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Infection of *Acanthamoeba castellanii* by *Chlamydia pneumoniae*

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***Chlamydia pneumoniae* is an intracellular respiratory pathogen, which, similar to *Legionella*, might have developed mechanisms to escape the intracellular bactericidal activity of both human host cells and amoeba. We therefore investigated the intracellular growth and survival of *C. pneumoniae* in *Acanthamoeba castellanii* by using cell culture, immunofluorescence microscopy, and electron microscopy. *A. castellanii* was incubated with purified elementary bodies of *C. pneumoniae* TW 183 at a concentration of 10^6 inclusion-forming units (IFU)/ml to give a ratio of approximately 1 IFU of *C. pneumoniae* per amoeba. Quantitative determination of chlamydial growth within *A. castellanii* revealed viable and infective *C. pneumoniae* in the range of 10^4 to 10^5 IFU/ml between days 7 and 14 postinfection. Immunofluorescence analysis and transmission electron microscopy with subsequent immunogold staining confirmed evidence of infection of the amoebae by *C. pneumoniae* and additionally revealed that *C. pneumoniae* entered the typical growth cycle. Our results show that amoebae allow the survival of *C. pneumoniae*, suggesting that amoebae may serve as an additional reservoir for *Chlamydia* or *Chlamydia*-related organisms.**

Legionella is the first human pathogen that has been demonstrated to multiply and persist in both macrophages and protozoa such as amoeba (18, 26, 27). In addition, *Legionella*-like amoebal pathogens which also cause pneumonia and which possibly use the amoebal cell to grow in the oligotrophic aquatic environment have been identified (28). If this supports the general concept that the capability to grow in amoebae also selects for growth in taxonomically unrelated cells such as macrophages or other professional phagocytes, further examples should be expected, as had been discussed recently (2, 3, 22, 25). Since *Chlamydia pneumoniae* and *Legionella pneumophila* share some clinical and bacteriological properties, *C. pneumoniae* could also be a candidate for endosymbiotic growth in amoebae. Both *L. pneumophila* and *Chlamydia* contain a macrophage infectivity potentiator (Mip), a factor which contributes to infectivity and intracellular multiplication of *L. pneumophila* in macrophages and amoebae (8, 9, 20, 21). In addition, the lipopolysaccharides (LPSs) of both pathogens show some similarities (6, 7, 31). *C. pneumoniae* is also an important respiratory pathogen; however, in contrast to Legionnaires' disease, *C. pneumoniae* diseases usually are mild and self-limited. Seroepidemiological studies have documented a prevalence of antibodies of more than 50%, suggesting that exposure to *C. pneumoniae* is quite common (1, 5, 16, 30). The agent presumably spreads via the respiratory route from person to person; however, direct evidence for this assumption is missing (13, 16). However, further modes of transmission and additional reservoirs of *C. pneumoniae* have, to the best of our knowledge, not been identified and have only rarely been discussed. We therefore investigated the survival and intracellular growth of *C. pneumoniae* in protozoa by using the free-living amoeba *Acanthamoeba castellanii*. Based on our results, amoebae might also be suitable hosts for *C. pneumoniae.*

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MATERIALS AND METHODS

Culture conditions. *A. castellanii* ATCC 30234 (American Type Culture Collection, Rockville, Md.) was grown at 37°C under axenic conditions in tissue culture flasks containing proteose-yeast-glucose (PYG) broth, as described previously (23). *C. pneumoniae* TW 183 (Washington Research Foundation, Seattle, Wash.) was continuously cultured on cycloheximide-treated HL cell monolayers (Washington Research Foundation) in six-well tissue culture plates by standard procedures (10). Aliquots of partially purified elementary bodies at a concentration of 10^7 inclusion-forming units (IFU)/ml were stored in 0.22 M sucrose–10 mM NaH_2PO_4–3.8 mM KH_2PO_4–5 mM glutamic acid (pH 7.4) (SPG) at −70°C until used for amoebal infection.

Chlamydial infection of amoebae. Amoebae were seeded at a density of 10^6/ml into a 24-well plate. Chlamydial stock solution containing 10^7 IFU/ml was diluted 1:10 in *C. pneumoniae* growth medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% L-glutamine [Gibco BRL, Eggenstein, Germany]) and added to *A. castellanii* to give a ratio of approximately 1 IFU of *C. pneumoniae* per amoeba. The plates were centrifuged for 1 h at 216 × *g*. After an incubation period of 1 h at room temperature (25°C), the supernatants were carefully aspirated and substituted by PYG broth containing 100 μg of vancomycin per ml, 50 μg of gentamicin per ml, and 2 μg of amphotericin B per ml. After coincubation periods of 7, 10, and 14 days, supernatant was removed and lysis of *A. castellanii* was performed by suspending the amoebae in an aqueous solution of 0.1% saponin (Sigma, Deisenhofen, Germany) and passing them three times through a 27-gauge syringe. Amoebal lysates were diluted 1:100 in *C. pneumoniae* growth medium, and chlamydial growth was determined by inoculating confluent HL cell monolayers in microtiter plates with serial 10-fold dilutions. Inoculated HL cells were stained after a coincubation period of 72 h with a fluorescein isothiocyanate-conjugated monoclonal chlamydial LPS antibody (Pathfinder) and quantitative titer determination was performed by counting chlamydial inclusions under an inverted fluorescence microscope at a magnification of ×200. Experiments measuring chlamydial growth within amoeba were repeated four times.

In addition, the intra-amoebal growth of *C. pneumoniae* was monitored daily by using cytospin slide preparations of infected *A. castellanii*. Slides were stained with the fluorescein isothiocyanate-conjugated chlamydial LPS antibody mentioned above. The morphology of *Chlamydia*-infected amoebae was examined by transmission electron microscopy and immunoelectron microscopy as described previously (12), with monoclonal antibody S25-23, which is directed against the genus-specific epitope of chlamydial LPS (15) and was a kind gift of H. Brade, Forschungsinstitut Borstel, Borstel, Germany.

RESULTS

Immunofluorescence analysis of cytospin slide preparations 12 h postinfection revealed that 10 to 20% of amoebae contained small inclusions, indicating chlamydial infection of *A. castellanii*. Our results of quantitative determination of chlamydial growth in *A. castellanii* showed that viable and infective *C. pneumoniae* cells were present at 10^4 to 10^5 IFU/ml between days 7 and 14 postinfection. However, on day 14 postinfection,

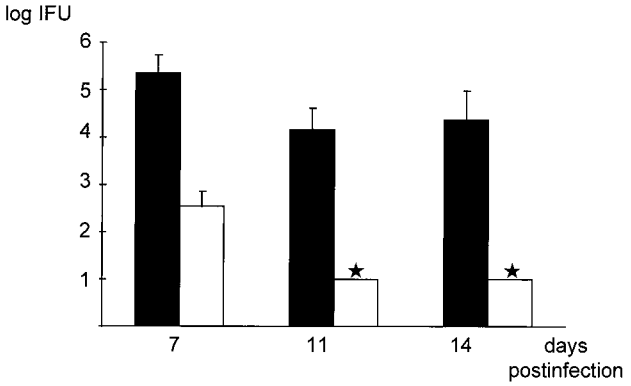

FIG. 1. *A. castellanii* amoebae at a density of 10^6/ml were infected with 10^6 IFU of *C. pneumoniae* per ml. Chlamydial growth in amoebae (■) was determined as described in Materials and Methods at subsequent time points postinfection. Data are shown in log IFU and represent the mean and standard deviation of four independent experiments. The growth of *C. pneumoniae* in PYG medium without amoebae (□) was also determined. Stars indicate that growth was below the detection level of 10 IFU/ml.

the viability of amoebal host cells had decreased to 28% and the loss of chlamydial target cells might have led to a decrease of chlamydial multiplication. When *C. pneumoniae* only was incubated in PYG broth, a sharp decline of viable *Chlamydia* cells was noted, indicating that the organisms retain their viability only within the protozoal host cell (Fig. 1).

Daily immunofluorescence analysis over the course of 14 days showed continued chlamydial infection of the amoebal cultures harboring all stages of the chlamydial growth cycle. From day 3, single large inclusions, which might have led to amoebal rupture and release of chlamydial elementary bodies, were seen (data not shown). The infectious cycle was perpetuated over 14 days, suggesting either persistent chlamydial infection or successive infection of amoebae which had not been invaded at the time of primary infection.

Transmission electron microscopy analysis of *A. castellanii* coincubated with *C. pneumoniae* revealed phagocytosis of chlamydial elementary bodies by amoebae (Fig. 2a) 4 h postinfection. Typical chlamydial inclusions containing elementary bodies and some reticulate bodies (Fig. 2b) adjacent to the nucleus of the host cell were identified at the beginning of day 3 postinfection. Inclusions observed from days 10 to 14 postinfection differed with respect to the morphology of their content (Fig. 2c). Only a few normal-shaped chlamydial forms, together with abundant organisms smaller than typical elementary bodies, were identified within the inclusions, suggesting that only restricted chlamydial growth was occurring within amoebae. Subsequent immunogold labelling confirmed this evidence of small *C. pneumoniae* elementary bodies within *A. castellanii* (Fig. 2d).

DISCUSSION

Our data show that *A. castellanii* allows chlamydial survival and induces the typical morphogenesis of *C. pneumoniae*. Thus, *C. pneumoniae* can be regarded as a second example of a respiratory pathogen which can use amoebae as host cells. This also complements the observation that endosymbionts related to the family *Chlamydiaceae* can be detected in acanthamoebae, as recently shown by Amann et al. (2) and that mixed infections with *Acanthamoeba* and *Chlamydia* had been reported (25). In the case of *L. pneumophila*, the adaptation to growth within amoebae is regarded as a training step for subsequent infection of animal cells and professional phagocytes. *C. pneumoniae* could also benefit from this adaptation process.

Although comparatively little is known about potential virulence factors of *C. pneumoniae*, similarities between *Chlamydia* and *Legionella* may help to identify factors of potential importance to the intracellular survival of *C. pneumoniae*. Both, *Legionella* and *Chlamydia* possess an unusual LPS. The *L. pneumophila* LPS is of the smooth type, exhibits low endotoxicity, and possesses long-chain fatty acids (27-oxo-octacosanoic acid [28:O (27-oxo)] and heptacosane-1,27-dioic acid (27:O-dioic) (31). The O side chain consists of a homopolymer of a nonulosonic acid (legionaminic acid). In contrast to *L. pneumophila*, *C. trachomatis* L2 LPS commonly is of the rough type but also possesses long-chain fatty acids (24). These represent normal, 3-hydroxylated, and branched fatty acids which belong mainly to the tetradecanoic, iso- and anteiso-branched tetradecanoic, hexadecanoic, and 3-hydroxyeicosanoic acids. The endotoxic activity of *C. psittaci* LPS (and possibly other chlamydial LPSs as well) is low when tested by lethal toxicity, pyrogenicity, or local Shwartzman reactivity (6). The low endotoxic activity may have resulted from an adaptation and selection process for intracellular life, and the long-chain and highly substituted fatty acids of both *Legionella* and *Chlamydia* may protect the bacteria from digestion by esterases and amidases of amoebae (11).

Mip contributes to the intracellular multiplication of *L. pneumophila* in amoeba and to the infection process of macrophages. The protein is a prolyl-peptidyl isomerase (14), which had been reviewed fairly recently (17). One of the effects may be an interference with intracellular signal transduction. *C. trachomatis* also produces a Mip-like protein, and it can be expected that this is true for *C. pneumoniae* too (20, 21). We therefore suggest that Mip, together with the unusual LPS, may also have enabled *C. pneumoniae* to live within *Acanthamoeba castellanii.*

The biological significance of intra-amoebal growth in the case of *Legionella* may be twofold. The oligotrophic environment does not offer sufficient nutrients for growth of *Legionella*, which obviously are delivered by the amoebae. In addition, amoebae protect against the bactericidal bacteria *Bdellovibrio* spp., which specifically attacks gram-negative bacteria (29). Regarding *C. pneumoniae*, the main advantage of phagocytosis by amoebae probably is the delivery of ATP from the host cell and protection from adverse affects in the environment.

After prolonged cocultivation of *C. pneumoniae* and *A. castellanii* in vitro, atypical inclusions harboring aberrant chlamydial forms were seen, suggesting suboptimal growth conditions for *C. pneumoniae*. However, when the amoebae were lysed 14 days after chlamydial infection and normal HL host cells were inoculated with released *C. pneumoniae*, the agent was infective and again entered its typical growth cycle. A similar mechanism was attributed to so-called persistent chlamydiae, which had been treated with gamma interferon, a cytokine known to inhibit the growth of *C. trachomatis* (4).

The clinical and epidemiological significance of intra-amoebal growth of *L. pneumophila* is very well characterized, and the environmental source of infections has been identified. Person-to-person spread of *Legionella* does not occur. Whether intra-amoebal growth of *C. pneumoniae* also plays a role in epidemiology is mere speculation. *C. pneumoniae* is believed to be transmitted from person to person by respiratory tract secretions, but direct evidence is lacking. It appears that many patients transmit *C. pneumoniae* ineffectively, and some persons colonized by *C. pneumoniae* are asymptomatic (5, 19). Detection of viable *C. pneumoniae* in respiratory specimens succeeds only rarely. On the other hand, several seroepidemio-

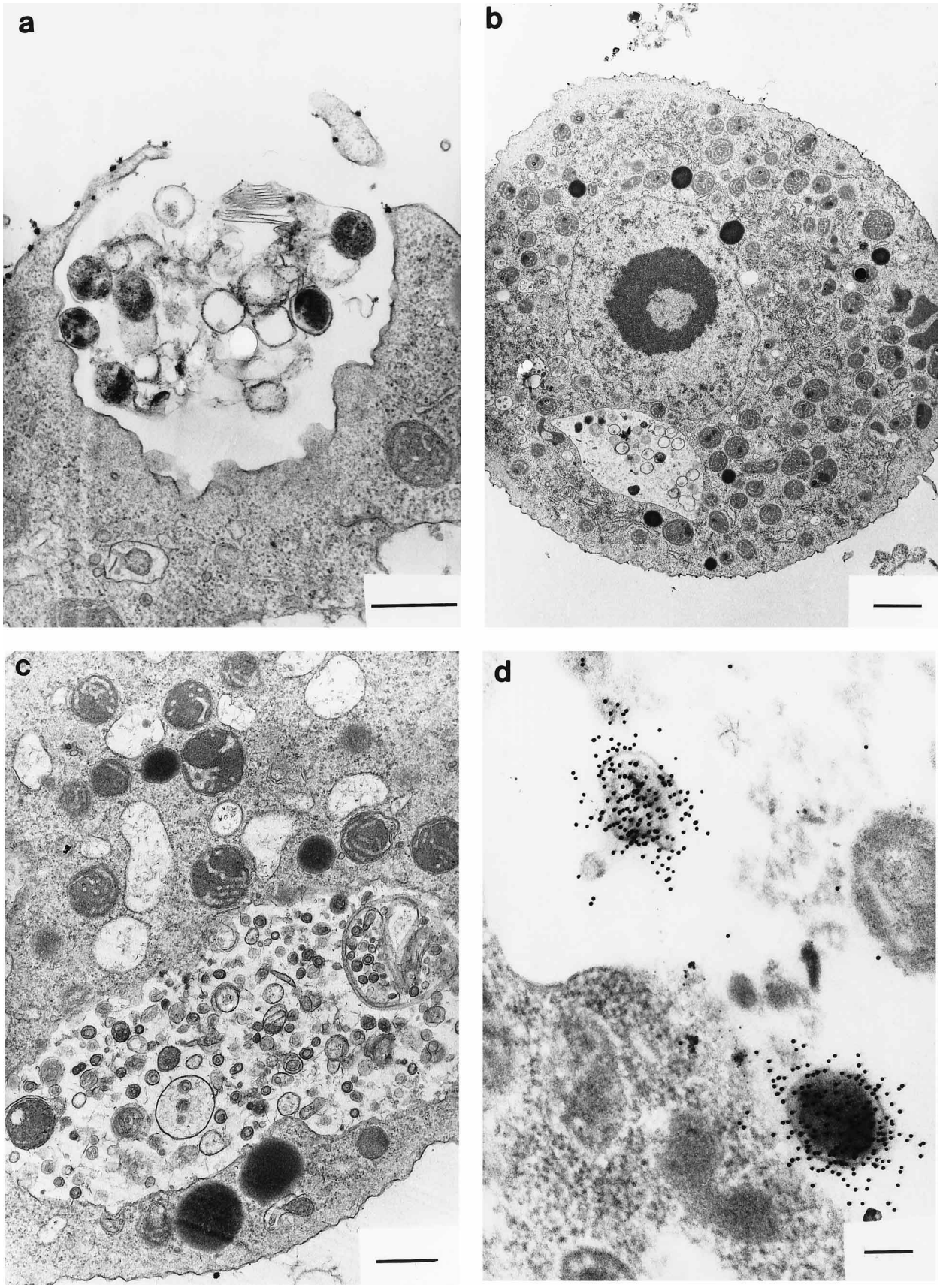

FIG. 2. (a) TEM 4 h postinfection. Phagocytosis of *C. pneumoniae* elementary bodies by *A. castellanii* is illustrated. Magnification, ×30,000. Bar, 0.5 μm. (b) TEM of *A. castellanii* infected with *C. pneumoniae* 2 days postinfection. Adjacent to the nucleus of the amoebal host, the arrow indicates a vacuole containing chlamydial elementary and reticulate bodies. Magnification, ×8,000. Bar, 1 μm. (c) TEM of *A. castellanii* and *C. pneumoniae* 10 days postinfection. An atypical inclusion with abundant forms smaller than cell culture-grown elementary bodies is visible. Magnification, ×23,000. Bar, 0.5 μm. (d) Colloidal immunogold staining of *C. pneumoniae* 10 days postinfection. Accumulation of gold particles at the chlamydial surface is visible. Magnification, ×80,000. Bar, 0.1 μm.

logical surveys revealed that 50 to 60% of sera show antibodies against *C. pneumoniae* (1, 5, 16, 30), suggesting that exposure to *C. pneumoniae* must be quite common.

It is tempting to speculate about whether the presence of *C. pneumoniae* in the environment fits the epidemiological data cited above. It could be a link between the observation of a rather inefficient transmission between persons, the difficulties in culturing *C. pneumoniae* from respiratory specimens, and the high prevalence of antibodies in the population.

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