Mouse resident peritoneal macrophages partially control in vitro infection with *Coxiella burnetii* phase II

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Abstract

*Coxiella burnetii*, the agent of Q fever in man and of coxiellosis in other species, is a small, dimorphic, obligate intracellular bacterium, sheltered within large, acidified, and hydrolase-rich phagosomes. Although several primary and established cell lines, macrophage-like cells, and primary macrophages from other species have been infected with *C. burnetii*, the infection of mouse primary macrophages has not been sufficiently characterized. In this report quantification of DAPI (4', 6-diamino-2-phenylindole) fluorescence images acquired by confocal microscopy, and transmission electron microscopy were used to compare the infection of three mouse-derived cells, L929 fibroblasts, J774 macrophage-like cells, and resident peritoneal macrophages, with a phase II clone of *C. burnetii* known to be non-virulent for mammals. Infected peritoneal phagocytes differed from L929 or J774 cells in that: (a) large vacuoles took longer to appear (3–5 d instead of 2), and were only found in a subset (20–30%) of macrophages, as opposed to in more than 70% of the other cells; (b) total and vacuole-associated relative bacterial loads in L929 and J774 cells were several-fold higher than in peritoneal macrophages; (c) estimated doubling times of the bacteria were about 68 h in the primary macrophages, 18 h in J774 and 22 h in L929 cells. Thus, mouse resident peritoneal macrophages control both the formation of the large vacuoles and the intracellular proliferation of *C. burnetii* phase II. © 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Keywords: *Coxiella burnetii* phase II; Macrophages; Intracellular infection

1. Introduction

The Gram-negative, dimorphic and obligate intracellular bacterium *Coxiella burnetii* is the agent of Q fever in man, a zoonosis of nearly worldwide distribution. *C. burnetii* belongs to the γ subdivision of Proteobacteria and has some homology to *Legionella* sp. [1–6]. Small and large cell variants (SCV and LCV) of the organism have been identified by transmission electron microscopy (TEM) of thin sections and were separated by gradient centrifugation. SCV, more resistant to environmental stresses, and assumed to be the infective forms, transform intracellularly into LCV, more active metabolically and more capable of replication [4,7,8].

Two phases of *C. burnetii* bacteria have been described. Highly virulent, phase I organisms are found in infected hosts and insect vectors. Phase II bacteria, avirulent for mammals, have been obtained in the laboratory by multiple passages through chicken embryos or cell cultures [1,4]. Phase I differs from phase II bacteria in the composition of the polysaccharide moieties of their lipopolysaccharide, which may explain the higher sensitivity of phase II organisms to damage by complement. In contrast to the plaque purified *C. burnetii* phase II bacteria used in the present work, other phase II preparations may not be free of phase I organisms, possibly accounting for often described phase reversions obtained after the inoculation of laboratory animal hosts [9].

Soon after their internalization by permissive host cells, *C. burnetii* are found in small phagosomes which contain one or a few organisms and proceed to fuse with each other.
and with other endocytic or phagocytic vesicles. By 2 or 3 d, infected cells display one or more large vacuoles that contain replicating organisms and are often apposed upon the cell nucleus [10–12]. Although growth of the vacuoles has been associated with membrane input, other mechanisms may contribute to the vacuolar size, such as reduced re-circulation of vacuolar membranes, or the production of osmotically active molecules by the bacteria. At the end of the log phase of growth, by day 5 or 6, depending on the cell type and inoculum, vacuoles may shelter hundreds of closely packed organisms [1,10–19].

Vacuolar contents are acidified, enriched in lysosomal hydrolases, and their membranes display lysosomal glycoproteins and rab7, but not transferrin receptors [5,12,13].

This infection pattern, involving the progressive development of large, bacteria-rich vacuoles in most infected cells, is found in primary or continuous lines of fibroblasts and epithelial cells from different species, as well as in macrophage-like cell lines, such as mouse J774 and P388D1, or human THP-1, acutely or persistently infected with phases I or II of *C. burnetii* [1,3,4,16–18,20,21].

Less is known on the infection of primary macrophages with *C. burnetii*. It has been shown that the infection depends on the animal species and strain of the macrophages, the virulence of the bacterium, and the degree of activation of the phagocytes; it is also to be expected that the infection may differ according to the macrophage population under study [22]. Thus, resident or elicited, but non-activated peritoneal macrophages from guinea-pigs and hamsters are reported to be quite susceptible to infection with phases I and II of *C. burnetii* [10–19].

In human monocyte-derived macrophages, however, phase II *C. burnetii*, although internalized in larger numbers, survived for only a few days, whereas the smaller numbers of virulent phase I organisms taken up were not killed and slowly multiplied [26,27]. These different fates were associated with the recognition of the two phases by discrete phagocytic receptors, the glycan-binding domain of the complement receptor CR3, for phase II bacteria, and the discrete phagocytic receptors, the glycan-binding domain of were associated with the recognition of the two phases by killed and slowly multiplied [26,27]. These different fates numbers of virulent phase I organisms taken up were not

**2. Materials and methods**

2.1. Cells

The cell lines used were J774 mouse macrophage-like cells, L929 mouse fibroblasts and resident peritoneal macrophages (PMφ). J774 cells were routinely passaged by scraping, whereas L929 were trypsinized. Resident PMφs were obtained from peritoneal washouts of BALB/c female mice, 7–9 weeks old, killed by cervical dislocation. Cells (2 \( \times 10^4 \)) suspended in Ca\(^{2+}\) and Mg\(^{2+}\)-free phosphate buffered saline were allowed to attach onto 13-mm diameter glass coverslips placed in 2 cm\(^2\) wells and kept in 0.5 ml antibiotic-free Dulbecco modified essential medium, containing 15 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 2 g/l sodium bicarbonate, 1 mM L-glutamine and 5% (v/v) fetal bovine serum. Cultures were kept at 36 °C in a 5% CO\(_2\)-95% air atmosphere. To stop cell multiplication, unless stated otherwise, before their final plating, L929 and J774 cells were exposed to 1000 rad (10 Gy), at a rate of 4.83 Gy/min, in a \( ^{137} \)Cs gamma irradiator.

2.2. Bacterial preparation and infection of host cells

Plaque purified, clone 4 phase II Nine Mile strain of *C. burnetii* bacteria, which are infective for cells but not for animals, were provided by Ted Hackstadt (Rocky Mountain Laboratories, NIAID, NIH, Hamilton, MT, USA) and handled in a biosafety level II facility [21]. Reference [29]. Recently, flow cytometry was used to compare the infection with virulent *C. burnetii* of lung alveolar macrophages from C57Bl/6, A/J and BALB/c mice, assumed to be, respectively, resistant, susceptible and of intermediate susceptibility to infection with the bacteria. Depending on the multiplicity and duration of the infection, relative loads of the organism were lower in C57Bl/6 macrophages than in the phagocytes from the more susceptible strains [30].

We have previously shown that the relative intracellular load of *C. burnetii* phase II, their intracellular distribution and doubling times, could be estimated in DAPI (4', 6-diamino-2-phenylindole)-stained cells by confocal laser fluorescence [19]. We have used this procedure to compare the development of the infection of three different mouse cells: resident peritoneal macrophages and J774 macrophage-like cells (both of BALB/c origin) and L929 fibroblasts (derived from C3H mice). In most experiments, J774 and L929 cells were irradiated to arrest their multiplication. Thin section TEM was performed to examine the intracellular distribution and morphology of the bacteria. Preliminary results were presented in abstract form [31].
provides information on some of the features of the phase II clone used in the present report. Infective inocula were prepared from confluent, persistently infected Vero cells as described [19]. Dilution assays indicated that stock suspensions contained $10^9$–$10^{10}$ infective bacteria/ml. At the time of infection, to disrupt aggregates, the suspensions were mildly sonicated at 35 kHz for 15 min at room temperature. One day after plating the cells, 10 µl of the suspensions were added per well containing a cell bearing coverslip in 0.5 ml complete medium. After 24 h, infected cultures were vigorously washed with Hanks saline and fresh medium was added.

2.3. Cell fixation and staining

At different times after infection, cultures were fixed for 1 in 3.5% formaldehyde containing 7.5% sucrose, stained

Fig. 1. Contrasting features of mouse peritoneal macrophages, L929 and J774 cells infected for 4 d with C. burnetii phase II. A, C and E: confocal superimposed images of DAPI fluorescence (cyan) and Nomarski differential interference contrast (gray): peritoneal macrophages (A); L929 cells (C); J774 cells (E). B, D and F: transmission electron microscopy of cells from the same experiment: peritoneal macrophages (B); L929 cells (D); J774 cells (F). Numbers over scale bars indicate µm.
for 15 min with 3.5 µM DAPI, washed and mounted in 50% glycerol (v/v).

2.4. Confocal microscopy, image acquisition and processing

Images were acquired in a Bio-Rad 1024UV confocal system attached to a Zeiss Axiocam 100 microscope equipped with a 40× water immersion objective N.A. 1.2 plan-apochromatic lens, with differential interference contrast. LaserSharp 1024 version 2.1A from BioRad was used for image acquisition and MetaMorph (Universal Imaging Corporation) version 3.5, was used for image processing. Polygons were drawn onto digitized images of infected cells and the relative fluorescence intensity of DAPI-stained bacteria within the circumscribed areas was determined. Under the measurement conditions used, the fluorescence intensity within each polygon is assumed to be proportional to the bacterial load in the region measured [19].

2.5. TEM

Infected host cells were fixed at room temperature with modified Karnovsky (2% formaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate pH 7.4) for 1 h and dislodged by scraping. Cell samples were post-fixed with 1% osmium tetroxide for 1 h at room temperature, gradually dehydrated and embedded in Araldite (Lab-Ladd). Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a JEOL 1200 EX II electron microscope at 80 kV.

3. Results

3.1. Large vacuoles containing C. burnetii phase II in infected PMφ, L929 and J774 cells

To provide an overview of Coxiella vacuoles in the late log phase of bacterial growth, parallel cultures of PMφ s, L929 and J774 cells were infected with similar numbers of organisms per well, and 4 d later fixed and processed for fluorescence and TEM. Fig. 1 emphasizes the paucity of bacteria in the large vacuoles of PMφ s (A, B), in contrast to the large numbers of organisms sheltered in the vacuoles of infected L929 (C, D) and J774 (E, F) cells.

3.2. Development of large parasitophorous vacuoles in PMφ s, L929 and J774 cells infected with C. burnetii phase II

Twenty-four hours after bacteria were added to the cultures, large vacuoles containing C. burnetii were rarely found in L929, J774 or PMφ s examined by phase contrast and TEM. Thereafter, the proportion of L929 and J774 cells with large vacuoles increased rapidly; it reached 60–80% or more on the third day, and remained almost stable between the fourth and eighth days of infection.

In contrast, after 3 d of infection, only about 25% of the PMφ s, developed large vacuoles, with only a modest increase by the eighth day of infection (Fig. 2). A striking feature of the infection of PMφ s was that very small numbers of C. burnetii bacteria were able to induce and maintain very large vacuoles in some of the infected cells.

3.3. C. burnetii in small vacuoles in PMφ that did not develop large parasitophorous vacuoles

Fluorescence microscopy of cultures infected with C. burnetii phase II for 4 d reveals that, although as shown in Fig. 1, large vacuoles develop in only a minority of PMφ s, the other cells display clusters of bacteria in small vacuoles dispersed in the cytoplasm (Fig. 3A–D). TEM observations confirmed that the bacterial clusters were enclosed in membrane-bounded vesicles (Fig. 3E, F) and, in addition, indicated that most bacteria appeared ultrastructurally well preserved in macrophages and in the other cells (Figs. 1B, inset, and 3F).

Since a large proportion of the bacteria in infected cultures were confined to small vacuoles, we wondered if the organisms identified by DAPI fluorescence in the small vacuoles could be dead. When heat killed C. burnetii were given to uninfected macrophages, DAPI-stained bacteria were clearly detected in macrophages fixed 4 h later. The organisms were weakly fluorescent in cells fixed at 24 h, but no fluorescence was detected at 48 h (results not shown).

3.4. Growth curves, estimated doubling times, and vacuolar bacterial density of C. burnetii in L929, J774 cells and PMφ

The fluorescence of DAPI-stained bacteria was measured to compare C. burnetii loads during the first 4 d of the
infection of L929, J774 and PM\(^\Phi\) cells. The results show that, at all times, the relative bacterial loads in PM\(^\Phi\) were lower by at least a log than in L929 or J774. Bacterial estimated doubling times were 20 h in L929; 18 h in J774; and 68 h inside PM\(^\Phi\). As a result, the net increase in bacterial load with time was much smaller in primary macrophages than in the other cells (Fig. 4).

A derived parameter, fluorescence density, which relates the fluorescence intensity to the section area of the vacuoles, was also used to compare the development of C. burnetii in the different host cells. Fig. 5 shows that the bacterial density did not appear to increase in PM\(^\Phi\) from days 2 to 4 of the infection, in contrast to the steep increase displayed in irradiated L929 and J774 cells.

We have previously shown that vacuole size and bacterial load were significantly correlated in C. burnetii-infected Vero cells [19]. Fig. 6 shows that a significant correlation (\(P < 0.001\)) was likewise found in non-irradiated, infected L929 cells (\(r = 0.78\)) and in PM\(^\Phi\) (\(r = 0.51\)). The figure also shows that within a similar range of vacuolar size, peritoneal macrophages displayed much lower relative loads of bacteria. Average area for the set of vacuoles shown in Fig. 6 was 173 ± 17 µm\(^2\) (mean ± standard error; \(n = 50\)) for L929 and 156 ± 12 µm\(^2\) for PM\(^\Phi\). In contrast, vacuolar
4. Discussion

Compared to L929 fibroblasts or J774 macrophage-like cells, phase II C. burnetii-infected PM\(\Phi\)s displayed the following features: (a) smaller intracellular loads of ultrastructurally well-preserved dimorphic bacteria; (b) most bacteria found in small vacuoles, each sheltering several organisms; (c) large vacuoles, containing few organisms, observed in about a quarter of the cells; (d) estimated doubling times in macrophages about three times longer than in the two other cell types.

The incomplete nature of the control of C. burnetii phase II infection by PM\(\Phi\)s is highlighted by the surprisingly small numbers of bacteria needed to induce and maintain large vacuoles in the cells. Since these vacuoles persisted at least for 8 d, the organisms should be metabolically viable, as also suggested by their ultrastructural preservation (Figs. 1 and 3). The identification of SCV and LCV of the bacteria in both small and large vacuoles (Figs. 1B, inset, and 3F), confirming earlier observations [32,33], reduces the possibility that the infection is controlled by inhibition of the transformation of SCV into LCV.

The cellular microbiology literature provides examples of wild type, attenuated or mutated organisms that survive and multiply in fibroblasts or epithelial cells, but may be restricted or killed in macrophage-like cells or primary macrophages [34,35]. Other organisms survive in macrophage cell lines but are restricted in primary macrophages [22,36]. It is likely that non-professional phagocytes should be more amicable to intracellular pathogens than their professional counterparts, and that macrophage-like cell lines may be deficient in specific phagocytic recognition and/or microbicidal mechanisms actually or potentially available to primary macrophages [37]. However, given the range of pathogen adaptations for intracellular survival and of facilitating and protective host cell responses to pathogens, it may be hazardous to predict the outcome of an intracellular infection solely on the basis of general features of the host cells and of the virulence of a particular organism to animals.

We hope the results presented will encourage the search for restriction mechanisms that control the C. burnetii phase II infection of mouse peritoneal macrophages.
tion of the bacteria and the development of large vacuoles might be slowed by host cell-related factors such as inappropriate vacuolar pH, interference with vesicle translocation or fusion, limited substrate or co-factor availability, or the production of microbistatic or microbialic effector molecules. These mechanisms and host cell functions may be positively or negatively modulated by cytokines, chemokines and other factors. Production of reduced oxygen or nitrogen products could be triggered directly by the interaction of the phagocytes with C. burnetii, or indirectly by cytokines or other factors produced by the cells. However, studies with cells from chronic granulomatous disease subjects indicated that oxygen reduction products may not be involved in the control of the bacteria in human monocyte-derived macrophages. Likewise, the production of NO by mouse alveolar macrophages may not account for the mouse strain dependence of the production of NO by mouse alveolar macrophages. 

Current studies in this laboratory examine the role of selected cytokines and effector microbicidal molecules in the control of phase II C. burnetii infection by primary mouse macrophages.

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