table 3.2.1 B. Considerable infectious progeny was obtained, when excess amino acids were removed 1, 2 or 3 days after treatment. The recovery of infectivity in **Leu**, **Ile** and **Met**-exposed cells gradually decreased over time, and in cells exposed for longer than 4 days to **Leu**, **Ile** or **Met**, infectivity restoration was not possible by additive removal for 2 days. However, withdrawal of the excess amino acids for longer periods (5 days) resulted in recoverable IFU in cells treated for 5 days (Table 3.2.1 C). Longer amino acid treatment (5 or 10 days) did abrogate this ability to recover. Removal of excess Phe led to an increase in the number of infectious chlamydiae, compared to that in continuously exposed cell monolayers. This indicates successful reversion of non-infectious bacteria to infectious forms.

	Treatment (days)	IFU/ml harvested from infected cell cultures exposed to:				
		None	**Leu**	**Ile**	**Met**	**Phe**
A	1	2×10^5	0	0	0	1×10^3
	2	9×10^7	0	0	0	6×10^3
	3	9×10^7	0	0	0	2×10^3
	4	9×10^7	0	0	0	1×10^4
	5	5×10^7	0	0	0	5×10^3
	6	1×10^7	0	0	0	4×10^3
	7	4×10^6	0	0	0	7×10^3
	8	4×10^5	0	0	0	7×10^3
	9	5×10^3	0	0	0	2×10^3
	10	2×10^3	0	0	0	5×10^3
	11	6×10^2	0	0	0	2×10^3
B	1 (+AA) 2 (-AA)		9×10^6	8×10^6	2×10^7	1×10^7
	2 (+AA) 2 (-AA)		3×10^7	7×10^5	1×10^6	5×10^6
	3 (+AA) 2 (-AA)		9×10^5	5×10^5	3×10^4	4×10^6
	4 (+AA) 2 (-AA)		7×10^3	0	0	4×10^5
	5 (+AA) 2 (-AA)		3×10^3	0	0	6×10^5
	6 (+AA) 2 (-AA)		0	0	0	2×10^5
	7 (+AA) 2 (-AA)		1×10^3	0	0	5×10^5
	8 (+AA) 2 (-AA)		0	0	0	6×10^4
	9 (+AA) 2 (-AA)		0	0	0	2×10^5
C	5 (+AA) 5 (-AA)		5×10^5	8×10^5	1×10^5	many
	10 (+AA) 5 (-AA)		0	0	0	many
	15 (+AA) 5 (-AA)		0	0	0	many

Table 3.2.1 – Upon treatment with Ile, Leu and Met, the progeny infectivity is abolished, but it can be rescued to a certain extent by amino acid removal

HEp-2 cells were infected for 2h with *C. trachomatis* at an MOI of 0,5 and then incubated continuously for various time intervals with IM containing 10mM Leu, Ile, Met, or Phe. As controls, host cells were infected and incubated in IM without any exogenous amino acids. Each specimen was represented by 2 wells of a 6-well plate.
A At the end of indicated time points, progeny infectivity was determined. **B** At the end of indicated time points, excess amino acid-containing medium was replaced with IM without exogenous amino acids and then cells were incubated for additional 2 days in an attempt to rescue the bacterial infectivity. This was followed by the determination of progeny infectivity. **C** Same conditions as B, but recovery time was increased to 5 days.
This experiment was performed at 2 different occasions with similar results. -AA: without added amino acid, +AA: with added amino acid.

3.2.4.5 Ultrastructure of chlamydiae

As revealed by TEM, **Leu**, **Ile** and **Met** and **Phe** overload clearly affected *C. trachomatis* ultrastructure in cells infected with relatively high MOI (15-50) for up to 4 days (figure 3.2.7). Internalized EBs transformed into ABs (black arrows). Multiple chlamydial compartments mostly containing single bacteria were detected, indicating that RBs failed to multiply normally and the vesicular homotypic fusion of inclusions was largely disrupted. RBs had irregular atypical morphology (black arrows), compared to the round-shaped control RBs (white arrows). Empty membrane-bound vesicles were sometimes noticed inside inclusions (black arrowheads), representing disrupted chlamydiae. **Phe**-treatment led to a considerable increase of inclusion size and to a distortion of RB structure. However, EBs can still be seen just like in the untreated control (white arrowheads).

We were not able to examine the ultrastructural characteristics of bacteria after 4 days p.i., because infected cells appeared to lyse at later times using high MOI. On the other hand, it was technically not possible to detect chlamydiae using lower MOI, as the number of small inclusions decreased over time, making it impossible to detect the bacteria in the ultrathin sections.

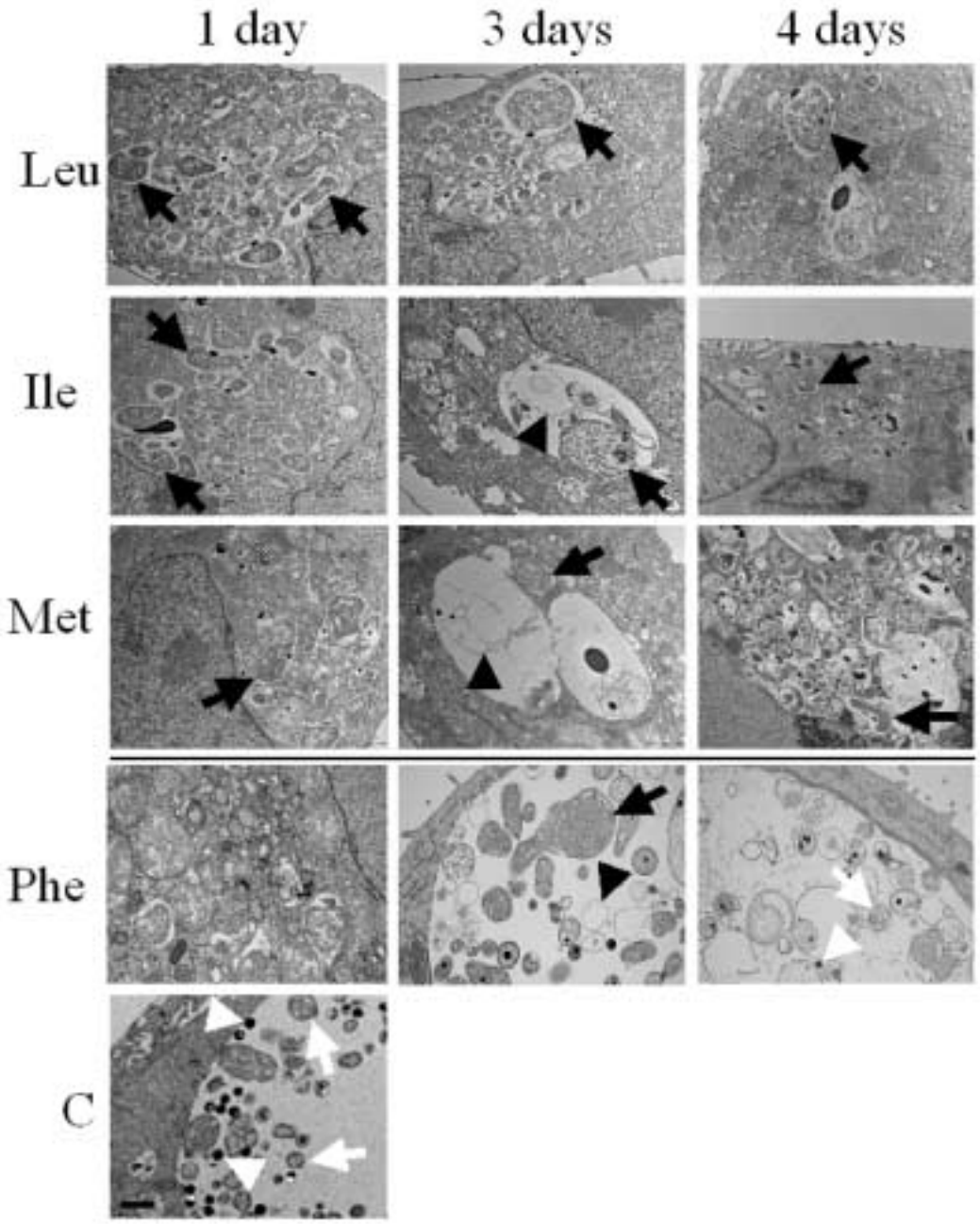


Figure 3.2.7 – Ile, Leu, Met and Phe treatment leads to aberrant development of bacteria

HEp-2 cells were infected for 2h and then supplemented with 10mM Leu, Ile, or Met for the indicated time periods. Cell cultures treated for 1, 3, or 4 days were infected at an MOI of 50, 20, and 15, respectively. Aberrant chlamydial forms (black arrows) and membrane-bound structures (black arrowheads), which could be disrupted bacteria can be noticed in treated cultures. White arrows indicate RBs, white arrowheads indicate EBs in the untreated control culture (infected at an MOI of 0.5) and in the Phe-treated culture. Bar represents 1µm.

Evidence that the structures within vacuoles revealed by TEM were in fact chlamydiae and not cytoplasmic organelles was obtained using immuno-EM targeting the Hsp60 protein (figure 3.2.8).

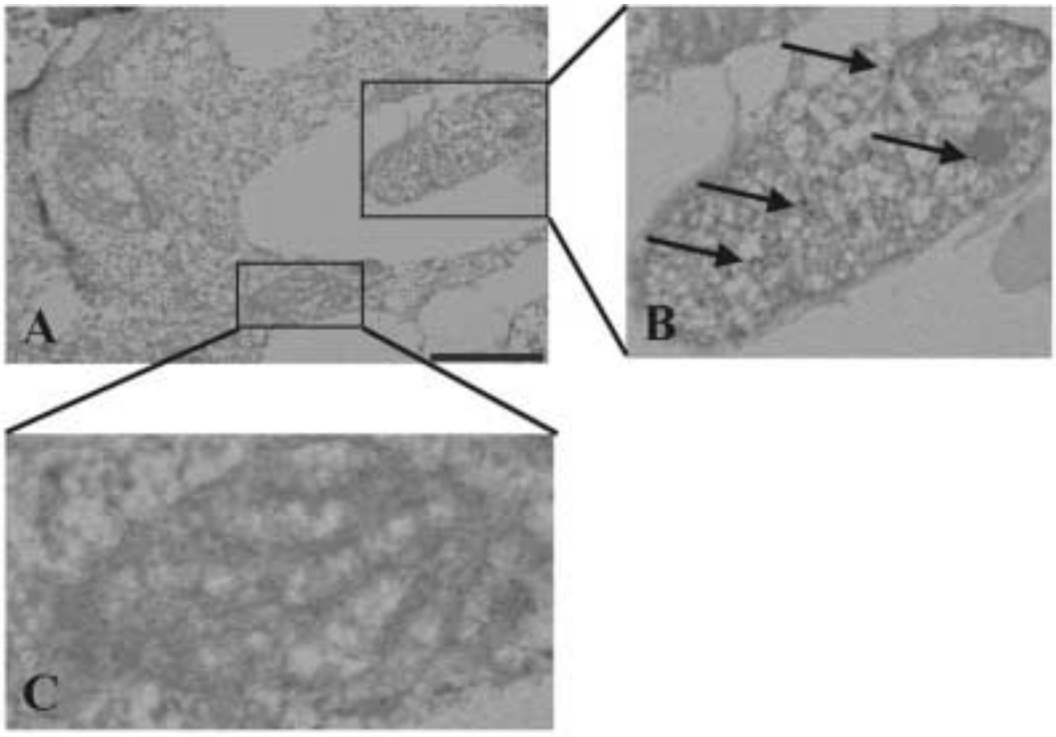


Figure 3.2.8 – Structures considered aberrant chlamydiae in Leu treated inclusions contain Hsp60 antigen

(A) Immunoelectron micrograph of a HEp-2 cell infected with *C. trachomatis* under conditions of high **Leu** concentration. (B) A longitudinal abnormal RB present within a vacuole was specifically immunostained with anti-chlamydial Hsp60 labeled gold particles (arrows). Other host cytoplasmic structures, such as mitochondria (C), were devoid of gold particles. Scale bar represents 1 μm.

3.2.4.6 Viability and gene expression of amino acid-treated chlamydiae

Lack of infectious chlamydial progeny and the formation of abnormally developed bacteria do not necessarily indicate that the bacteria are not viable. Therefore, the production of the short-lived transcripts of bacterial 16S rRNA was examined. This gene is a widely used marker to demonstrate presence and viability of chlamydiae in clinical samples and in *in vitro* persistence models or to assess chlamydiostatic activities of antibiotics (Cox *et al.*, 2003;Khan *et al.*, 1996;Koehler *et al.*, 1997). RT-PCR demonstrated the presence of 16S rRNA transcripts in treated cells throughout the 15-day period, indicating viability of bacteria (figure 3.2.9 A). Since RT-PCR can only determine presence or absence of transcripts and not

relative amounts, qRT-PCR (real-time PCR) was performed (figure 3.2.10) to confirm the results.

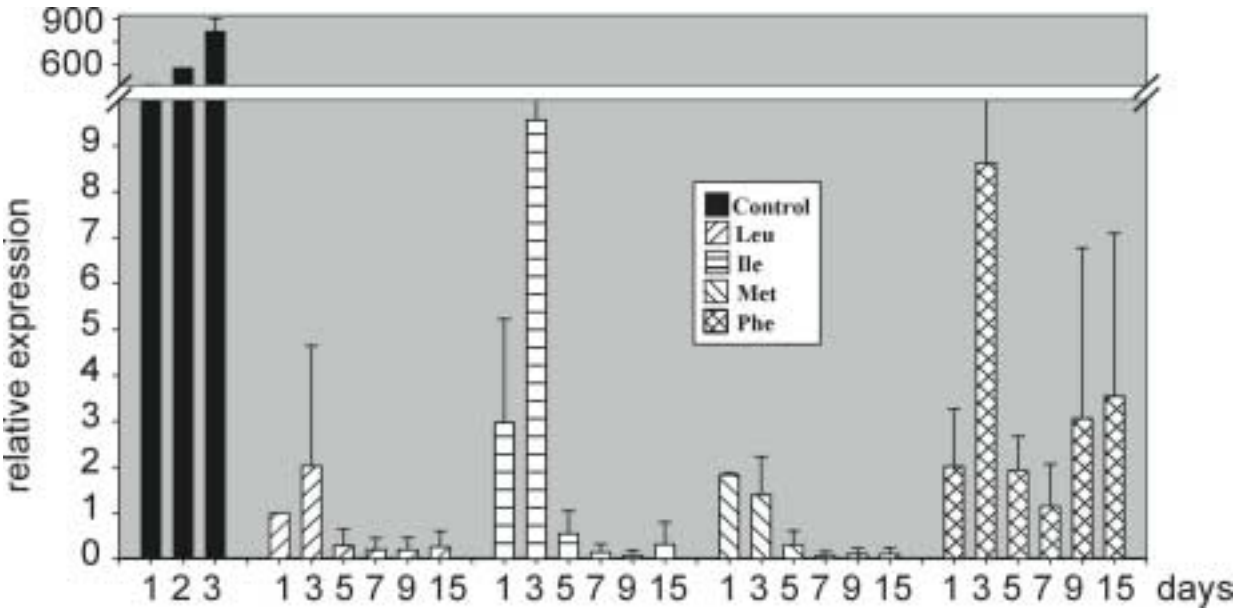


Figure 3.2.9 – Quantitative (real-time) RT-PCR shows attenuated but constant presence of 16S rRNA transcripts

RNA from HEp-2 cells infected with *C. trachomatis* and treated with 10mM of amino acids for the indicated times was isolated, reverse transcribed, and real-time PCR with appropriate primers was performed. The expression of 16S in relation to GAPDH was set to 1 for the 1-day-**Leu** sample.

The mRNA expression of the key chlamydial genes *omp1* (MOMP), *groEL* (Hsp60) and *omcB* was analyzed. The level of ***omp1*** transcripts in **Ile**, **Leu** and **Met**-exposed infected monolayers was constantly attenuated until it vanished (figure 3.2.9 C). Suppression of ***groEL*** expression over time was even more effective (figure 3.2.9 B). The ***omcB*** gene appeared to be very strongly suppressed at all time points examined (figure 3.2.9 D).

In **Phe** exposed infected cells, mRNA transcripts of all analyzed genes were always detected with no significant changes in their expression levels, except for *omcB* which was highly attenuated by 5 days post-treatment. Not surprisingly, gene transcription in control infected cell cultures was detected at all time points, despite the almost complete destruction of cell monolayers and the regular changing of the medium. This was due to the sporadic presence of infected cells in the wells throughout the entire course of the experiments.

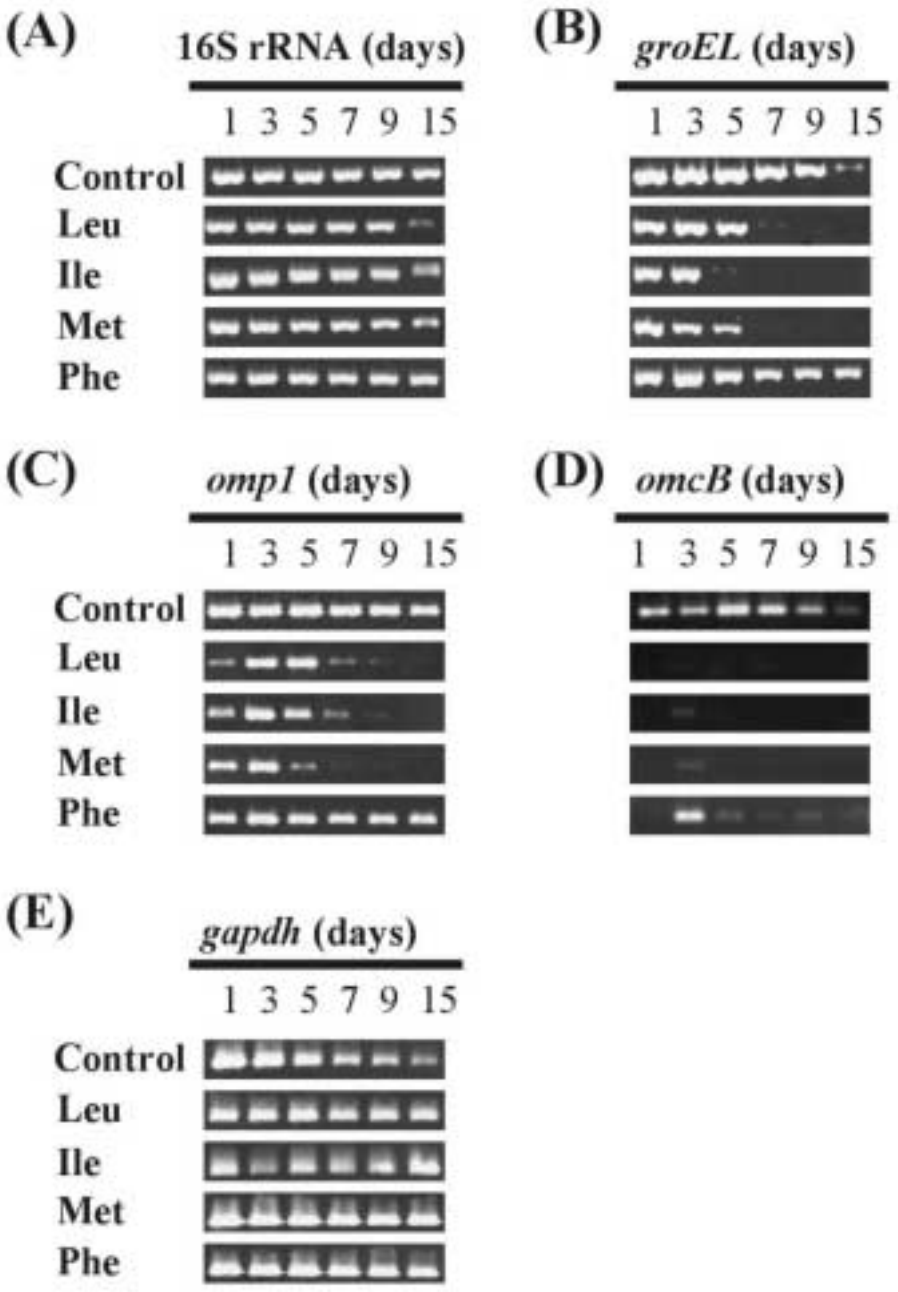


Figure 3.2.10 – Amino acid treatment causes downregulation of certain immunologically important chlamydial genes

HEp-2 cells were infected for 2h with *C. trachomatis* at an MOI of 0.5 and then continuously exposed to 10 mM **Leu**, **Ile**, **Met**, or **Phe** for periods up to 15 days. Infected control cells were incubated with IM without excess amino acids. At indicated timepoints, the production of transcripts of chlamydial 16S rRNA **(A)** was examined to determine chlamydial viability. To characterize the gene expression pattern, *groEL* **(B)**, *omp1* **(C)**, and *omcB* **(D)** genes were also examined. The *gapdh* gene **(E)** of host cells in amino acid-treated and untreated cultures was expressed at all the time points. The amplification products were: 333 bp for 16S rRNA, 484 bp for *groEL*, 193 bp for *omp1*, 147 bp for *omcB*, and 250 bp for *gapdh*.

3.2.4.7 Protein expression pattern in amino acid-treated chlamydiae

Protein synthesis of *groEL* and *omp1* products was studied. No Hsp60 was detected when cells were exposed to either **Ile**, **Leu**, or **Met** for longer than 3 days (figure 3.2.11). In contrast, MOMP could never be detected in these cells. These antigens were present in untreated infected cells for up to 5d p.i., before the monolayers were lysed. Interestingly, Hsp60 was abundant in **Phe**-exposed cells infected for 15 days, unlike MOMP that had low levels 3d p.i. and was then attenuated over time until it disappeared.

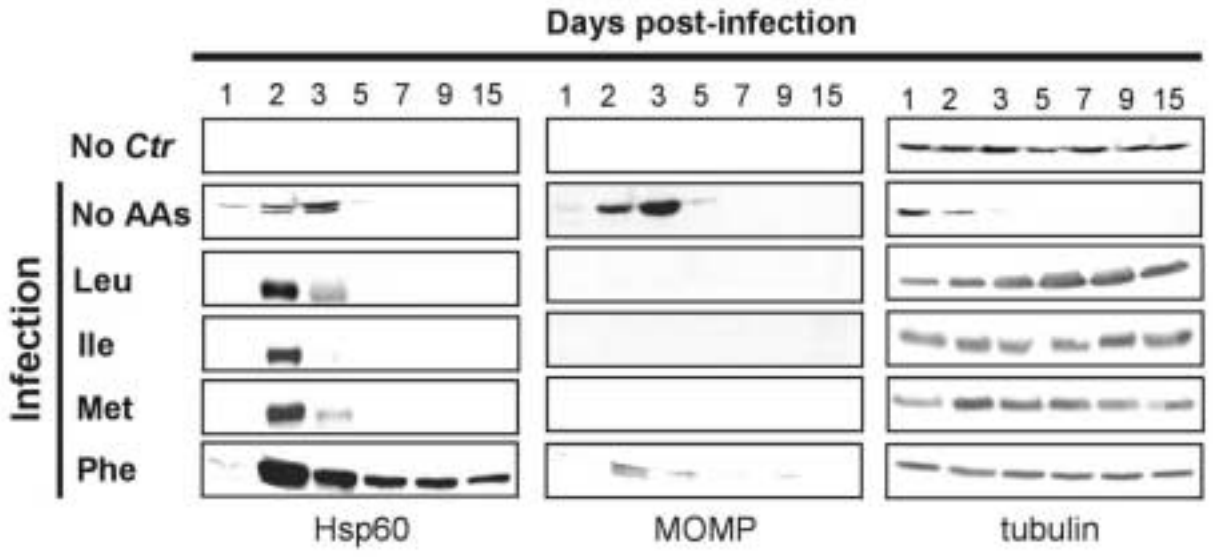


Figure 3.2.11 – Protein expression is altered in amino acid-treated chlamydiae

HEp-2 cells were infected for 2h with *C. trachomatis* at an MOI of 0.5 and then exposed to 10mM of single amino acids. Uninfected and infected control cells were incubated with IM only. At indicated time intervals, cells were harvested, solubilized and then loaded onto SDS-PAGE gels followed by immunoblotting using anti-chlamydial MOMP or Hsp60 antibodies. Anti-host α-tubulin antibody was used as a control for equal loading.

The Western blot performed could only determine the total amount of protein present in the culture. Since the number of inclusions decreases over time after treatment with **Ile**, **Leu**, **Met** and **Phe** (figure 3.2.6), it is difficult to conclude that any decrease is resulting only from a downregulation of protein synthesis. The effect could also be due to the decrease in total bacteria. In order to solve this problem and to determine gene expression on a cell-to-cell basis, immunostaining against LPS and Hsp60 was performed and single infected cells were examined (figure 3.2.12). The results show that long-term treatments with **Ile**, **Leu** and **Met** result in an increasing number of structures, that are LPS positive (and therefore represent chlamydial inclusions or bacteria), but that have lost their Hsp60 signal.

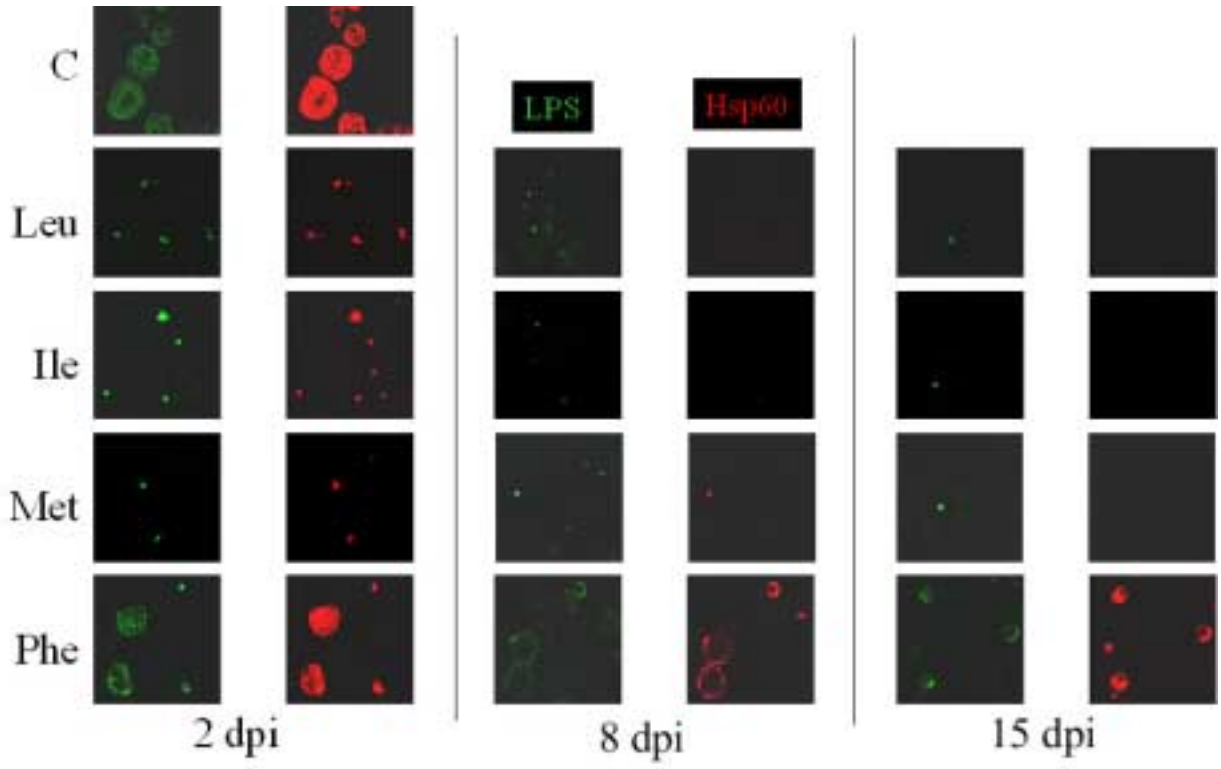


Figure 3.2.12 – Amino acid treatment leads to decrease of Hsp60 expression

HEp-2 cells were infected with *C. trachomatis* for 2h at an MOI of 0,5 and then incubated with 10mM of **Ile**, **Leu**, **Met** and **Phe**. After indicated time points, the cultures were stained using antibodies against LPS (green) and Hsp60 (red). Confocal imaging reveals an increase over time of LPS-positive structures that have lost their Hsp60 signal. Micrographs show 80x80µm.

3.3 Influence of amino acids on chlamydiae *in vivo*

3.3.1 Introduction

As discussed in the previous section, the effect that **Leu**, **Ile** and **Met** have on chlamydial infections might be used as a therapeutic approach, as other natural substances administered to infected animals have already been shown to work effectively against chlamydiae: Vitamin E administration could improve the recovery of lambs intratracheally infected with *C. pneumoniae* (Stephens *et al.*, 1979), and infected mice could be successfully treated intraperitoneally with antibacterial flavonoids (Tormakangas *et al.*, 2005a). Also, excess tryptophane was suggested against *C. trachomatis* infections (Singla, 2007). Therefore, the antichlamydial effect of the inhibitory amino acids was tested in an *in vivo* system.

3.3.2 Establishment of an *in vivo* model

We applied the mouse system widely used for modeling lung infections with *C. pneumoniae* (Kaukoranta-Tolvanen *et al.*, 1993). After establishing the infection model, the effect of excess amounts of inhibitory amino acids on **uninfected** animals (Balb/C mice due to availability) was determined. The natural substances were orally administered through the drinking water in which they were solubilized until saturation.

No LD_{50} values for **Leu**, **Ile** and **Phe** were available for oral administration, and the value for **Met** was very high (Ross, 2000). So although the concentrations in the water were not toxic according to the literature, we wanted to exclude any negative effects. We performed a feeding assay over the course of 10 days, where the animals had access to supplemented water and food ad libitum. Water consumption as well as body weight of the mice was determined over the course of 10 days.

As shown in figure 3.3.1 A, the administration of **Leu** or **Ile** did not have any negative effect on water consumption or animal weight compared to control mice. On the other hand, mice that were fed with **Met** or **Phe** supplemented drinking water (figure 3.3.1 B) showed aberrant behavior: The animals refused to drink the water and lost weight. Already after one day of treatment, they seemed irritated and aggressive when handled outside the cage, their fur started to ruffle, and they were sensitive to noise and touch. As further treatment would have presented unnecessary stress to the animals, the experiments with **Met** and **Phe** were prematurely aborted.

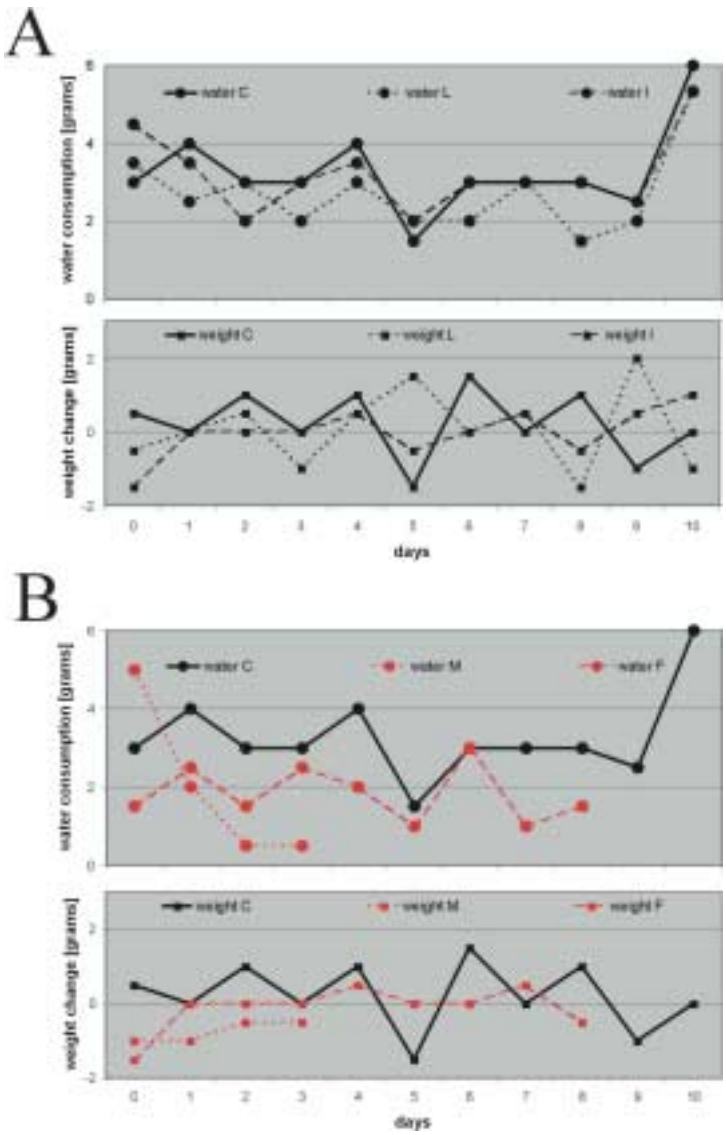


Figure 3.3.1 – Long term administration of Leu and Ile exhibits no negative effect on mice whereas Met and Phe administration leads to reduced body weight and water consumption

Amino acids were dissolved until saturation in sterile tap water. Resulting concentrations were Leu: 166mM, Ile: 269mM, Met: 226mM, Phe: 179mM. Balb/C-Mice were divided into groups of two. Supplemented water and food were provided to the animals *ad libitum*, and drinking water consumption per animal (-●-) as well as weight change per animal (-■-) were determined daily. **(A) Leu** and **Ile** supplemented drinking water caused no change compared to normal drinking water. **(B)** Mice strongly refused to drink **Met** supplemented water, and their body weight decreased. Therefore, the experiment was aborted after 3 days. **Phe** supplemented water was also less consumed compared to the control (water C).

3.3.3 Mouse infection with *C. pneumoniae* and oral leucine treatment.

Based on these results, we decided on a long-term administration of **Leu** to infected mice (C57/BL6). As an indicator for animal health, water consumption and weight were recorded every 3,5 days.

As expected, the animals reduced their water consumption after the infection, as the animals had to deal with the bacteria (figure 3.3.2). Also, a weight loss was recorded. After one week, the infection started to clear and water consumption as well as body weight change returned to normal.

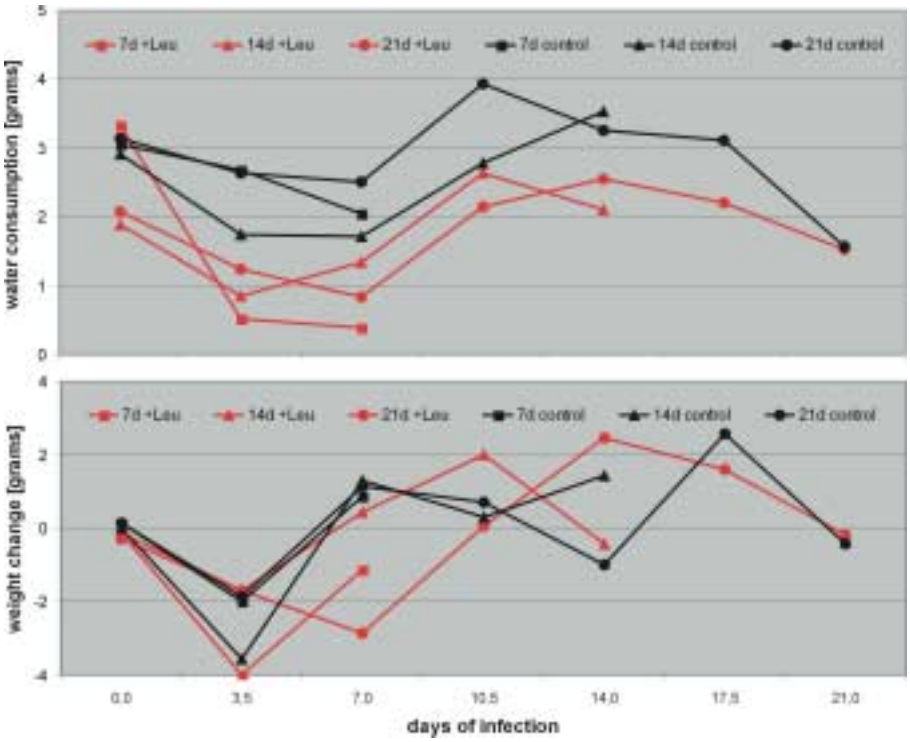


Figure 3.3.2 – Water consumption and animal weight decrease after *C. pneumoniae* infection until animals recover

Each group of 7 mice was treated with **Leu**-supplemented or normal drinking water (control) for one week before being intranasally infected with 10^7 IFUs *C. pneumoniae* (day 0). Water consumption and weight change (both averaged to yield value per 1 animal) were determined on a regular basis. One treated and untreated group was sacrificed on days 7 (-■-), 14 (-▲-) and 21 (-●-) to determine the bacterial load in the lungs.

Figure 3.3.3 shows the number of IFUs recovered from each mouse lung. Consistent with the literature the infection was mostly cleared after three weeks. However, after two weeks, the **Leu**-treated mice had mostly cleared the infection whereas in the control animals' lungs, a high number of bacteria could still be found.

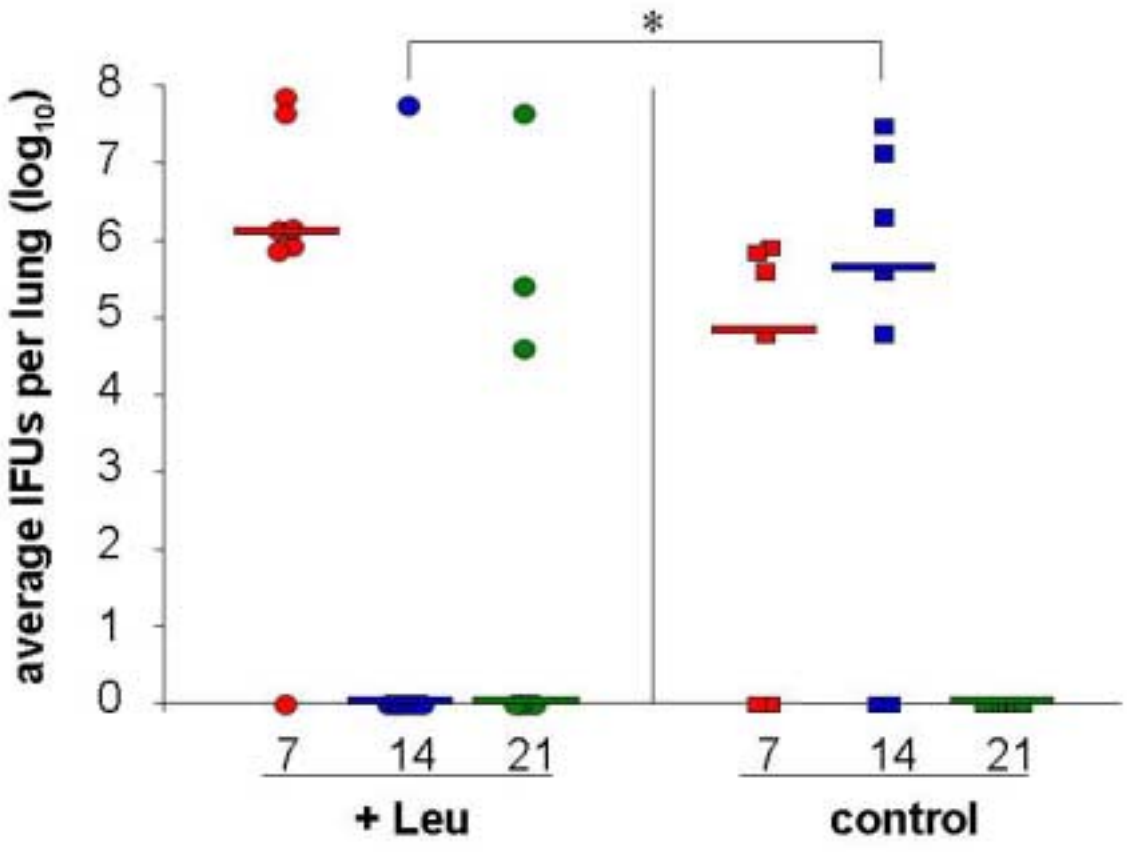


Figure 3.3.3 – Leu-supplemented drinking water has beneficial long-term effects on murine *C. pneumoniae* infections

Groups of 7 mice (5 in the group 14d +**Leu**) were treated with **Leu**-supplemented water, intranasally infected with 10^7 IFU of *C. pneumoniae*, and sacrificed after one, two or three weeks. Subsequently, the number of infectious bacteria in the lung was determined. * A statistically significant difference ($p<1\%$) between treated and untreated mice was observed 14 days after the infection. Lines represent median of the measurements.

To check if the observed effect was due to an increased **Leu**-concentration in the blood stream, the serum concentration was determined. However, no statistically significant difference of the **Leu** serum concentrations could be detected (figure 3.3.4).

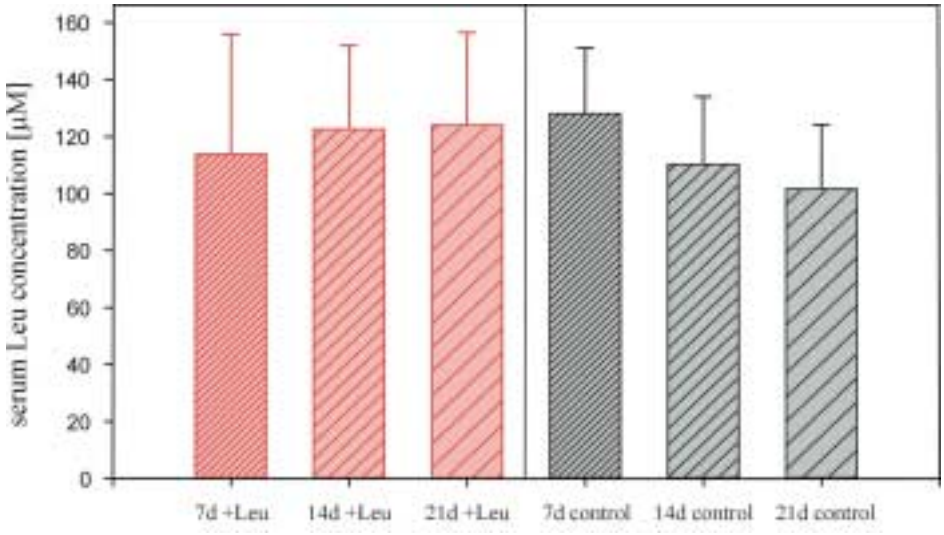


Figure 3.3.4 - Leu administration in drinking water does not significantly increase Leu concentration in serum

After sacrificing the animals (for details see figure 3.3.3), the **Leu-**concentration of the serum was determined. Apart from a slight trend, no statistically significant difference was observed. **Leu-**concentration in serum reported in the literature is 100µM.

4. Discussion

Effect of elevated concentrations of amino acids on chlamydial infections

4.1 Overview of amino acid influence on chlamydiae

Part 1 of this thesis investigated the differential effects that the 20 proteogenic amino acids have on *Chlamydia trachomatis* and *Chlamydophila pneumoniae* when added in excess – at a concentration of 10mM - to infected cells, either at the beginning of the infection (when bacteria are present in their EB form) or in the middle of the infection (when most bacteria have differentiated into RBs). The inhibitory influence was measured by the change of inclusion size determined by confocal fluorescence microscopy and infectivity assays of the chlamydial progeny in relation to untreated infected cells (figures 3.1.1 and 3.1.2). **Leu**, **Ile** and **Met** completely inhibited chlamydial growth at 10mM. To further differentiate these effects, dose dependent (0.1 – 5mM) growth inhibition experiments were performed (figure 3.1.3). The differential effect of **Met** (considerably less inhibitory to *C. pneumoniae* versus *C. trachomatis*) was determined by electron microscopy of infected and **Met**-treated cells (figure 3.1.4).

The amino acids can be grouped according to their inhibitory effects (table 3.1.1):

4.1.1 Ile, Leu, Met and Phe exhibit the strongest inhibition of inclusion development and progeny infectivity

All amino acids that elicit the strongest inhibition on chlamydiae at high concentrations contain hydrophobic side chains: **Phe, Met** and the branched chain amino acids (BCAAs) **Leu** and **Ile**). A relationship between these amino acids and chlamydial growth has been reported in the literature but only relating to omission from culture medium: Growth inhibition of *C. psittaci* by the omission of single BCAAs resulted from competition between residual amounts of omitted amino acids still present in the cytoplasm, and other amino acids (Coles and Pearce, 1987). The omission of **Ile** abrogated growth inhibition by **Val** depletion, indicating that **Ile** is an antagonist of **Val**. **Ile** and **Val** together were shown to be antagonists of **Leu**. This antagonism could take place either at an enzyme or transporter with affinity for these substances. The antagonistic effects of amino acid supplementation are discussed in section 4.4.1.

4.1.2 Asp and Glu promote growth of both chlamydial species

The amino acids **Asp** and **Glu** which promote the growth of both *C. trachomatis* and *C. pneumoniae* are thought to be used as an additional carbon source since genes that encode key enzymes involved in glucose formation and energy-production pathways are present in the chlamydial genomes (Stephens *et al.*, 1998). It could be shown that in the absence of glucose, other gluconeogenic carbon sources, such as **Glu**, can support chlamydial growth (Iliffe-Lee and McClarty, 2000). Evidence that some amino acids may be needed in greater amounts than normally present has come from the studies of (Ojcius *et al.*, 1998), which demonstrate the stimulation of **Glu** synthesis in *C. psittaci* infected cells.

4.1.3 Met differentially influences *C. trachomatis* and *C. pneumoniae*

The most striking difference observed in our experiments was the inhibition of *C. trachomatis* but not of *C. pneumoniae* by **Met** when added in the middle of the infection (figures 3.1.1 and 3.1.3). The differential influence of **Met** was further examined by electron microscopy (figure 3.1.4), and it was shown that it strongly inhibited RB proliferation in *C. trachomatis* but not in *C. pneumoniae*. One possible molecular mechanism behind this phenomenon, the differential affinity of the BrnQ-transporter has recently been suggested in our group and is described below (section 4.4.1)

4.1.4 Arg, Asn, Gly, His, Hyp, Lys, Pro, Ser, Thr and Tyr exhibit only weak inhibitory effects on chlamydiae

These effects were not further investigated, but possible mechanisms include antagonism of enzymes, transporters or receptors, or the use of these amino acids as carbon source. It could also be possible that the addition of some amino acids activates certain responsive mechanisms of chlamydial and/or host gene regulation which may negatively affect bacterial growth. Evidence of such a mechanism of transcriptional regulation in chlamydiae has been published (Schaumburg and Tan, 2006), indicating that the **Arg** transport system *glnPQ* could be transcriptionally regulated *in vitro* by the recombinant ArgR repressor in response to **Arg** levels. Our results show, however, that **Arg** has only weak influence on chlamydial infectivity. This could be due to a saturated **Arg** transport system which cannot be upregulated any further.

Summary part 1

In part 1, the screen for amino acids inhibiting chlamydial growth *in vitro* was performed. The most potent inhibitory amino acids were **Leu**, **Ile**, **Met** and **Phe**. These amino acids were then used in further experiments.

4.2 Natural amino acids elicit long-term anti-chlamydial effects

Part 2 of this thesis investigated the effects of long-term administration of elevated concentrations of **Leu**, **Ile**, **Met** and **Phe** on chlamydiae. Any negative influences that high amino acid concentrations might have on the host cells were excluded by WST-1, LDH and mitotracker assays (figure 3.2.1). To determine the effect of amino acids on an already established infection as well as on freshly infected cells, immunofluorescence (figure 3.2.2 and 3.2.4) and progeny infectivity (figure 3.2.3) assays were performed over the course of 15 days. Also, the condition of the monolayer of infected cells was recorded (figure 3.2.5) and the number of inclusions was counted over time (figure 3.2.6). Infectivity recovery assays were performed to assess if the treated bacteria were dead or could be reactivated (table 3.2.1). EM (figure 3.2.7) and immuno-EM pictures (figure 3.2.8) determined the ultrastructure of treated chlamydiae, and qRT-PCR (figure 3.2.9) verified their viability. To elucidate the expression pattern, transcription (figure 3.2.10) and protein expression (figure 3.2.11) of key chlamydial genes were determined as well as *in situ* presence of Hsp60 (figure 3.2.12).

4.2.1 Amino acids show no remarkable effect on host cells

As a prerequisite for further studies, it was verified that long-term exposure to increased concentrations of amino acids did not have negative effects on the host cells. Apart from the visual integrity that the monolayers maintained just like control cells, WST-1, LDH and mitotracker tests were performed.

The WST-1 test can be used for cell proliferation or cell viability assays. The cleavage rate of WST-1 (a water-soluble tetrazolium salt) by mitochondrial dehydrogenases correlates with the number of viable cells in the culture and can be determined via spectroscopy. The number of viable cells after 15 days was not changed by amino acid supplementation (figure 3.2.1 A). To detect increased cell death by cell lysis, the LDH test was performed. This colorimetric assay is based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant (figure 3.2.1 B). Also, no aberrant behavior of

amino acid treated cells could be determined. To finally exclude increased apoptotic events that would not release mitochondrial enzymes into the supernatant, the Mitotracker assay which contains a mitochondrial membrane potential-sensitive dye was performed. This dye passively diffuses across the plasma membrane and accumulates in active mitochondria. Both treated and untreated cells showed comparable mitochondrial potentials (figure 3.2.1 C). To determine amino acid induced influences on the host, genome wide DNA microarrays had been performed earlier (Braun, P., unpublished data). These experiments did not reveal differential gene regulation in mRNA levels of uninfected HeLa cells treated for 24 hours with 10mM of **Leu** compared to the untreated control. Proteome comparisons had also been performed (Braun, P., unpublished data) and did not show alterations in the protein expression pattern. We therefore conclude that elevated concentrations of amino acids do not influence the host cells and have a direct influence on intracellular chlamydiae.

4.2.2 Amino acids induce chlamydial persistence

Prolonged exposure to excess concentrations of the amino acids **Leu**, **Ile** and **Met** starting 2h after the infection completely suppressed bacterial growth (figure 3.2.4) and infectivity (table 3.2.1) and decreased inclusion counts over time (figure 3.2.6). The monolayers of infected and treated cells looked healthy and dense until day 15 (figure 3.2.5) unlike infected and untreated monolayers that detached after only a few days. There are two explanations for these results. Either, the bacteria were damaged and killed over time, or they underwent persistence.

Persistent chlamydiae have the following three characteristics: 1) They are **viable and metabolically active**. These features are typically demonstrated by the presence of the short-lived transcripts of bacterial 16S rRNA transcript. This gene is a widely used marker to provide evidence for the presence and viability of chlamydiae in clinical samples and in *in vitro* persistence models or to assess chlamydiostatic activities of antibiotics (Cox *et al.*, 2003;Khan *et al.*, 1996;Koehler *et al.*, 1997). 2) They are **non-infective** and form **aberrant bodies**. In contrast to acute infections, where chlamydiae are present either as EBs or RBs, persistent bacteria have retransformed into non-infective forms which, just like RBs, do not infect other host cells. 3) Persistent bacteria can be **reactivated** upon removal of the stimulus. In the case of antibiotics, persistent forms are shown to be refractory even to prolonged courses of treatment with antibiotics, because chlamydial growth resumes after completion of the treatment (Hooton *et al.*, 1990;Roblin and Hammerschlag, 1998;van der Willigen *et al.*, 1988;Xu *et al.*, 2000).

These three persistence properties were tested for amino acid treated chlamydiae: **1) Viability and metabolic activity**: The 16S rRNA transcript was found throughout the whole incubation period (figure 3.2.10). The amount of transcripts in relation to the host cell's GAPDH was also measured to determine the relative decrease over time (figure 3.2.9). It was therefore concluded that a small population of viable bacteria was present throughout the whole incubation period. **2) Abolished infectivity** and **3) possibility for reactivation:** Bacteria treated with excess concentrations of certain amino acids are not infectious. However, they form aberrant bodies (figure 3.2.7), and their infectivity can be reactivated by removal of excess amino acids, as shown in table 3.2.1. The longer the bacteria were treated, the longer it took them to be reactivated (chlamydiae treated for 5 days with high concentrations of amino acids could only be reactivated by 5 days growth in normal medium).

Interestingly, these effects are similar to *in vitro* effects of some antibiotics frequently used on *C. trachomatis in vitro*. Ciprofloxacin and ofloxacin, at concentrations higher than the minimum bactericidal concentration, fail to eradicate chlamydiae in an established infection (2–3 days post-inoculation), and induce a persistent infection throughout 20 days of culture. These non-infective bacteria can be reactivated upon removal of the antibiotics (Dreses-Werringloer *et al.*, 2000). Similarly, prolonged treatment with azithromycin does not reveal a clear elimination of *C. trachomatis* from host cells and generates a persistent state (Dreses-Werringloer *et al.*, 2001). Similar to these *in vitro* data, failure to treat chlamydiae has also been described *in vivo*, even after prolonged courses of treatment with antibiotics (Hooton *et al.*, 1990;Roblin and Hammerschlag, 1998;van der Willigen *et al.*, 1988;Xu *et al.*, 2000).

We conclude that natural nutritional supplements such as **Leu**, **Ile** and **Met** modulate chlamydial biology and cause formation of a small population of persistent chlamydiae. The effects of these amino acids appear not to be HEp-2 cell- or chlamydiae species-specific, as similar results were obtained with *C. pneumoniae* and in HeLa cells (unpublished data from our group).

4.2.3 Persistence induced by elevated amino acid concentrations shows an altered gene expression pattern

It is known that persistent chlamydiae show a modulated gene expression pattern when compared to the acute infection. Antibiotics as well as iron starvation result in bacteria that show an increase of the immunopathological Hsp60 (*groEL*), at the same time downregulating

other chlamydial genes, notably the immunoprotective MOMP (*omp1*) and *omcB*. These two regulations are thought to play a role in many autoimmune processes harming the host. An excess production of the immunopathological Hsp60 compared with a decrease in expression of the immuno-protective MOMP can contribute to hypersensitivity or autoimmunity (Gerard *et al.*, 1998;Morrison *et al.*, 1989a). The reason for the pathogenic and tissue-destroying infections is the immunological host reaction, namely the continuous secretion of inflammatory cytokines causing chlamydiae to go into persistence (Knight *et al.*, 1995).

Amino acid supplementation *in vitro* over a period of 15 days of amino acid exposure showed a suppression of synthesis of all of these proteins (figure 3.2.11) - Hsp60 was also shown to be downregulated (figure 3.2.11 and 3.2.12). If this pattern of reduced immunopathological Hsp60 could be mimicked *in vivo*, the danger of autoimmune damage (rheumatoid arthritis, trachoma) would theoretically be greatly reduced.

Summary part 2

Part 2 of this thesis suggests that proper use of amino acids, over a longer time period, combined with an antibiotic therapy, might represent a new and effective treatment strategy for chlamydial infections. The potential ability of amino acids to facilitate chlamydial eradication and to reduce immunopathologic disorders associated with infections might be of substantial interest, especially from the point of view of their proven safety as nutritional supplements.

Amino acids as therapy

4.3 Influence of amino acids on chlamydiae *in vivo*

Part 3 of this thesis examines the impact of amino acid supplementation in a mouse model. First, healthy animal were subjected to a diet consisting of normal feed and amino acid supplemented drinking water *ad libitum* (figure 3.3.1). Then, the effect that **Leu** supplemented drinking water had on a murine lung infection with *C. pneumoniae* was assessed over a period of three weeks (figure 3.3.3). Finally, the concentration of **Leu** in the blood serum was measured to see how it was affected by high amino acid concentrations in the drinking water (figure 3.3.4).

4.3.1 Ile + Leu do not show negative impact on mice organism

A negative systemic impact of administering supraphysiological amino acid concentrations to mice in the drinking water was excluded by feeding assays with healthy animals. **Ile** and **Leu** did not show negative effects (figure 3.3.1). **Met** and **Phe** were not further taken into consideration, as long term feeding lead to decreased weight and reduced water consumption of uninfected mice. Since **Phe** and **Met** are not toxic at these concentrations when given orally, a decrease in water consumption followed by general discomfort and weight loss might simply be due to an unpleasant gustatory experience for the animals.

4.3.2 Leu, administered orally, has an inhibitory effect on chlamydiae

To see the effect of **Leu** on a chlamydial infection *in vivo*, mice infected with *C. pneumoniae* were provided with drinking water saturated with **Leu**. After one week, lungs of infected control mice (drinking water containing no amino acids) showed the presence of about 1% of the bacteria used for the infection (figure 3.3.3). This number then increased about 8-fold after two weeks indicating that the bacterial development, i.e. replication, had not been completely inhibited by the immune system. Only after three weeks, the infection was overcome and no infectious particles could be recovered any more.

In **Leu** treated mice, a comparable amount of bacteria was present after one week of the infection (figure 3.3.3). However, after two weeks, a virtually complete lack of infectious bacteria was recorded, indicating that bacterial development and reinfection had been inhibited.

The absence of infectious particles in lungs of mice treated with **Leu**, however, does not prove that the chlamydiae are completely eliminated. Since high concentrations of amino acids induce persistence *in vitro* (see part 2 of this thesis), it is possible that the same happens *in vivo*. The transformation of RBs into EBs would be inhibited and the bacteria would form ABs with modified metabolism caused by the excess intake of **Leu**. So the formation of at least a small persistent population cannot be excluded. However, *in vitro* persistence is induced without the effect of an immune system (e.g. in cell cultures without adaptive immunity, no IFN-γ is produced by lymphocytes). We therefore infer that the combined effects of the immune system and the nutritional imbalance would more efficiently eliminate the bacteria.

4.3.3 Leu serum concentration is not significantly elevated after continuous ingestion of supplemented drinking water

Force feeding rats with **Leu** has been shown to cause an increase in **Leu** concentration in the serum up to 229% after 3 hours (Peng, Gubin, Harper, 1973). However, no statistically significant difference in blood **Leu** concentrations was observed while feeding supplemented drinking water *ad libitum* (figure 3.3.4). But as the serum concentrations were not specifically determined post prandially, and only small amounts of amino acids were ingested at each drinking event, we conclude that slight increases in **Leu** concentration were quickly equilibrated by the organism and too small to be measured with our method.

Anti-chlamydial effects can be elicited at concentrations lower than 10mM. Our previous work demonstrated a dose-dependent inhibition in *C. trachomatis* growth using concentrations ranging from 1 to 5 mM (section 1 of this thesis and Al Younes *et al.*, 2004;Coles and Pearce, 1987). Relatively small modifications of the total available amino acids in the growth medium have also been reported to lead to the development of less infective and aberrant chlamydial forms (Harper *et al.*, 2000). We conclude that a slight increase in the serum elevated the **Leu** concentration in the lung epithelium to a sufficiently high level to affect chlamydiae. These effects are self-limiting because **Leu** promotes its own disposal by activating the BCKDC (α-ketoacid dehydrogenase complex), tightly regulating the **Leu**-concentration in the blood. Thus, a continuous supply of **Leu** in the diet seems to be necessary for the anti-chlamydial effects.

In summary, an inhibitory influence of **Leu** on lung infections with *C. pneumoniae* was observed. Constant administration of this chlamydiostatic substance for a long period of time therefore might have the potential for treating acute chlamydial infections, possibly assisting antibiotic therapies.

4.4 Conclusion

4.4.1 Inhibition of chlamydial development by elevated concentrations of branched chain amino acids is caused by lack of Val

By comparing the effects that different amino acids have on chlamydial development, it was shown that the branched chain amino acids were most effective. Recently, our group showed that **Val** abrogated the inhibitory effect of **Leu**, **Ile**, **Met** and **Phe** (Braun, Al-Younes, Gussmann *et al.*, submitted). Therefore, a role of a common transporter was examined which would either be situated in the inclusion membrane, the bacterial outer membrane or the bacterial inner membrane.

So far, neither homologies for specific transport systems for amino acids in the membranes of inclusions have been described (Rockey *et al*., 2002), nor is anything known about pores in the inclusion membrane or free diffusion of small molecules such as amino acids (Heinzen, Hackstadt, 1997). However, in other intracellular parasites such as *Toxoplasma gondii* and *Plasmodium falciparum*, pores have been identified to allow passive transport of small molecules (Schwab *et al*., 1994; Desai *et al*., 1993). A competition at the inclusion membrane was therefore excluded.

The bacterial outer membrane possesses pore-forming proteins such as MOMP or PorB (Jones *et al*. 2000, Kubo and Stephens, 2000). This would allow the free diffusion of compounds such as amino acids across the outer membrane. A competition based on transporter activity is therefore assumed to take place in the inner membrane of the chlamydiae.

The underlying mechanism could recently be identified: Our group showed that the heterologously (in *E. coli*) expressed transporter for branched chain amino acids, BrnQ, encoded by both *C. trachomatis* and *C. pneumoniae* could be competitively inhibited by high concentrations of **Leu**, **Ile**, **Met** and **Phe** thereby leading to a reduction in **Val** uptake and a lack of **Val** concentrations within the bacteria (Braun, Al-Younes, Gussmann *et al.*, submitted). The addition of **Val** compensated this effect. Other amino acids such as **Ser**, **Gly** and **Thr**, all hydrophilic and most likely not transported by the BrnQ-transporter, did not competitively inhibit **Val** transport.

The anti-chlamydial effect of **Met** is most probably also based on the BrnQ-transporter inhibition - strong in the case of *C. trachomatis*, but considerably weaker with *C. pneumoniae*. We assume that this is due to different specificities for **Met** of the BrnQ-

transporters of the two species (59% homologous): The *C. trachomatis* BrnQ-transporter seems to have a high affinity for **Met** and can be inhibited by this amino acid. *C. pneumoniae* growth, on the other hand, is not strongly inhibited by high concentrations of **Met**, indicating a rather weak affinity. *C. pneumoniae* (not *C. trachomatis*) also possesses the **Met**-binding protein MetQ (*cpn0279*) of a respective ABC-transporter (*metD*-Locus, Merlin *et al*, 2002). It is therefore reasonable to assume that the uptake of **Met** is mostly mediated by the ABC-transporter and that the organism is less dependent on the BrnQ-transporter (resulting in decreased affinity for the uptake of **Met**). This transporter is therefore less inhibited by an increased concentration of this amino acid.

Ingestion of an unbalanced meal containing large amounts of **Leu** induces a marked decrease in the body concentration of free **Val** and **Ile** (Peng *et al*., 1973). This phenomenon, termed the "**leucine paradox**" (Rogers *et al*., 1967), can be explained in part by promotion of the BCAA oxidation through activation of the branched-chain α-keto acid dehydrogenase complex (BCKDC) by dephosphorylation. This activation then also depletes the cells of **Ile** and **Val**. On the other hand, **Leu** administration also stimulates protein synthesis and inhibits protein degradation, both of which also impact the **Val** and **Ile** concentrations.

So in our model, two effects lead to a nutritional deficient condition: 1. Elevated **Leu** concentration in the cells hosting chlamydiae resulting in the inhibition of **Val**-transport via the BrnQ-transporter and 2. the general decrease in **Val** concentration in the serum via BCKDC activation, stimulated protein synthesis and inhibited protein degradation. The resulting lack of **Val** inhibits chlamydial growth *in vivo*.

4.4.2 The differing expression profile of amino acid induced persistence might be useful for therapeutic applications, alone or in combination with antibiotics

Antibiotics are often not the ideal treatment against chlamydiae. It was shown that chlamydial infections in some cell types are refractory to antibiotic treatment, for example human monocytes and lymphocytes. These circulating blood cells could be the vehicle for transfer of the pathogen from the respiratory organs (primary infection) to the vascular cells. There, a reinfection can take place, promoting the development of chronic diseases (Gieffers et al., 2001;Yamaguchi et al., 2003). There are also general arguments against antibiotic treatment. Some drugs should not be taken by pregnant or breastfeeding women or by individuals under the age of 16. The use of antibiotics can also cause significant side effects, for example gastrointestinal intolerance (Guaschino et al., 2003;Marrazzo and Stamm, 1998) – over 20%

of patients abort the treatment, although some antibiotics need to be taken for up to three weeks, to prevent recurring infections (Roblin et al., 1994).
Usage of amino acids which do not harm the patient (Kadowaki and Kanazawa, 2003) could represent a therapeutic method which is more effective in completely curing chlamydial infections. Amino acids are consumed in large amounts by body builders and athletes before and after strength training workouts, promoting greater gains in muscle mass and strength. Amino acids can be taken from protein-rich diets or, alternatively, they can be purchased as tablets, powders, capsules or liquids in the form of individual amino acids. Excessive amounts of free amino acids can be taken orally or can be administered through injections, and they are safe with no known side effects (Kadowaki and Kanazawa, 2003). Ingestion of exogenous amino acids to increase plasma and tissue concentrations could trigger or accelerate treatment of chlamydial diseases.

The protein expression level in persistent chlamydiae following treatment with high concentrations of amino acids differs from the antibiotics-induced pattern, mainly in the absence of Hsp60. This protein is the major cause for immunostimulatory processes in the host and is the underlying cause of diseases such as trachoma, refractory arthritis or chronic lung diseases (COPD). Amino acid treatment could therefore induce persistence with a non-immunogenic profile which would be less harmful to the host, avoiding autoimmune sequelae. Alternatively, a combination treatment with antibiotics could be more effective than both methods alone, damaging the bacteria to a level where reactivation is not any more possible - further experiments would need to be performed to confirm this hypothesis. This idea has been filed as a patent by our group (Al Younes *et al*., 2003).

4.4.3 Modified amino acid molecules might have a more pronounced effect on chlamydiae *in vivo*

For several reasons, single amino acids might not be the ideal compounds to treat chlamydial infections. Therefore, other molecules eliciting similar responses could be more useful:
In the organism, the concentrations of **Leu** and other branched chain amino acids (BCAAs) are tightly regulated. Without this regulation, increased concentrations of these amino acids in the blood are toxic. Maple syrup urine disease for example is caused by a long-term building up of unprocessed BCAAs through genetic defects in the enzyme which decarboxylates the carbon skeletons (α-ketoacid dehydrogenase complex, BCKDC). Affected infants suffer from poor feeding, vomiting, lack of energy and developmental delay. The distinctive sweet odor

of the urine, much like burned caramel, gives the condition its name. Today, individuals can lead normal lives if a certain BCAA-poor diet is strictly followed.

In healthy individuals, the keto acid derived from leucine (α-ketoisocaproate) inhibits BDK (BCKDC kinase) which normally inhibits the BCKDC by phosphorylation. This leads to the degradation of **Leu** and the other BCAAs, limiting the effect that elevated **Leu**-concentrations can have on chlamydiae as the concentration can only be elevated shortly and to a limited extent. In order to increase efficacy of a treatment with **Leu**, elevated concentrations of this amino acid would need to be induced. One solution could be to inhibit BDK which reduces degradation of **Leu** by interfering with BCKDC activity. Due to the negative effects of long-term elevated BCAA concentrations (as in maple syrup urine disease), this would of course only be possible in short-term treatments.

However, since the anti-chlamydial effects are based on transporter competition, other substances with corresponding effects might be used. From the *in vitro* and *in vivo* results, we conclude that possible chemicals for the treatment of acute chlamydial infections must have the following properties:

1. Limited toxicity. As the treatment would last for several days or even weeks in order to cover the full chlamydial development cycle, negative implications of elevated concentrations of this compound must be minimized. Also, a risk-benefit ratio regarding side-effects would need to be assessed.

2. Sufficient transport to the site of action. In order to reach the infected cells, the compound would need to be stable throughout the digestive tract (in the case of oral administration). Then, passive diffusion through the inclusion membrane and outer chlamydial membrane must occur, in order to reach the site of action - the inner chlamydial membrane – at high concentrations.

3. High affinity transport by BrnQ. If the compound blocks the BrnQ-transporter it would inhibit **Val**-transport into the cytoplasm of the bacteria. This lack of **Val** would then lead to chlamydial starvation, block of RB to EB conversion and elimination of most infective bacteria. If as *in vitro* a small population of persistent chlamydial forms is induced, this population would not cause hypersensitivity reactions in the host because of their reduced Hsp60 levels. A combination treatment with conventional antibiotics might even completely clear the infection.

4. Favorable pharmacokinetic properties. In order to be more efficient and to ensure a convenient application with long time spans between administrations the compound would need to be stable in the blood.

4.5 Outlook

The data presented in this thesis are a basis for further studies. The experimental setups – cell culture methods, growth inhibition assays, electron microscopy and animal study assays which have been applied here could be used.
The first approach would be to see if amino acids that inhibit chlamydial growth together with antibiotics cause less bacteria to go into persistence – or maybe even eliminate them completely. If this is the case, further animal studies would be needed to see if a combination therapy can even more efficiently eliminate the chlamydial infection.

The second type of follow-up studies would include the search for amino acid analogues or other compounds which also inhibit the BrnQ-transporter and cause **Val**-depletion in chlamydiae, but at the same time do so more efficiently than **Leu**.

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