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Characterization and intracellular trafficking pattern of vacuoles containing *Chlamydia pneumoniae* in human epithelial cells

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Summary

Chlamydiae are obligate intracellular pathogens that reside within a membrane-bound vacuole throughout their developmental cycle. In this study, the intraphagosomal pH of *Chlamydia pneumoniae* (*Cpn*) was qualitatively assessed, and the intracellular fate of the pathogen-containing vacuole and its interaction with endocytic organelles in human epithelial cells were analysed using conventional immunofluorescence and confocal microscopy. The pH-sensitive probes acridine orange (AO), LysoTracker (LyT) and DAMP did not accumulate in the bacterial inclusion. In addition, exposure of cells to bafilomycin A1 (BafA1), a potent acidification inhibitor, did not inhibit or delay chlamydial growth. The chlamydial compartment was not accessible to the fluid-phase tracer Texas Red (TR)-dextran and did not exhibit any level of staining for the late endosomal marker cation-independent mannose-6-phosphate receptor (Ci-M6PR) or for the lysosomal-associated membrane proteins (LAMP-1 and -2) and CD63. In addition, transferrin receptor (TfR)-enriched vesicles were observed close to *Cpn* vacuoles, potentially indicating a specific translocation of these organelles through the cytoplasm to the vicinity of the vacuole. We conclude that *Cpn*, like other chlamydial spp., circumvents the host endocytic pathway and inhabits a non-acidic vacuole, which is dissociated from late endosomes and lysosomes, but selectively accumulates early endosomes.

Introduction

The genus *Chlamydia* represents obligate intracellular pathogens that reside in a membrane-bound compartment called an inclusion body, which has a unique biphasic developmental cycle that alternates between an infectious, metabolically inactive elementary body (EB) and a non-infectious, metabolically active reticulate body (RB). Four species of *Chlamydia* have been recognized: *C. trachomatis*, *C. psittaci*, *C. pneumoniae* (*Cpn*) and *C. pecorum* (Peeling and Brunham, 1996). *Cpn* is considered to be a significant cause of respiratory tract infections, such as pneumonia, asthma, bronchitis, sinusitis and sarcoidosis, and has also been implicated in other non-pulmonary diseases, such as atherosclerosis, reactive arthritis, meningoencephalitis and Alzheimer's disease (Balin *et al*., 1998).

Intracellular pathogens have evolved certain strategies that enable them to survive and multiply even in the face of efficient host defence mechanisms. Some pathogens, such as *Coxiella burnetii*, *Leishmania* spp. (Garcia-del Portillo and Finlay, 1995a) and *Cryptococcus neoformans* (Levitz *et al*., 1999), are adapted to live in the hostile low pH of the phagolysosome. Other microorganisms avoid acidification and subsequent degradation by residing in a modified parasitophorous vacuole, such as *Legionella pneumophila*, *Toxoplasma gondii*, *Mycobacterium* spp. (Garcia-del Portillo and Finlay, 1995a), the human granulocytic ehrlichiosis agent (Webster *et al*., 1998), *Ehrlichia chafeensis* (Barnewall *et al*., 1997), and *Histoplasma capsulatum* (Strasser *et al*., 1999). A third group of organisms includes *Listeria monocytogenes*, *Trypanosoma cruzi* and *Shigella flexneri*; members of this group escape the phagosome to the cytosol to avoid the consequences of fusion with lysosomes (Garcia-del Portillo and Finlay, 1995a).

The characterization of *C. trachomatis*- and *C. psittaci*-containing vacuoles and their fate in the host cell have been the subject of intensive research. More than two decades ago, Friis (1972) was the first to conclude that phagosomes harbouring *C. psittaci* do not fuse with lysosomes within L cells. This notion was supported by several subsequent experiments performed mainly on macrophages infected with the same species (Wyrick and Brownridge, 1978; Zeichner, 1982; 1983). Similarly, more recent studies on the trafficking routes of *C. psittaci* or *C. trachomatis* in the target epithelial cells have pointed to a complete disconnection of chlamydial inclusions from the endocytic pathway and the lack of phagosomal acidification (Heinzen *et al*., 1996; Schramm *et al*., 1996; Taraska *et al*., 1996). However, a single group suggested an interaction of

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early and late endosomes with *C. trachomatis* vacuoles in epithelial cells infected under the same experimental conditions, but with a higher multiplicity of infection (MOI) (van Ooij *et al*., 1997).

To date, there have been no reports analysing the intracellular accommodation and the maturation of vacuoles inhabited by *Cpn*. To address this issue, we assessed the intraphagosomal pH of *Cpn* in the HEp-2 epithelioid cell line after staining with different lysosomotropic dyes, using conventional epifluorescence and confocal laser scanning microscopy. We also characterized the bacteria-containing inclusion at different post-infection time points using a fluid-phase tracer, a variety of antibodies directed against endosomal and lysosomal markers and pharmacological agents to follow the trafficking of this pathogen in either HEp-2 or HeLa cells. This enabled us to draw comparisons of the intracellular fate of *Cpn* and its interaction with the endocytic cascade of host cells with other species of *Chlamydia* and also with other intracellular pathogens.

Results

Qualitative assessment of the pH of Cpn-*containing vacuoles*

The intravacuolar pH of *Cpn* in HEp-2 cells was examined at middle (40 h after infection) and late (70 h after infection) stages of the bacterial developmental cycle with various pH-sensitive probes. At these time points, the red fluorescence of acridine orange (AO), indicative of an acidic environment, was excluded from the *Cpn*-harbouring vacuole (Fig. 1A). This staining pattern was not altered when cells were labelled with a combination of AO and LyT, both of which stain lysosomes red (data not shown). Likewise, LyT alone (data not shown) and DAMP (Fig. 1B) were sequestered in acidified vesicles of the host cell cytoplasm, but not in the chlamydial vacuole. These experiments collectively provide evidence that *Cpn* is present and multiplies within a non-acidified compartment of epithelial cells.

Effect of BafA1 on chlamydial growth

To address further the nature of the *Cpn* vacuole, 10 nM BafA1, a potent inhibitor of the vacuolar proton ATPase (H^+-ATPase) responsible for acidifying endocytic vesicles (Mukherjee *et al*., 1997), has been used. This concentration was found to inhibit the acidification of intracellular organelles greatly, as confirmed by AO labelling (Fig. 1C and D). As described in the legend to Fig. 2, cells were not incubated with the drug for periods exceeding 15 h after infection because of toxic effects exerted by BafA1, which resulted in the detachment of some cells (data not shown).

Application of BafA1 1 h before infection up to 15 h after infection neither inhibited the formation nor delayed the

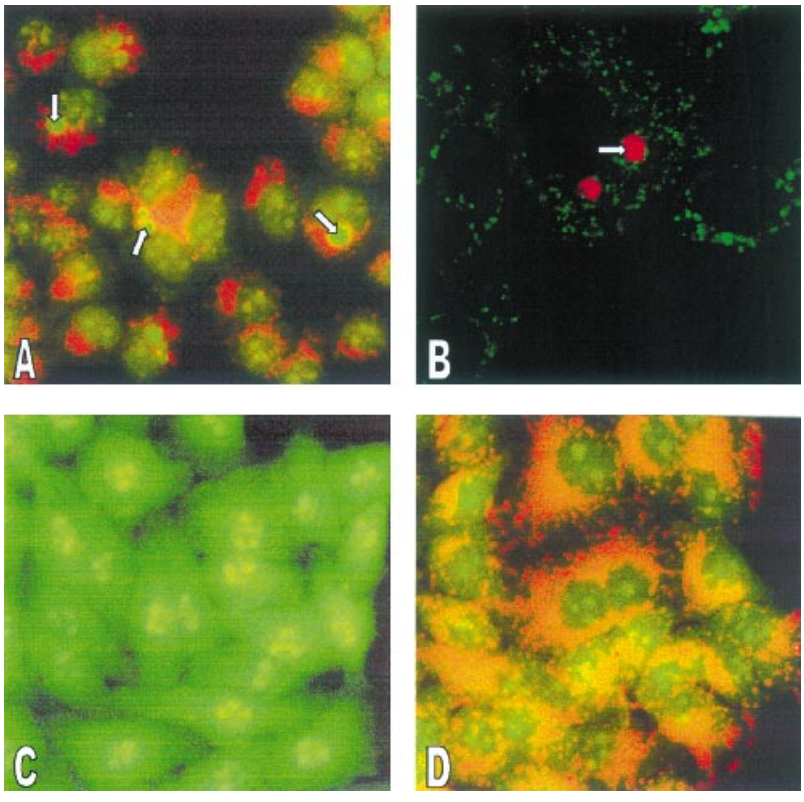

Fig. 1. Determination of pH of *Cpn* vacuoles and the effect of BafA1 on the acidification of intracellular compartments. HEp-2 cells infected for 40 h were incubated with either AO (A) or DAMP (B) and examined using fluorescence microscopy. Chlamydial inclusions (arrows) did not accumulate detectable amounts of the lysosomotropic weak bases, suggesting non-lowered pH. On the other hand, treatment of HEp-2 cells with 10 nM BafA1 (C) greatly inhibits acidification and, thus, diminishes the orange–red fluorescence of AO in acidic organelles compared with the control non-treated cells (D).

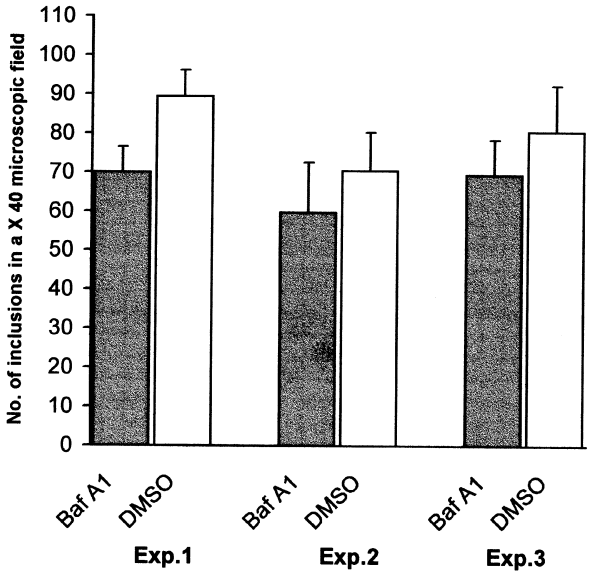

Fig. 2. Effect of BafA1 on the formation of *Cpn* infection in HEp-2 cells. Host cells were preincubated for 1 h with 10 nM BafA1, infected with *Cpn* in the presence of BafA1, washed and then incubated for an additional 15 h at 37°C and 5% CO_2 in the presence of the same drug concentration. By 15 h after infection, BafA1 was removed by extensive washing with fresh IM, and monolayers were then incubated for a further 20 h in the absence of BafA1. Control cell cultures were treated and infected as described in the presence of comparable amounts of DMSO alone, in which BafA1 was dissolved. At the end of these experiments (35 h after infection), cells were fixed and stained for chlamydial inclusions. In each experiment, at least three replicates of tests and controls were made.

growth of chlamydial vacuoles when compared with DMSO-treated cells (data not shown). However, owing to loss of infected cells, the overall number of inclusions in BafA1-treated monolayers was smaller compared with those within monolayers exposed to DMSO alone (Fig. 2); this was probably a consequence of the combined effects of drug treatment and infection on the target cells. These data suggest that the absence of acidification, even during the very early stages, is compatible with the normal development of *Cpn* inclusions. Moreover, the use of the ion pump inhibitor BafA1 might indirectly indicate that the H^+-ATPase is either absent from or inactive in the chlamydial phagosomal membrane.

Distribution of the fluid-phase tracer TR-dextran

TR-dextran can be internalized into the cells by pinocytosis and delivered to and retained by various compartments of the endocytic pathway (Swanson, 1989). As shown in Fig. 3A, dextran accumulated efficiently in HEp-2 cells and showed a dotted to vacuolar appearance discrete from *Cpn* vacuoles. The absence of the fluorescent marker indicated that inclusions did not fuse with pinosomes and/or endocytic vesicles containing the tracer.

Early endosomes are intimately associated with Chlamydia *vacuoles*

TfR is a 180 kDa glycoprotein consisting of two identical disulphide-linked 90 kDa subunits (Schneider *et al.*, 1984). This receptor is expressed on the cell surface and on the early endosomes, which are the first compartments

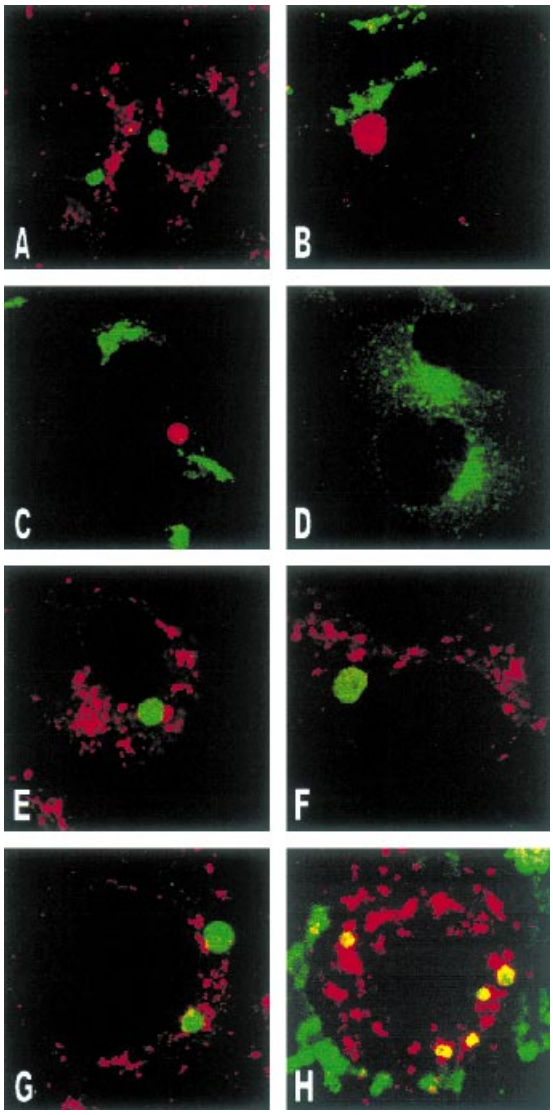

Fig. 3. *Chlamydia pneumoniae* vacuoles in epithelial cells do not sequester TR-dextran and avoid interaction with late endosomes and lysosomes.
A. Immunofluorescence staining of HEp-2 cells that have been infected with *Cpn* (green) and incubated with TR-dextran (red).
Distribution of the late endosomal marker Ci-M6PR (green) in HeLa (B) and HEp-2 (C) cells inoculated with the pathogen (red) for 40 h.
D. Normal distribution of Ci-M6PR in uninfected HeLa cells.
Distribution of the lysosomal markers (red) LAMP-1 (E), LAMP-2 (F) and CD63 (G) in HEp-2 cells infected with the bacteria (green) for 40 h.
H. Co-localization of LAMP-1 (red) with *N. gonorrhoeae* (green)-containing phagosomes. Intracellular gonococci that co-localize with LAMP-1 appear more yellow than green.

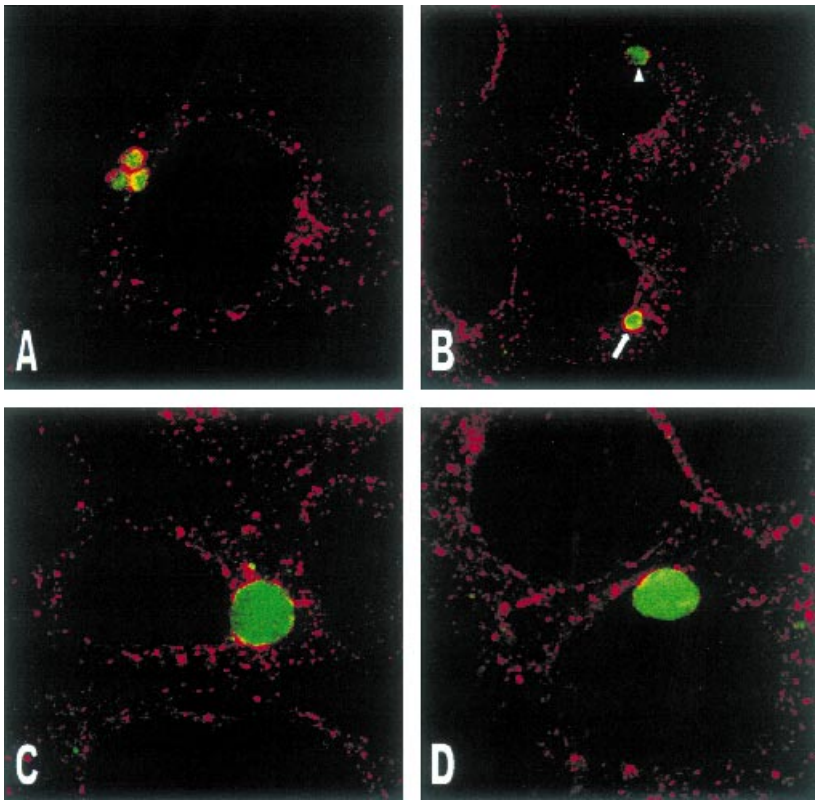

Fig. 4. Double immunofluorescence labelling of TfR and *Cpn*. HEp-2 cell monolayers on coverslips were infected for 20 h (A), 40 h (B) and 70 h (C and D), dually stained with antibodies specific to TfR and to the bacteria and then visualized on a confocal microscope. Vesicles containing TfRs were found concentrated as aggregates close to the inclusion (A, arrow in B and C) or had a granular pattern of distribution surrounding the bacterial vacuole (arrowhead in B). Note that a slight association of these receptors was observed in a subpopulation of cells that were infected for 70 h (D).

receiving endocytosed materials. Early endosomes usually comprise two distinct compartments: the sorting endosomes, part of the endocytic pathway, which contain internalized ligands that will be delivered to lysosomes and degraded; and the recycling endosomes, which contain molecules to be returned back to the cell surface (Mukherjee *et al*., 1997).

To shed light on the distribution of TfR in relation to *Cpn*-containing inclusions, HEp-2 cultured cells were infected for 20, 40 or 70 h and then stained for TfR and pathogens. Based on microscopical observations, TfR-enriched vesicles are smaller in size when compared with lysosomes and are abundant within the cytoplasm of HEp-2 cells. Indeed, two staining patterns of TfR were observed, particularly at 20 h and 40 h after infection. TfR-positive structures were found either as aggregates intimately associated with the parasitophorous vacuoles, staining a considerable part of its circumference, as seen in Fig. 4A and B (arrow), or exhibited a granular discontinuous fluorescence distribution, which was also closely adjacent to the inclusion (Fig. 4B, arrowhead). The granular pattern of TfR labelling was sustained in most of the cells infected for 70 h (Fig. 4C), whereas a small population of infected cells showed a reduced accumulation of TfR-containing vesicles at the periphery of the inclusion, as demonstrated in Fig. 4D. In general, this significant concentration of TfR adjacent to the inclusion may reflect a degree of translocation of early endosomes within the cytoplasm to the proximity of bacterial vacuoles, suggesting a possible interaction between these vacuoles and early endosomes. Because of the absence of confluent circumferential staining, TfR is very likely not a constituent of the vacuolar membrane, at least not at the time points examined.

Cpn *inclusions do not interact with late endosomes*

The 275 kDa Ci-M6PR plays an important role in the transport and targeting of newly synthesized mannose residues-containing enzymes from the Golgi to the lysosomes via the late endosomes. In addition to its presence on late endosomes, this receptor is also found on the plasma membrane and in the trans-Golgi network (Mukherjee *et al*., 1997).

A recent study revealed that Ci-M6PR is not retrieved and does not accumulate in late endosomes of HEp-2 cells (Hirst *et al*., 1998). Therefore, we analysed the interaction of *Cpn* inclusion with late endosomes in HeLa epithelial cells that were used successfully in co-localization experiments with Ci-M6PR as a marker for late endosomes (Garcia-del Portillo and Finlay, 1995b; Pizarro-Cerdá *et al*., 1998). HeLa monolayers were infected for 20 h, 40 h or 70 h and then processed for confocal microscopy as described for HEp-2 cells. Figure 3B shows that the *Cpn* vacuole has no detectable association with Ci-M6PR-positive compartments at any time point assessed.

Although Ci-M6PR does not accumulate in late endosomes of HEp-2 cells (Hirst *et al*., 1998), we examined whether *Cpn* can intersect the MPR biosynthetic pathway in this cell type at either 20 h, 40 h or 70 h after infection. The results of fluorescence distribution in infected HEp-2 cells were analogous to those in HeLa cells (Fig. 3C).

Interestingly, the distribution pattern of the Ci-M6PR was altered in infected HEp-2 and HeLa cells, similar to that observed previously in *C. trachomatis*-infected HeLa cells by van Ooij *et al*. (1997). In uninfected cells, Ci-M6PR showed dotted cytoplasmic staining as well as immunofluorescent labelling concentrated in a perinuclear region, which may be characteristic of Golgi staining (Fig. 3D). In infected cells, the dotted cytoplasmic labelling strongly decreased, and the Ci-M6PR staining was mainly localized in the perinuclear region (Fig. 3B and C). Overall, these experiments reveal that *Cpn* is present in a vacuole that evades the fusion with late endosomes (as confirmed in HeLa cells) and does not intersect the Ci-M6PR loading pathway in HEp-2 cells.

Cpn-*containing inclusion avoids fusion with lysosomes*

LAMP and CD63 proteins are considered as major components of the lysosomal membranes (Berditchevski *et al*., 1995; Mukherjee *et al*., 1997). Microscopic examination of cells infected for different periods of time demonstrated no co-localization of bacteria and LAMP-1, -2 and CD63 (Fig. 3E–G). The absence of these lysosomal components from the chlamydial compartment membrane suggests that it does not share the characteristics of a phagolysosome and remains discrete from the late stages of the endocytic traffic.

To exclude the possibility that the absence of fusion of *Cpn*-containing vacuoles with the endocytic compartments may be a result of abnormal endocytic trafficking in these non-professional phagocytes, HEp-2 cells were infected with *Neisseria gonorrhoeae*, previously reported to be trafficked along the endocytic pathway of Chang epithelial cells and HL-60 monocytic cells (Hauck and Meyer, 1997). Confocal microscopic analysis demonstrated the co-localization of LAMP-1 with the membrane of neisserial phagosomes (Fig. 3H), indicating the fusion of lysosomes with the bacteria-containing phagosome. Thus, the fusion of bacteria-containing vacuoles with endocytic compartments was determined by the pathogen rather than by the host cell.

Influence of chloramphenicol on fusion of Cpn *inclusions with lysosomes*

To test the importance of ongoing chlamydial protein synthesis in the inhibition of fusion of the parasite vacuole with the degradative compartments, HEp-2 cell cultures were infected with *Chlamydia* for 36 h, incubated with chloramphenicol, an inhibitor of bacterial protein synthesis, and then stained for bacteria and LAMP-1. The concentrations of chloramphenicol ranged from 50 to 200 $\mu g\ ml^{-1}$, which were applied for a further 24 h, 48 h and 72 h. As a clear consequence of the chloramphenicol treatment, the inclusions were arrested in their growth, and a degree of destruction and release of chlamydial particles occurred.

Microscopic examination of the preparations did not detect co-localization of LAMP-1 with the chlamydial vacuoles exposed to any of these drug concentrations for 24 h or 48 h. On the contrary, when infected cell cultures were treated with the same concentrations for 72 h, co-localization of LAMP-1 with chlamydiae was detected in $\approx$ 25% of the examined infected cells (Fig. 5). Although *Cpn*–lysosome interactions were observed in some cells after exposure to chloramphenicol for 72 h, the data presented provide evidence that inhibition of ongoing protein synthesis of intracellular *Chlamydia* did not dramatically alter the trafficking pattern of the parasitophorous vacuole.

Discussion

In the current work, we have examined the nature of the internal environment and the intracellular pathway of *Cpn* vacuoles mainly in HEp-2 human epithelial cells using conventional immunofluorescence and confocal laser microscopy. This technique has been used successfully before to describe the intracellular route of *C. trachomatis* (Heinzen *et al*., 1996; van Ooij *et al*., 1997), *C. psittaci* (Taraska *et al*., 1996), *Salmonella typhimurium* (Rathman *et al*., 1997), *Brucella abortus* (Pizarro-Cerdá *et al*., 1998), *C. burnetii* (Heinzen *et al*., 1996), *E. chaffeensis* (Barnewall *et al*., 1997) and others.

One parameter determined was the pH of the *Cpn* inclusion, which was clearly not acidic. A similar result has been obtained for *C. trachomatis*, which also does not reside in an acidic compartment (Heinzen *et al*., 1996; Schramm *et al*., 1996). Although the mechanism of how *Cpn* prevents acidification is not known, the exclusion of the proton pump H^+-ATPase from the inclusion is the proposed mechanism of *C. trachomatis* (Heinzen *et al*., 1996) and *M. tuberculosis* (Sturgill-Koszycki *et al*., 1994). The proton pump H^+-ATPase is acquired from organelles of the endocytic cascade during maturation. Its absence from the *Cpn* inclusion would be consistent with the notion that the *Cpn* compartment is distinct from other host cell compartments. The prevention of acidification is very likely a survival strategy of *Chlamydia* spp., as *C. trachomatis* and *C. psittaci* do not survive, e.g. in the monocytic cell line THP-1 and in dendritic cells, the inclusions of which do acidify (Ojcius *et al*., 1997; 1998).

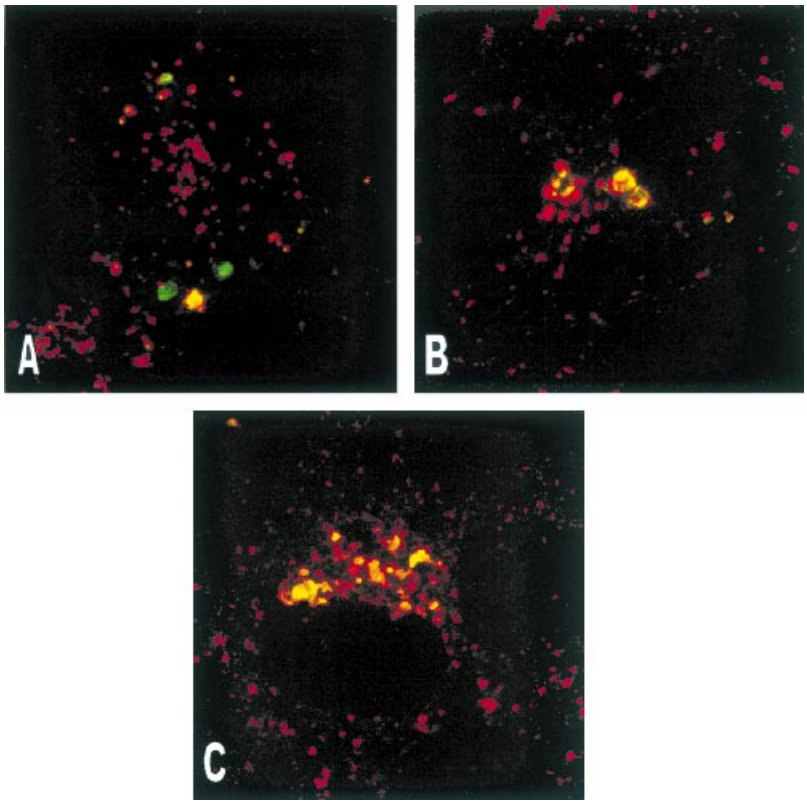

Fig. 5. Co-localization of LAMP-1 with *Cpn* in chloramphenicol-treated host cells. HEp-2 cells that had been infected for 36 h were incubated with 200 μg ml^{-1} chloramphenicol for an additional 72 h. At the end of the incubation time, cells were stained for LAMP-1 and pathogens as described in *Experimental procedures* and examined using confocal microscopy. Approximately 25% of the infected cells showed co-localization (yellow) of the lysosomal marker with the inclusions (A and B) and with chlamydial particles (C), which might be released after the destruction of some inclusions.

In earlier experiments testing acidification of *C. trachomatis*-containing vacuoles, Schramm *et al*. (1996) found that bacteria were not present in an acidified environment, and the pH remained above 6.0 at 2 h, 4 h and 12 h after infection. Similarly, the absence of acidity at 22 h for *C. trachomatis* (Heinzen *et al*., 1996) and at 40 h and 70 h after infection, as observed in the current work, strongly suggests that these bacteria are not trafficked to acidified compartments over time. For some intracellular pathogens, such as *S. typhimurium* and *B. suis*, the early acidification serves as an inducer for the expression of genes essential for intraphagosomal survival and for pathogen replication (Alpuche *et al*., 1992; Porte *et al*., 1999). Such a drop in pH, if any, could possibly induce the expression of genes controlling the life-cycle of *Chlamydia*. However, the neutralization of all acidic compartments by BafA1 did not influence the growth of *Cpn*. Thus, acidification is clearly not a signal for the transition from EB to RB in *Cpn*.

A local accumulation of early (Scidmore *et al*., 1996a; Taraska *et al*., 1996; van Ooij *et al*., 1997) and late endosomes (Taraska *et al*., 1996; van Ooij *et al*., 1997) has been shown close to the bacterial vacuole in studies published earlier for *C. trachomatis* and *C. psittaci*. However, Heinzen *et al*. (1996) demonstrated an absence of late endosomes near the vacuole. Among these studies, a single report (van Ooij *et al*., 1997) suggested a significant interaction between early and late endosomes and this vacuole. In comparing the trafficking pattern of *Cpn* with those described previously for other chlamydiae, early endosomes are also closely concentrated around the chlamydial compartment, but not late endosomes. We believe that the redistribution of TfR vesicles to the inclusion is probably a specific process induced by the pathogen, as it was observed in all infected cells examined, while the late endosomes and lysosomes did not accumulate in a similar way. It is also very unlikely that the local concentration of TfR-containing vesicles resulted from displacement by the growing inclusion, as the most considerable overlap of TfR staining and the chlamydial vacuole was noticed in cells infected for 20 h or 40 h. At these time points, the vacuoles are small in size compared with the inclusions observed 70 h after infection, at which time a subset of inclusions showed a slight overlap with the TfR staining (Fig. 4D). Whether this probable interaction between parasite vacuole and TfR-enriched vesicles implies a functional relationship remains to be determined.

Our findings obtained with confocal microscopy demonstrated that early endosomes do not fuse with the inclusion and show a punctate or aggregate pattern of distribution around the pathogen vacuole. These results correlate with and strengthen those obtained by other investigators using confocal and electron microscopy. For instance, no signs of fusion of these vesicles have been documented at

18 h (Scidmore *et al*., 1996a) and 24 h after infection (Taraska *et al*., 1996) or even at very early phases (30 min, 2 h or 4 h) of infection (Hackstadt *et al*., 1998). The recruitment of these vesicles and their failure to fuse might reflect two independent steps of the maturation process, whereby the fusion rather than the docking is actively prevented by the pathogen. Inhibition of the early endosomal fusion might be crucial for the subsequent fate of the inclusion, as it prevents the delivery of signalling molecules to the inclusion membrane, which may be required for the interaction with late endosomes.

Several theories have been evoked to explain how *Chlamydia* can influence the maturation of their inclusion and inhibit phagolysosome formation. The mode of entry is considered to play a role, as opsonized *C. psittaci* underwent lysosomal fusion (Friis, 1972; Wyrick and Brownridge, 1978). In contrast to this, Scidmore *et al*. (1996b) recently demonstrated that antibody-coated *C. trachomatis* EBs internalized via the Fc receptor, avoiding lysosomal compartments in HeLa and CHO cells. Other reports indicated that factors responsible for the lack of fusion with lysosomes are present on the EB, as internalization of heat-treated EBs, but not UV- or chloramphenicol-treated EBs or even non-treated purified EB envelopes, led to fusion with lysosomes (Friis, 1972; Eissenberg *et al*., 1983; Prain and Pearce, 1989). However, when *C. trachomatis* EBs were first allowed to enter their host cells before the addition of chloramphenicol, virtually all of the EB-containing vacuoles fused with lysosomes, indicating that early protein synthesis of bacteria is required to avoid lysosomes (Scidmore *et al*., 1996b). In contrast to *C. trachomatis*, *Cpn* demonstrates relatively low infectivity for cell culture, even when highly concentrated inocula of EBs were applied using centrifugation. Therefore, we were not able to study the consequences of protein synthesis inhibition at very early phases of infection, because the microscopic detection of EBs internalized per cell, which are $\approx 0.2\ \mu m$ each, was problematic. However, we were able to block the continuous protein synthesis at a middle stage (36 h after infection), which led to fusion of only $\approx 25\%$ of the inclusions with lysosomes. Thus, our data, along with those reported by Scidmore *et al*. (1996b), suggest the importance of early protein synthesis in the modification of the inclusion and, thus, in segregation from the degradative pathway.

It is becoming increasingly evident that proteins of the inclusion membrane are provided, at least in part, from the chlamydiae themselves. Antibodies directed against membranes isolated from *C. psittaci*-infected cells were capable of staining the cytoplasmic surface of the inclusion membrane (Taraska *et al*., 1996). Similarly, Rockey and Rosquist (1994) identified three chlamydial proteins in lysates of *C. psittaci*-infected cells but not in lysates of uninfected cells or EBs. Immunofluorescent labelling of infected cells with sera raised against these proteins demonstrated specific staining of the vacuolar membrane. Other proteins of chlamydial origin have recently been identified and localized to this membrane (Rockey *et al*., 1995; Bannantine *et al*., 1998a,b). Again, these proteins were present uniquely in *Chlamydia*-infected cells, but not in purified EBs and uninfected cells. Although the function of these proteins is unknown, they may be involved in nutrient acquisition and in pathogen–host interaction (Bannantine *et al*., 1998b).

Parasitophorous vacuoles can be modified by the addition of elements of pathogen origin. For example, the protozoans *T. gondii* (Nichols *et al*., 1983; Beckers *et al*., 1994) and *P. falciparum* (Stewart *et al*., 1986) have specific secretory organelles called rhoptries, which are capable of secreting proteins and lipids to the parasitophorous vacuoles (Sibley *et al*., 1986; Dluzewski *et al*., 1992). Another example is the outer membrane protein PorB of *N. gonorrhoeae*, which translocates from the outer membrane of the gonococci into host cell membranes (Rudel *et al*., 1996). PorB is able to modify the maturation of latex beads containing phagosomes in primary human macrophages (Mosleh *et al*., 1998), indicating a possible role of this protein in the modulation of neisserial phagosome maturation. On the other hand, there is now accumulating evidence that pathogens harbour type III secretion systems that deliver effector proteins or enzymes essential for survival within cells. Four putative type III secretion genes from *C. psittaci* have recently been proposed to encode virulence factors because of sequence similarities to effector proteins of other bacteria (Hsia *et al*., 1997). Although export of these proteins to their final destination (i.e. cytosol, inclusion membrane, etc.) has not been demonstrated, we hypothesize that these proteins, in addition to other inclusion elements of chlamydial origin (mentioned above), which may also be products of this type III secretion, may interfere with the intracellular membrane trafficking, conferring survival and replication of chlamydiae within the modulated vacuoles.

In the present study, we have described the trafficking pattern of *Cpn* through a human epithelial cell line and found it to be similar to that of *C. trachomatis* and *C. psittaci* described in other studies (Heinzen *et al*., 1996; Scidmore *et al*., 1996a; Taraska *et al*., 1996). Obviously, *Cpn* neither interacts with late endosomes nor is sheltered in a phagolysosome. However, the close approximation of early endosomes to the bacterial compartment may indicate an interaction with the early endosomal part of the endocytic pathway. Inhibition of inclusion maturation into a phagolysosome suggests that *Cpn*, like other chlamydial spp., is able to modulate the maturation of its phagosome to ensure a safe niche within target host cells.

Experimental procedures

Chemicals, fluorescent probes and antibodies

The following mouse monoclonal primary antibodies were used: anti-human TfR diluted 1:100 and anti-*Cpn* at a dilution of 1:5 (Dako Diagnostica); anti-human LAMP-1 and -2 (1:35) and anti-human CD63 at a dilution of 1:40 (Developmental Studies Hybridoma Bank, University of Iowa, IA, USA). The rabbit polyclonal genus-specific antichlamydial (diluted 1:60 and obtained from Milan Analytica) and anti-Ci-M6PR (1:50; a generous gift from Albert Haas, Biocenter, University of Würzburg, Würzburg, Germany) antibodies were also used. The labelled antibodies used were fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG (1:100; Jackson ImmunoResearch Laboratories) and FITC- conjugated rabbit anti-dinitrophenol (DNP) IgG (1:10; Molecular Probes). TR-dextran (molecular weight of 10,000, lysine fixable) and the lysosomotropic dyes AO, LyT Red DND-99 and 3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine (DAMP) were also purchased from Molecular Probes. Unless otherwise noted, all reagents were obtained from Sigma-Aldrich.

Host cells and Cpn *strain*

Unless otherwise specified, the host cells were HEp-2 cells (ATCC-CCL23), a human epithelioid cell line derived from a larynx carcinoma. The *Cpn* strain used was TW-183 (ATCC VR2282) obtained from the Washington Research Foundation (WRF).

Media

The media used in these experiments were basically minimal essential media (MEM) containing Earle's salts (Gibco BRL) with slight differences in their composition. Cell growth medium (CGM) was used for the propagation and growth of uninfected host cells and was composed of MEM supplemented with 1% non-essential amino acids, 2 mM L-glutamine (Biochrom), 10% fetal calf serum (FCS), 10 mM HEPES and 10 μg ml^{-1} gentamicin. Maintenance medium (MM) comprises all the constituents of CGM except FCS. In addition, MM contains 1 μg ml^{-1} cycloheximide. This medium was used during cultivation and harvest of chlamydiae. Infection medium (IM) was also MEM containing the same amounts of amino acids, glutamine and HEPES, but only 5% FCS. In addition, neither cycloheximide nor gentamicin was included in the IM, which was used in the experiments of intracellular trafficking of the vacuole housing *Cpn* and its characterization in the epithelial host cells.

Growth and purification of Chlamydia

Before harvest, bacteria were propagated in HEp-2 cells, grown in six-well plates, with the aid of centrifugation. Seventy-two to 96 h after infection, MM was poured off, and the cells were detached in cold HBSS (Biochrom) using 3-mm-diameter glass beads or a rubber policeman. The cell suspensions were centrifuged at 500 × *g* and 4°C for 10 min (Hermle Labortechnik), and the pelleted cells were then combined together and ruptured using glass beads and a Dounce homogenizer (Wheaton) to ensure destruction of cells. The lysates were centrifuged as before to sediment nuclei and cell debris. The supernatants were removed and recentrifuged at 20 100 × *g* for 40 min at 4°C in a SS34 rotor (Sorvall Instruments) to pellet chlamydiae. The crude bacterial preparations were suspended in 30% Percoll (v/v) (Pharmacia) in 10 mM HEPES, 145 mM NaCl, pH 7.4 (Newhall *et al.*, 1982), by repeated passages in a 22-gauge needle and centrifuged again at 90 720 × *g* (TFT65.13 rotor, Kontron Instruments) for 40 min at 4°C. The harvested bacteria were washed once in sucrose–phosphate–glutamate buffer (SPG, pH 7.4), diluted and resuspended in the same buffer. The *Chlamydia* suspension was then aliquoted and frozen at −75°C and freshly thawed for each experiment.

Titration of infectivity of Cpn

Infectivity titre determination of purified bacteria was made with and without the aid of centrifugation. In centrifugation-assisted determination, fivefold serial dilutions of thawed EB stocks were prepared in 0.5 ml of IM. The inocula were centrifuged at 920 × *g* for 1 h at 37°C (Hermle Labortechnik) onto HEp-2 cell monolayers grown on coverslips in 12-well plates, followed by a 1 h period of incubation at 37°C and 5% CO_2. For the infectivity titration without centrifugation, cells seeded in 24-well plates were inoculated with twofold serial dilutions of bacterial stocks made in 100 μl of IM and then incubated statically for 2 h under the conditions mentioned above.

In both methods of pathogen inoculation, the inocula were removed, and the infected cells were washed to remove unadsorbed chlamydiae and then incubated in fresh IM at 37°C and 5% CO_2. By 40–48 h after infection, developed chlamydial inclusions were detected using immunofluorescent staining with suitable antibodies, as described below. For each dilution, inclusions in 15 microscopic fields at × 400 magnification were enumerated using a Leica epifluorescence microscope, and the average number was then calculated. The last dilution capable of producing $\geq$ 25, in infections not assisted by centrifugation, or $\geq$ 50 inclusions, in centrifugation-assisted infections, per one microscopic field was selected to be used in subsequent experimental infections.

Infection of epithelial cells for microscopy

Glass coverslips (13 mm diameter) in 24-well plates were seeded with epithelial cells suspended in CGM and incubated overnight at 37°C and 5% CO_2 to allow adherence. Before infection, appropriate chlamydial dilutions (predetermined earlier) made in 100 μl volumes of IM were added to the wells. The plates were then incubated statically for 2 h at 37°C and 5% CO_2. Thereafter, the inocula were removed, and the infected cells were washed, overlaid with fresh IM and incubated for the specified time periods. For centrifugation-assisted infections, the appropriate bacterial dilution prepared in 500 μl of IM was inoculated onto cells cultured in 12-well plates. The plates were then immediately centrifuged at 920 × *g* for 1 h at 37°C followed by a 1 h incubation period at 37°C and 5% CO_2. After aspiration of the inocula, monolayers were washed and overlaid with IM and incubated as described.

Double immunofluorescence microscopy

For double staining of *Chlamydia* and intracellular markers, the following protocol was used. Briefly, infected cells were fixed for 40 min in 4% paraformaldehyde (PFA) (Merck), 120 mM sucrose (Roth) in PBS (pH 7.4), followed by quenching the fixation with 50 mM NH_4Cl (Merck) in PBS for 10 min. The cells were then incubated with the blocking and permeabilizing solution, which consists of 0.2% bovine serum albumin (BSA) and 0.2% Triton X-100 (Merck) in PBS for 40 min. Next, the cells were serially incubated for 60 min with primary antibodies and then with secondary antibodies diluted in BSA-PBS solution. Finally, the coverslips were washed and mounted in Mowiol mounting medium and inverted on glass microscopic slides. The labelled preparations were analysed using a Leica TCS NT laser scanning confocal microscope equipped with krypton–argon mixed gas laser. Images were obtained and processed using Photoshop 5.0 (Adobe Systems).

Estimation of the pH of Cpn*-containing vacuoles*

AO vital staining produces a green colour at neutral pH. When it accumulates within acidic vesicles, it produces a bright orange to red colour (Swanson, 1989). Staining with AO was performed as described before (Yoshimori *et al*., 1991). Vital fluorescence visualization of AO in the preparations and photography were conducted with the Leica epifluorescence microscope equipped with a Leica camera (loaded with Fuji Sensia II-400 film) using the filter set responsible for simultaneous observation of fluorescein and rhodamine.

Labelling of acidic compartments with LyT vital staining was performed according to the manufacturer's instructions and viewed by the immunofluorescence microscope using a rhodamine filter. In some experiments, cells were exposed to a combination of AO and LyT for 30 min and then examined as described for AO alone.

Labelling with DAMP, a DNP-containing compound that can be fixed with aldehydes, was basically made according to a method described elsewhere (Anderson *et al*., 1984). The cells were washed and prepared for microscopy as described earlier using rabbit FITC-conjugated anti-DNP IgG, mouse anti-*Cpn* antibodies and rhodamine-labelled goat anti-mouse IgG.

Treatment with the acidification inhibitor BafA1

HEp-2 cells were treated with BafA1, infected and stained to visualize infection, as detailed in the legend to Fig. 2. To demonstrate the effects of acidification inhibition on the establishment of infection and on chlamydial growth, the growth pattern of the bacterial inclusions in the drug-exposed cells was qualitatively compared with the controls. In addition, the inclusions were counted in at least 10 microscopic fields at $\times 400$, and the average number of inclusions per one field was determined.

TR-dextran labelling

To label endocytic organelles, host cells infected with *Chlamydia* for 25 h or 55 h were incubated with TR-dextran (0.8–1 mg ml^{-1} medium) for an additional 15 h at 37°C and 5% CO_2. The cells were then washed extensively, fixed, permeabilized as described above and labelled sequentially with *Chlamydia* genus-specific rabbit polyclonal antibodies and FITC-conjugated goat anti-rabbit IgG. The preparations were then visualized using the confocal microscope.

Treatment with chloramphenicol

HEp-2 cells were infected for 36 h and then incubated with IM containing either 50, 100 or 200 μg of chloramphenicol (Fluka), an efficient chlamydial protein synthesis inhibitor, for different time intervals (24 h, 48 h and 72 h). At the end of the incubation periods, monolayers were washed and doubly stained for *Chlamydia* and LAMP-1 and then examined under the confocal microscope.

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