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Host Ferritin Translocates into the Chlamydial Inclusion: A Clear Alteration in its Subcellular Distribution as a Result of Infection

Hesham M. Al-Younes and Thomas F. Meyer*

ABSTRACT

In the present study, the subcellular distribution of the major iron storage protein ferritin was investigated in cell cultures infected for various times with *Chlamydia trachomatis* or *Chlamydia pneumoniae*. In *C. trachomatis*-infected cells, confocal microscopy showed that ferritin was localized in the cytoplasm and did not overlap with the chlamydial staining, when examined 2, 5 and 10 h post-infection (pi). Interestingly, 18 h pi, the immunostaining indicated translocation of ferritin to the chlamydial inclusion. At 44 h pi, the localization of ferritin indicated a dramatic cytoplasmic redistribution and recruitment to *C. trachomatis* inclusion. For *C. pneumoniae*, ferritin did not colocalize with the inclusion until 40 h pi, when it was reduced in the cytoplasm and superimposed strongly with the chlamydial staining. At late stage of *C. pneumoniae* infection (70 h pi), predominant ferritin staining colocalized with the inclusion. Serial optical sections made by the confocal microscopy and the immunogold labeling technique confirmed the presence of ferritin inside of the chlamydial vacuole. The evidence presented in this work indicates an existence of a translocation system used by Chlamydiales to attract and acquire host proteins as large as ferritin through the inclusion membrane.

Keywords: *Chlamydia,* Ferritin, Iron, Protein Translocation.

1. INTRODUCTION

Chlamydia pneumoniae and *Chlamydia trachomatis* are obligate intracellular pathogens responsible for a large spectrum of diseases in humans. *C. pneumoniae* is an important cause of respiratory diseases, accounting for approximately 10% of cases of community-acquired pneumonia and has been linked to other nonrespiratory diseases like atherosclerosis and Alzheimer's disease (Balin *et al*., 1998; Campbell *et al*., 1998; Cosentini *et al*., 1999). Serovars of *C. trachomatis* are associated with eye infection and with the sexually transmitted diseases (Schachter and Caldwell, 1980; Guaschino and De Seta, 2000; Byrne, 2010). Chlamydiae exist in 2 developmental distinct forms: the extracellular invasive elementary bodies (EBs) that attach to the host cell surface and are internalized into a vacuole. Within 8-10 h, EBs transform to the intracellular, noninvasive, metabolically active reticulate bodies (RBs), which undergo several division cycles within the same membrane-bound vacuole that is known also as the inclusion. The RBs differentiate back into EBs that are released from the host cell after 2 to 4 days, depending on the bacterial genus and strain (Moulder, 1991).

Iron represents a paradox for living systems; it is essential for a wide variety of metabolic processes like O_2 transport, electron transport and DNA synthesis. On the other hand, free iron can interact with reduced forms of oxygen through Fenton reaction, resulting in the formation of reactive oxygen species (ROS) known to cause lipid peroxidation, protein modification, and DNA damage. Because of its oxidative properties, intracellular concentration of free iron should be tightly regulated and complexed with high-affinity-iron-binding proteins, such as ferritin. This protein stores excess iron and has the capacity to sequester up to 4500 iron atoms. Ferritin (M. wt. ~ 450 kDa) is composed of 24 subunits of heavy (H) and light (L) chains. The H chain has ferroxidase activity that oxidizes Fe^{2+} to Fe^{3+}, whereas the L chain is involved in the iron nucleation and long-term iron storage (Aisen *et al*., 2001; Orino *et al*., 2001; Li and Qian, 2002).

To date, the best understood pathway for iron acquisition in eukaryotic cells is that mediated by the iron-loaded transferrin (Tf) and the cell surface Tf

* Department of Biological Sciences, Faculty of Science, University of Jordan, Amman, 11942, Jordan; Max Planck Institute for Infection Biology, Department of Molecular Biology, Charitèplatz 1, D-10117 Berlin, Germany. Received on 8/11/2009 and Accepted for Publication on 13/10/2010.

receptor (TfR). Tf bound to TfR is internalized and cycled through recycling early endosomes, where iron is released to the cytosol, with the iron-free Tf–TfR complex returning back to the extracellular surface. Iron released from Tf enters the so-called cytoplasmic labile iron pool before being used for metabolic activities or being bound by the cytoplasmic iron storage protein ferritin (Li and Qian, 2002; Dunn *et al*., 2007; Richardson *et al*., 2010).

Pathogen interactions with host proteins related to iron homeostasis have been previously described. These interactions have most likely evolved to supply the microorganisms with the iron required for growth and survival. For example, *Neisseria* (Gray-Owen and Schryvers, 1996), *Moraxella* and *Staphylococcus* (Modun *et al*., 2000) produce surface receptors specifically binding Tf. Some microorganisms, like *Neisseria*, bind lactoferrin, an iron-binding glycoprotein present in mucosal secretions and released by leukocytes at the sites of inflammation (Schryvers and Stojiljkovic, 1999; Ekins *et al*., 2004). Bacteria, including *Haemophilus*, *Vibrio* and *Yersinia*, express specific receptors that bind heme and hemoglobin (Schryvers and Stojiljkovic, 1999; Wandersman and Stojiljkovic, 2000). Other pathogens that live within an early endosomal-like compartment, such as *Mycobacterium tuberculosis*, *Ehrlichia chaffeensis* and *Francisella tularensis*, may have an access to Tf-iron (Clemens and Horwitz, 1996; Sturgill-Koszycki *et al*., 1996; Barnewall *et al*., 1999; Olakanmi *et al*., 2007; Pan *et al*., 2010).

Iron is an element essential for chlamydial growth (Raulston, 1997; Al-Younes *et al*., 2001; Freidank *et al*., 2001; Dill *et al*., 2009). It has been shown that both Tf and TfR were found in close apposition to chlamydial inclusions in both *C. trachomatis* and *C. pneumoniae* infection models (Scidmore *et al*., 1996; Taraska *et al*., 1996; van Ooij *et al*., 1997; Hackstadt *et al*., 1998; Al-Younes *et al*., 1999; 2001), suggesting that these microorganisms could exploit the host iron uptake system to obtain iron (Raulston, 1997; Al-Younes *et al*., 2001). Moreover, the internalization of TfR into the chlamydial inclusion and an enhanced recruitment of this receptor to the inclusion grown under iron-restricted conditions were observed (Al-Younes *et al*., 2001). To research the possibility of interaction between Chlamydiales and other iron-carrying host proteins, the localization of ferritin in the infected cells was investigated in this study. Using monoclonal and polyclonal anti-ferritin antibodies and confocal and electron microscopy as a read out, the localization of ferritin during different stages of infection was followed.

2. MATERIAL AND METHODS

2.1. Bacteria Strains and Stock Preparation

Chlamydial isolates used were *C. trachomatis* LGV-434 (serovar L2) and *C. pneumoniae* strain CWL-029 (ATCC VR-1310). When required, organisms (EB form) were purified as previously described (Al-Younes *et al*., 2001). Bacterial stocks were aliquoted and stored at -80°C. Infectivity of purified EBs in the stocks was determined by number of inclusion-forming units (IFU) per ml (Al-Younes *et al*., 2001).

2.2. Tissue Culture Cells and Infection

The human epithelial cell line HEp-2 (ATCC-CCL23) was plated on 12 millimeter diameter coverslips placed in 12-well plates and incubated overnight at 37°C in RPMI 1640 medium (GIBCO-Invitrogen Corporation, Paisley, Scotland) supplemented with 10% fetal bovine serum (FBS) and 10 µg gentamicin per ml.

To ensure the detection of *C. trachomatis* at early times after the infection (2 and 5 h), cells were infected with bacteria at a multiplicity of infection (MOI) of 50 diluted in RPMI 1640 medium supplemented with 5% FBS, while cells examined at 10, 18 and 44 h post-infection (pi), at which times the inclusion is microscopically visible, were infected with an MOI of 0.5. The MOI of 0.5 of *C. trachomatis* was selected to produce a moderate infection, avoiding formation of big inclusions resulting from entry of more than one EB per cell and subsequent homotypic fusion of individual EB-containing phagosomes. For the infection with *C. pneumoniae*, an MOI of 1 diluted in RPMI 1640 medium supplemented with 5% FBS was selected. Cell monolayers were infected with either chlamydial species as described earlier (Al-Younes *et al*., 2001). After infection, the cells were washed twice with the medium. Fresh RPMI 1640 medium plus 5% FBS was then added and incubation was continued at 37°C and 5% CO_2 for the indicated times.

2.3. Double Immunofluorescence Staining

Infected cells were rinsed two times with phosphate-buffered saline (PBS; pH 7.4) and fixed for 30 min in 4% paraformaldehyde (PFA; Merck, Darmstadt, Germany)-

120 mM sucrose in PBS, followed by quenching the fixation with 50 mM NH_4Cl in PBS for 10 min. After two washings with PBS, the cells were permeabilized for 30 min with 0.2% Triton X-100-0.2% bovine serum albumin (BSA) in PBS at room temperature. Next, cells were first incubated with genus-specific anti-chlamydial rabbit antiserum raised against EBs and recognizing lipopolysaccharides and some major outer membrane proteins (Milan Analytica AG, Switzerland) and then with one of the following antibodies: mouse monoclonal anti-human ferritin (clone M94156a; Fitzgerald Industries International, Inc., USA), rabbit polyclonal anti-human ferritin or rabbit polyclonal anti-horse ferritin (Sigma) that cross-reacts with the human ferritin. The secondary antibodies used were a goat anti-rabbit IgG Cy2, a goat anti-rabbit Cy3 and a goat anti-mouse Cy3 conjugates (Jackson ImmunoResearch Laboratories, USA), respectively. Coverslips were then mounted onto glass slides using Mowiol mounting medium and viewed as previously described (Al-Younes *et al*., 1999).

2.4. Transmission Electron Microscopy

Infected host cells grown in six-well plates were washed twice with cold PBS and then fixed with 4% PFA. After fixation, cells were gently removed by scraping the wells. The cells were pelleted, embedded in agarose and infiltrated with 2.3 M sucrose-20% polyvinyl pyrrolidone in PBS. The blocks were trimmed, frozen and sectioned with an RMC cryo-microtome. The cryosections were incubated first with mouse anti-ferritin antibodies and then with goat anti-mouse IgG conjugated with 12-nm gold particles (Jackson ImmunoResearch Laboratories, USA). The stained electron microscopy specimens were viewed and photographed using a Zeiss EM 10 electron microscope.

3. RESULTS

3.1. Ferritin Translocates into *C. trachomatis* Inclusion

The subcellular distribution of the cytoplasmic ferritin in host HEp-2 cells infected with *C. trachomatis* serovar L2 was investigated at different times (2, 5, 10, 18 or 44 h) pi. At 2 h (Figure 1A) and 5 h (Figure 1B) after infection, ferritin (red) did not colocalize with bacteria (green) and exhibited an abundant granular fluorescence distribution throughout the cytoplasm, similar to its appearance in uninfected control cells (Figure 1F). At 10 h pi, ferritin labeling remained distinct from the inclusion in the majority of infected cells (Figure 1C). Interestingly, at 18 h pi, ferritin staining became less abundant in the cytoplasm and strongly coincided with the inclusion staining (Figure 1D). At 44 h pi, ferritin was predominantly, if not exclusively, limited to the inclusion (Figure 1E). The translocation of ferritin was reconfirmed using rabbit polyclonal antibodies against either human or horse ferritin (data not shown). Overall, confocal microscopy data demonstrated that during the infection, ferritin is recruited from the cytoplasm to the inclusion.

To confirm the intravacuolar localization of ferritin, infected cells were stained with mouse anti-human ferritin monoclonal antibodies and then analyzed through optical sections by confocal microscopy. As depicted in Figure 2, sequential analysis of several optical sections that correspond to the bottom, the middle and the top of a 48-h-old inclusion demonstrates that ferritin staining (red) heavily overlaps with the inclusion (green), confirming the presence of ferritin in the chlamydial inclusion.

3.2. Ferritin Translocates into *C. pneumoniae* Inclusion

The possibility of recruitment and internalization of the cytoplasmic ferritin into the *C. pneumoniae* inclusion in host cells was examined. At 20 h pi, ferritin staining (red) exhibited a granular pattern in the cytoplasm (Figure 3A), similar to the staining pattern observed in uninfected control cells (Figure 1E) and the vast majority of developed inclusions showed no overlap with the anti-ferritin staining. At 40 h pi (Figure 3B), the punctate cytoplasmic staining of ferritin considerably decreased and ferritin colocalized strongly with the inclusion. In the cell cultures infected for 70 h pi, the cytoplasmic staining corresponding to ferritin has almost vanished in the infected cells and was restricted exclusively to the inclusion (Figure 3D).

3.3. Distribution of Ferritin in the Inclusion

Examination of enlarged merged images of the intact chlamydial inclusions demonstrated a dotted distribution pattern of the red-stained ferritin (arrows) that could not be completely superimposed with the green-stained developing bacteria (Figure 4A), suggesting that ferritin molecules are close to but not internalized by individual bacteria in the inclusion. This notion was supported when disrupted inclusions in the stained preparations were investigated. As shown in Figure 4B, most of ferritin molecules appear as red dots (arrows) against the center

of the lysed inclusion. Interestingly, examination of areas away from the center of the lysed inclusion revealed a presence of red dots, which were smaller than the released bacteria and were either scattered and dissociated from the bacteria (arrows, Figure 4C) or were closely associated with the released bacteria (arrowheads, Figure 4C). Strong overlapping of ferritin staining with some freed chlamydiae, which appears as yellow in Figure 4C, confirms the close association of this human protein with bacteria.

To achieve a detailed ultrastructural analysis on the ferritin distribution in the inclusion, infected cells exposed to the mouse monoclonal antibodies against ferritin were analyzed using immunoelectron microscopy. Staining with gold-labeled secondary antibodies showed that this protein was found (1) in the lumen of the inclusion (arrowheads, Figure 5), (2) associated with the inner face of the inclusion membrane (double arrows, Figure 5), and (3) associated with the chlamydial forms developed in the inclusion (single arrows, Figure 5). Noteworthy, immunogold particles, indicative of presence of ferritin in the lumen of the inclusion, were either individually scattered or present as aggregates attached to small electron dense structures, as illustrated in Figure 5.

Taken together, confocal and immunoelectron microscopical analyses revealed that ferritin is translocated into the inclusion in a time-dependent fashion and that the translocated ferritin closely associated with the developing bacteria, indicative of a direct interaction.

4. DISCUSSION

In the present report, examination of the subcellular distribution of the host ferritin in cells infected with *C. trachomatis* or *C. pneumoniae* revealed that ferritin is present predominantly in the inclusion at the middle and late stages of infection, as indicated by confocal and immunoelectron microscopy. The presence of ferritin within the chlamydial inclusion is not an artificial phenomenon based on following reasons. Firstly, the respective isotypic control (purified mouse IgG1) demonstrated no cross-reactivity with the inclusion. Secondly, fluorescence microscopical examination detected colocalization of ferritin with inclusions at middle and late stages of infection (40 and 70 h pi for *C. pneumoniae* and 18 and 44 h pi for *C. trachomatis*), but not at early stages (20 h pi for *C. pneumoniae* and 2, 5 and 10 h pi for *C. trachomatis*). Thirdly, the ferritin label was associated with structures smaller than the bacteria, as seen in Fig. 4B and C. Finally, protein homologues for either human ferritin or other known bacterial ferritins could not be found in the genomes of *C. pneumoniae* and *C. trachomatis*.

The role of ferritin in the chlamydial inclusion remains at this stage only speculative. This protein might play a central role in the maintenance of the "intravacuolar" iron balance, similar to its function in the cytoplasm. Ferritin might serve as a protective protein, abolishing or minimizing oxygen free radical-mediated damage of the inclusion membrane and developing bacteria by sequestering "excess intravacuolar" iron. ROS were proposed to play a central role in the pathogenesis of chlamydial infection, as they, for instance, cause host cell membrane damage via lipid peroxidation (Azenabor and Mahony, 2000) and enhance the inflammatory response of infected cells (Visseren *et al*., 2002). It is also reasonable to hypothesize that replicating chlamydiae could, in part, mobilize iron from ferritin to overcome the difficulty of obtaining iron in the confines of the inclusion, in addition to the Tf-iron (Scidmore and Hackstadt, 1995; Raulston, 1997; Al-Younes *et al*., 2001). Of note, microscopical data showed that ferritin could be in direct contact of the bacterium, a strategy that could be used by chlamydiae to directly remove iron from ferritin. Several previous studies support the view that ferritin could be a potential iron source for pathogens. It has been suggested that ferritin could be the major target from which an intracellular bacterial pathogen may acquire iron (Wilson and Britigan, 1998; Ratledge and Dover, 2000). Addition of ferritin enhances the growth of *Vibrio vulnificus* (Simpson and Oliver, 1987) and *V. parahaemolyticus* on iron-limited agar plates (Wong *et al*., 1996). High affinity iron-binding siderophores of *Mycobacterium tuberculosis* have the capacity to remove iron from ferritin (Gobin and Horwitz, 1996; Ratledge, 2004; 2007). Furthermore, an extracellular ferric reductase characterized in *Mycobacterium paratuberculosis* was able to mobilize ferric iron effectively from environmental sources such as ferritin, suggesting that ferritin could be an iron source for this bacterium (Homuth *et al*., 1998).

The mechanism by which cytoplasmic ferritin is trafficked and internalized into the chlamydial inclusion is unclear. Normally, recycling of iron from ferritin

involves transport of the existing ferritin from the cytosol into the lysosome, followed by lysosomal degradation (Roberts and Bomford, 1988; Radisky and Kaplan, 1998). Therefore, pathogen phagosomes that fuse usually with lysosomes, like those of *Leishmania* (Duclos and Desjardins, 2000) and *Coxiella* (Heinzen *et al*., 1996), may contain ferritin. Indeed, presence of ferritin in the lumen of the phagolysosome has been confirmed by biochemical analysis of purified phagolysosomes containing latex beads using 2-dimensional PAGE analysis (Garin *et al*., 2001), supporting the view that ferritin could be provided to the parasitophorous vacuole by lysosome fusion. However, chlamydial inclusions do not fuse with lysosomes. Also, inclusion membrane cannot allow diffusion of this large protein, since it is not passively permeable to molecules as small as 520 Da from the cytoplasm (Heinzen and Hackstadt, 1997). These reasons point to the existence of a unique transport and delivery system of ferritin into the inclusion. The ability of other intracellular pathogens especially those that reside in non-phagolysosomal compartments (e.g. *Mycobacterium* and *Ehrlichia*) or those that survive freely in the cytoplasm (e.g. *Listeria* and *Shigella*) to interact with ferritin might, therefore, be worth to investigate.

In summary, data obtained by confocal microscopy, supported by immunogold labeling studies, demonstrated for the first time an internalization of the cytosolic ferritin into the chlamydial inclusion at middle and late times of the developmental cycle. It will be important to determine whether ferritin is transported via a general or a unique mechanism and to investigate the role of host ferritin in the chlamydial biology.

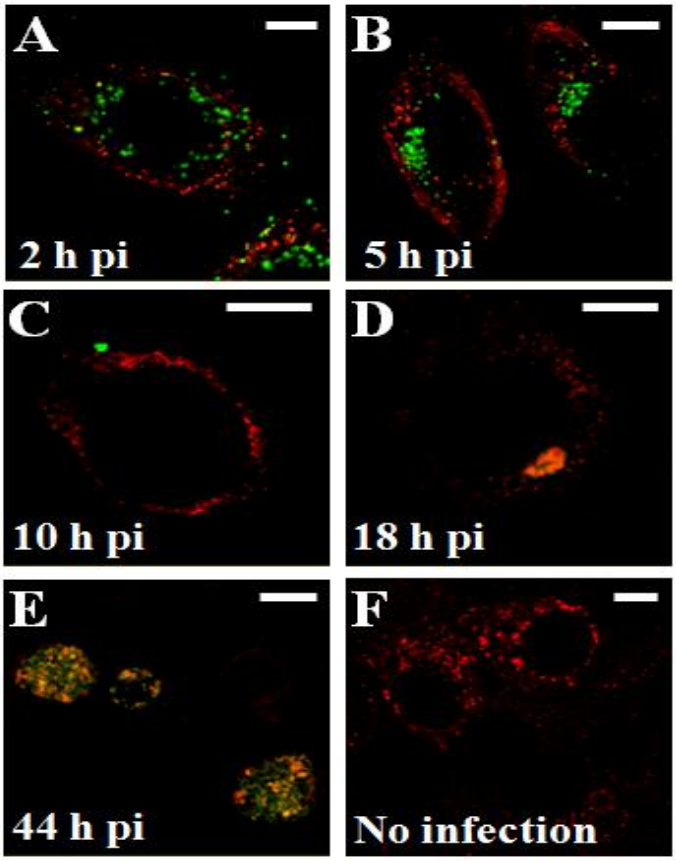

Figure 1

Immunofluorescence staining showing translocation of ferritin into *C. trachomatis* inclusion over time. At 2 (A), 5 (B) and 10 h pi (C), ferritin label (red) did not colocalize with the chlamydial vacuoles (green). As of 18 h pi (D), ferritin was found within the inclusion but also found in the cytoplasm, whereas at 44 h pi (E) the cytoplasmic ferritin staining almost disappeared and was restricted to the inclusion. (F) ferritin staining in the uninfected control HEp-2 cells. Immunodetection of ferritin in panels A-F was performed in HEp-2 cells that were incubated with the mouse monoclonal antibodies. Scale bars represent 10 µm.

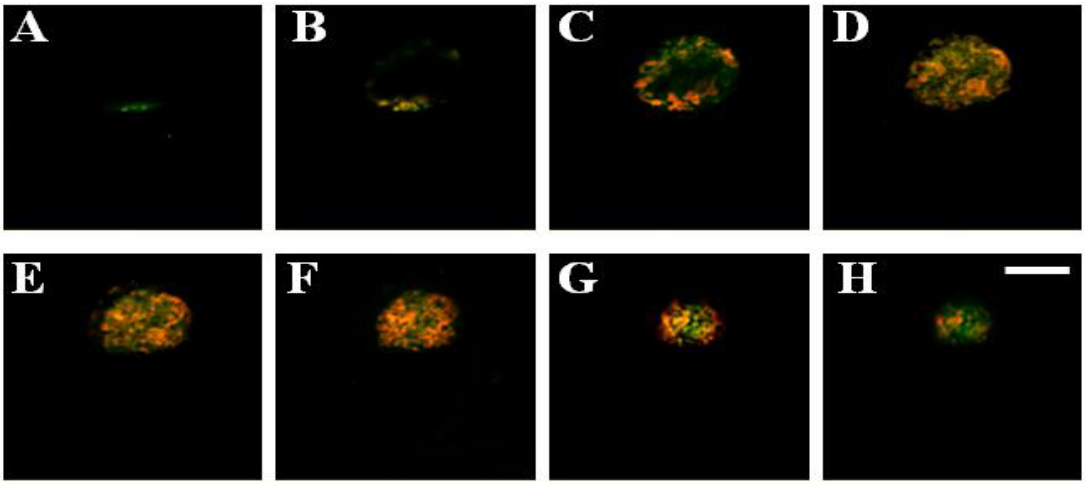

Figure 2

Sequential analysis of ferritin staining in a 48-h-old inclusion of *C. trachomatis* developed in HEp-2 cells. Series of optical sections were performed using confocal microscopy. Shown are sections that correspond to the bottom, the middle and the top of the inclusion demonstrating the presence of ferritin (red) inside the bacterial inclusion (green). Scale bars represent 10 µm.

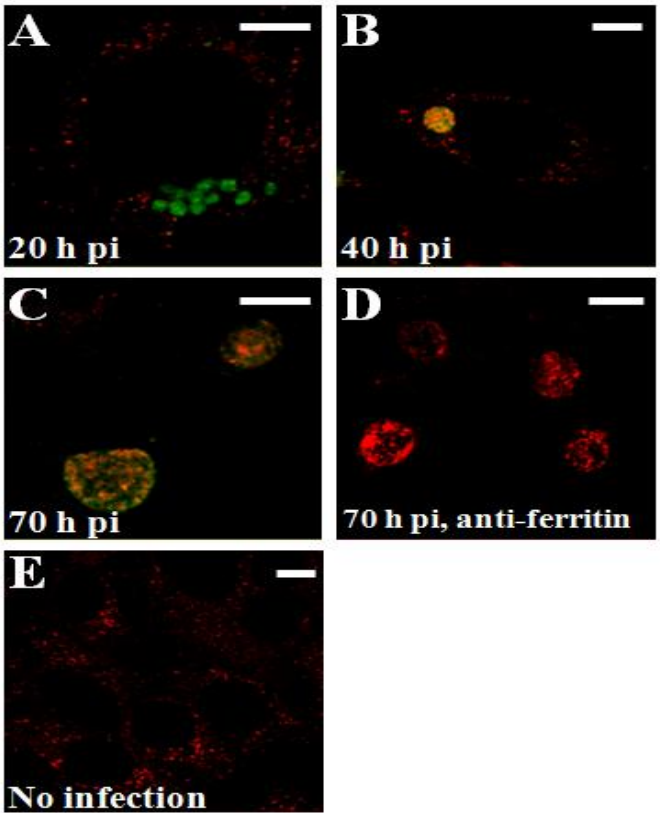

Figure 3

Double immunofluorescence staining showing translocation of ferritin into *C. pneumoniae* inclusion over time. At 20 h pi (A), ferritin (red) demonstrated a granular staining pattern in the cytoplasm that did not coincide with the inclusions (green). At 40 h pi (B), ferritin stain was detected in the cytoplasm and heavily overlapped with the inclusion. When examined 70 h pi (C), the staining for ferritin considerably decreased in the cytoplasm and colocalized predominantly with the inclusion. (D) Single immunofluorescence staining of ferritin shows predominant immunoreactivity restricted to the *C. pneumoniae* inclusion. (E) immunofluorescence staining of ferritin (red) in uninfected control HEp-2 cells. Scale bars represent 10 µm.

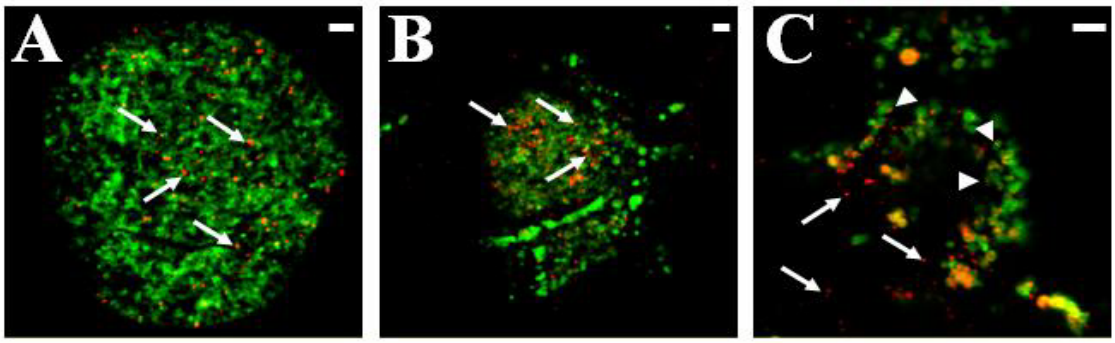

Figure 4

Distribution of ferritin in the inclusion. (A) Immunofluorescence micrograph of an enlarged intact *C. trachomatis* inclusion reveals the dotted distribution pattern of ferritin stain (red) within the inclusion that mostly did not overlap with the bacterial signal (green). (B and C) Micrographs of lysed inclusions confirm the granular appearance of ferritin in the inclusion. Ferritin stain was detected close to released chlamydiae (arrowheads) or superimposed with the bacterial stain (yellow), indicating that ferritin may be associated with the bacteria. However, ferritin label was also found disconnected from the freed pathogens (arrows in C). Scale bars represent 2 µm.

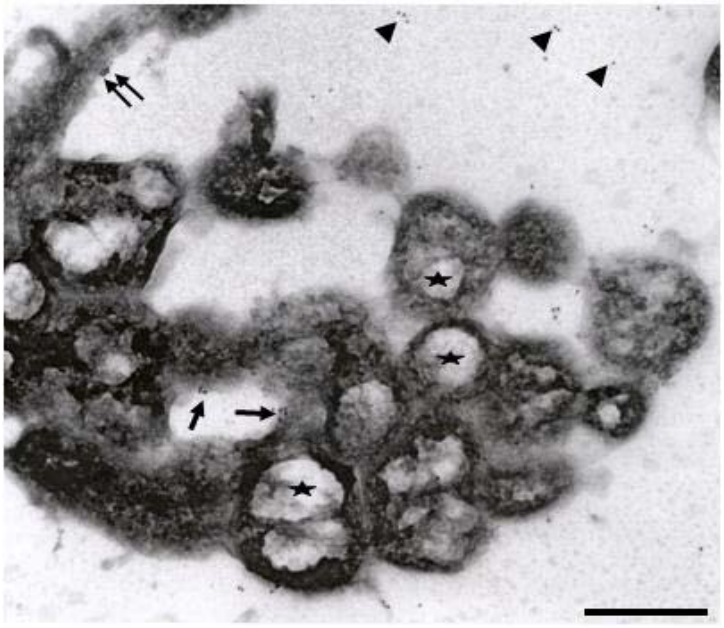

Figure 5

Ultrastructural features and distribution of ferritin in 20-h-old *C. trachomatis* inclusions. Ultrathin sections were stained with mouse anti-ferritin antibodies followed by addition of gold-labeled secondary antibodies. Colloidal gold particles were found scattered individually or aggregating in the inclusion lumen (arrowheads). Gold particles were also associated with the lumenal side of the inclusion membrane (double arrows) or attached to the developing bacteria (single arrows). Asterisks indicate bacteria. Scale bar represents 500 nm.

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انتقال البروتين فيريتين من سيتوبلازم الخلية الثديية إلى حويصلة تكاثر البكتيريا كلاميديا

هشام محمود اليونس وتوماس ماير*

ملخـص

يعتبر البروتين فيريتين الخازن الرئيس في الخلية لعنصر الحديد الأساسي في تفاعلات أيضية مختلفة. تم في هذه الدراسة، فحص توزيع الفيريتين في بيئات خلايا إنسانية بعد عدواها لفترات زمنية مختلفة بالبكتيريا كلاميديا تراكوماتس أو كلاميديا بنومونيي. أشار الميكروسكوب الليزري المتألق بعد استخدام الأجسام المضادة المناسبة إلى عدم وجود إعادة تموضع للفيريتين في الخلايا المصابة بكلاميديا تراكوماتس لفترات أقل من 18 ساعة. على العكس من ذلك تماماً، وجد انتقال واضح للفيريتين من سيتوبلازم الخلية العائل إلى داخل حجرة التكاثر البكتيري ابتداء من 18 ساعة بعد العدوى. أما بالنسبة لتأثير الإصابة بكلاميديا بنومونيي، تم الكشف عن وجود نقل كثيف للفيريتين إلى داخل الحجرة البكتيرية ابتداء من 40 ساعة بعد العدوى. وقد تم تأكيد نتائج إعادة توزيع الفيريتين في الخلايا والناجمة عن العدوى بواسطة تقنية المجهر الالكتروني. تشير نتائج هذه الدراسة بشكل واضح إلى انتقال فيريتين الخلية العائل إلى داخل حويصلة البكتيريا كلاميديا والذي يحتمل حدوثه عن طريق نظام خاص يستخدم لجذب ونقل الفيريتين عبر غشاء الحويصلة البكتيرية. إن وجود الفيريتين قريباً من البكتيريا قد يكون مهماً جدا، وذلك من أجل حصول البكتيريا على عنصر الحديد واللازم لسير بعض عملياتها الأيضية ولضمان التكاثر الناجح للكلاميديا في الخلية العائل.

الكلمـات الدالـة: كلاميديا، فيريتين، الحديد، نقل البروتين.

* قسم علوم الأحياء، كلية العلوم، الجامعة الأردنية، عمان، الأردن؛ معهد ماكس بلانك، قسم علم الأحياء الجزيئي، تشاريتبلاتز، برلين، ألمانيا. تاريخ استلام البحث 2009/11/8 وتاريخ قبوله 2010/10/13.