A Proposed Model to Explain Persistent Infection of Host Cells with *Coxiella burnetii*

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L929 mouse fibroblast cells and J774 macrophage-like cells are both susceptible to persistent infection with the Q fever agent *Coxiella burnetii*. Previously this laboratory has shown that persistently infected cell populations multiply with unaltered generation times or cell cycle progression. It has also been reported by others and us that highly infected cells typically exhibit one large parasite-containing vacuole. We now report that lightly and heavily infected cells are capable of division and in the process segregate the parasite-containing vacuole into one of the emerging daughter cells; the companion daughter cell emerges parasite-free. This asymmetric division of infected cells, revealed via photomicrography of stained cells, accounts for the appearance of uninfected cells within persistently infected host cell populations that were previously 100% infected. Some of the persistently infected L929 populations were maintained in culture for over two years without the addition of normal cells.

INTRODUCTION

The rickettsial agent of Q fever, *Coxiella burnetii*, occasionally causes persistent infection in man and experimental animals. This obligate intracellular prokaryotic parasite has been detected in placental tissue from women who had previously experienced Q fever and who had overtly recovered (Syrucsek *et al.*, 1958). Chronic endocarditis and granulomatous liver involving the Q fever agent are well known (reviewed by Baca & Paretsky, 1983). Reactivation of Q fever in laboratory animals has been obtained after treatment with ionizing radiation (Sidwell *et al.*, 1964*a*) or cortisone (Sidwell *et al.*, 1964*b*), or as a result of parturition (Sidwell & Gebhardt, 1966). Reasons for persistence are not known although defects in the immune system may be, in part, responsible. The types of cells that harbour the persisting rickettsiae remain unidentified. Several cell lines have been persistently infected with *C. burnetii*, including L929, J774 and P388D1 cells (Baca *et al.*, 1981; Burton *et al.*, 1978). The cell populations continue to divide without the addition of uninfected cells. Recently we reported (Baca *et al.*, 1985) that persistent infection of L929 cells with *C. burnetii* results in infected cell populations with generation doubling times equal to those of uninfected cells. Furthermore, the cell cycle progression of the infected cell population was identical to that of uninfected cells. Those observations, in part, prompted us to investigate the mechanism of persistent infection. Another observation that led us to undertake these investigations was that persistently infected populations frequently contain normal uninfected cells. Several explanations may account for these observations, including (i) the possibility that some cells are not susceptible to infection; (ii) infected cells may cure themselves of the infection; (iii) an infected cell may divide, resulting in an infected and an uninfected daughter cell; or (iv) combinations of the three.

METHODS

C. burnetii propagation and infection of L929 cells. The Nine Mile strain of *C. burnetii*, phase I (clone 7) and phase II (clone 4) were originally obtained from M. Peacock of the Rocky Mountain Laboratory, US Public Health Service, Hamilton, Montana, USA. A 50 µl sample of a 10% (w/v) yolk sac homogenate heavily infected with the

rickettsiae was added to 5.0 ml L929 cells ($2.5 \times 10^5 \text{ ml}^{-1}$) held in Corning 60 mm tissue culture plastic Petri dishes. The infected L929 cells were grown in antibiotic-free Eagle's essential medium, spinner modified, with L-glutamine, NaHCO_3 (0.22%, w/v) and 5% (v/v) heat-inactivated calf serum. The normal L929 cells were originally obtained from R. Erickson, University of Colorado Medical School, Denver, USA, and were adapted for growth in suspension culture. The Petri dishes were kept at 37°C in a 10% CO_2 atmosphere. At 3 d postinfection the cell concentrations were adjusted back to $2.5 \times 10^5 \text{ ml}^{-1}$ with fresh growth medium and 10 ml of the suspended cells were transferred to 50 ml screw-cap Erlenmeyer flasks and incubated at 35°C in a New Brunswick G24 Incubator Shaker (100 r.p.m.). Eventually the cell suspension was increased to 25 ml in 125 ml screw-cap Erlenmeyer flasks. The cell concentrations were adjusted to $2.5 \times 10^5 \text{ ml}^{-1}$ three times a week. Viable cell counts were determined by the dye exclusion technique using erythrosin B and a haemocytometer (Phillips, 1973). Routinely, duplicate samples were counted from replicate flasks.

C. burnetii propagation and infection of J774 cells. J774 macrophage cells were infected with both phases of the Nine Mile strain of *C. burnetii*. A 50 μl sample of a 20% (w/v) yolk sac homogenate heavily infected with the rickettsiae was added to 5.0 ml J774 cells (1.0×10^5 cells) held in Corning 60 mm tissue culture plastic Petri dishes. Cell cultures were maintained as previously reported (Akporiaye *et al.*, 1983).

Staining of C. burnetii-infected cells. Two or three drops of infected L929 or J774 cells were centrifuged onto glass microscope slides in a Shandon Cytospin 2 centrifuge at 1000 r.p.m. (approx. 100 g) for 5 min. After drying, the prepared slides were stained by the method of Gimenez (1964). The slides were stained with primary carbol fuchsin stain for 10 min followed by two 7 s exposures to the malachite green counterstain. The stained cells were examined with a Leitz SM Lux photomicroscope. Photographs were taken on either Kodak Ektachrome or Plus-X film.

RESULTS

Long-term cultivation and viability of persistently infected cell populations

Several independently infected and maintained populations of L929 and J774 cells were used in these studies. Some L929 fibroblast cell populations were exposed to both phases of *C. burnetii*

Fig. 1. Percentage of L929 cells infected with phase I *C. burnetii* and percentage cell viability.

Fig. 2. Percentage of L929 cells infected with phase II *C. burnetii* and percentage cell viability.

Fig. 3. Proposed model for persistent infection of host cells by *C. burnetii*. N, nucleus.

and maintained in continuous culture for over two years without the addition of normal uninfected cells. Cell counts were made three times weekly together with determinations of cell viability and the percentage infection of the cell population. The viability of L929 cells persistently infected with phase I remained high, exceeding 90% (Fig. 1). During the 250 d span shown, the proportion of cells infected fluctuated from around 25% to over 90%. Phase II-infected cells also remained highly viable; the proportion of cells infected varied from around 60% to close to 100% (Fig. 2). With this and other cell populations, we have periodically observed 100% infection. For example, at days 540 and 493 postinfection with phase I and phase II *C. burnetii*, respectively, all the cells contained rickettsiae (data not shown).

During the early stages of infection (1–60 d) the L929 phase I-infected cells maintained a high viability (over 90%, during which the proportion of infected cells fluctuated from around 40% to close to 90%). Phase II-infected cells also exhibited over 90% viability and the proportion of cells infected varied from 70% to 100%. At 2 d postinfection the proportion of cells infected with phase I *C. burnetii* was close to 80% while almost 100% of the cell population was infected with phase II rickettsiae.

The J774 macrophage-like cells were exposed to both phases of *C. burnetii* and maintained in continuous culture for over 300 d. During this time, no normal cells were added to the infected cultures. Twice a week cell counts were made along with determination of cell viability and percentage of cells infected. The cell viability remained over 70% during this period; the proportion of the cells infected varied from 70% to 100% in both phase I- and phase II-infected cell populations.

The viabilities of the infected L929 and J774 cells were comparable to those of their uninfected counterparts.

Model for persistent infection and experimental evidence

Fig. 3 depicts the proposed model for persistent infection of cells by *C. burnetii*, and Figs 4–7 are photomicrographs of infected L929 and J774 cells, providing visual evidence that substantiates the model. The model shows that a normal cell becomes infected, resulting in one or more rickettsiae-containing vacuoles. The infected cell subsequently undergoes mitosis, giving rise to two daughter cells, which may be infected. With progressive infection, the

Fig. 4. L929 cells infected with phase I *C. burnetii* in metaphase and telophase at 336 d postinfection. Note the cell in telophase giving rise to an infected daughter cell with three vacuoles and a rickettsiae-free daughter cell. Bar, 5 μ m.

rickettsiae-containing vacuoles eventually fuse to form a single large highly infected vacuole (Fig. 3*f*). Such a heavily infected cell would undergo mitosis followed by cytokinesis, giving rise to an infected daughter cell and a normal uninfected daughter cell (Fig. 3*g-i*). Fig. 4 shows an L929 phase I-infected cell in telophase giving rise to an infected daughter cell with three vacuoles and a rickettsia-free daughter cell. Close by is a heavily infected cell in metaphase. These cells were from a population that had been continuously infected for 336 d. An L929 phase I-infected cell (331 d postinfection) in late telophase is shown in Fig. 5. This heavily infected cell is in the process of giving rise to a heavily infected daughter cell and a normal uninfected daughter cell. A heavily phase II-infected L929 cell in telophase (Fig. 6) at 302 d postinfection is in the process of giving rise to a heavily infected daughter cell and a rickettsia-free daughter cell. A J774 cell at 42 d postinfection with phase I *C. burnetii* is shown in Fig. 7; the cell is also in telophase with an emerging heavily infected daughter cell and a lightly infected companion daughter cell.

The model (Fig. 3) indicated that heavily infected cells are larger than normal uninfected cells. That conclusion is supported by actual measurements of infected L929 cells (Table 1). Heavily infected cells (containing ≥ 100 rickettsiae per cell) were approximately 1.5 times larger than normal uninfected cells. Also the emerging infected daughter cells were larger than their counterpart normal emerging uninfected daughter cells.

Mitotic indices of normal and infected cells

The mitotic indices of normal uninfected and infected L929 cells in exponential growth were similar. For example, at 452 d postinfection, 9% and 7% of phase I- and phase II-infected cells,

Fig. 5. L929 cells infected with phase I *C. burnetii* in telophase with an emerging heavily infected daughter cell (arrowed) and a rickettsiae-free daughter cell. Nearby is a normal uninfected cell. The photomicrograph was taken of cells continuously infected for 465 d. Bar, 5 μ m.

Table 1. *Diameter measurements of infected and normal L929 cells*

Cell	Diameter (μ m)*	
	Range	Mean $\pm$ SE
Normal	13-32	22 $\pm$ 4.2
Infected†	20-43	28 $\pm$ 4.2
Heavily infected‡	24-58	39 $\pm$ 6.4
Emerging daughter cells in mitosis:§		
Infected/normal	14-30/9-20	24 $\pm$ 4.3/15 $\pm$ 3.2
Infected/infected	12-29/11-28	23 $\pm$ 4.2/22 $\pm$ 4.2
Normal/normal	11-20/9-20	16 $\pm$ 2.8/15 $\pm$ 2.6

* One hundred cells were measured.

† A cell was designated infected if one to fifty rickettsiae were observed associated with the cell.

‡ A cell was designated heavily infected if it contained more than one hundred rickettsiae.

§ Cells in late telophase were measured. A cell was designated infected if it contained one or more rickettsiae.

respectively, were in mitosis. Parallel cultures of normal uninfected cells contained 11% of cells in mitosis.

DISCUSSION

Previously we (Baca *et al.*, 1981, 1985) and others (Burton *et al.*, 1971) have reported that various cell lines, including the L929 and J774 cell lines, can become persistently infected with

Fig. 6. L929 cells infected with phase II *C. burnetii* at 302 d postinfection. Note the cell just off centre in late telophase with a narrow cytoplasmic bridge connecting the emerging daughter cells. One of the emerging daughter cells is heavily infected; the other emerging daughter cell is rickettsiae-free. Arrows point to rickettsiae-containing vacuoles. Bar, 3 μ m.

the Q fever agent for long periods and without the addition of normal cells. Persistently infected L929 cells continue to divide for over a year with unaltered generation times and cell cycle progression (Baca *et al.*, 1985). This suggested to us that infected cells could undergo mitosis and cytokinesis. The results presented in this report unequivocally demonstrate that, indeed, infected cells, including those that contain enormous numbers of rickettsiae, can divide. A lightly or heavily infected cell with one rickettsiae-containing vacuole may divide asymmetrically, one daughter cell inheriting the parasite-containing vacuole and the other emerging parasite-free.

After entry of *C. burnetii* by phagocytosis (Burton *et al.*, 1971) the phagocytic vacuole fuses with lysosomal granules (Akporiaye *et al.*, 1983; Burton *et al.*, 1971) and the parasites proceed to multiply. More than one such vacuole may occur within a cell (Fig. 4); the cell may subsequently divide, giving rise to two infected daughter cells (Fig. 7). With progressive infection the cells eventually exhibit one large rickettsiae-containing vacuole (Burton *et al.*, 1978; Baca *et al.*, 1981), which presumably results from the fusion of several rickettsiae-containing vacuoles. Such a cell, upon division, gives rise to an infected and an uninfected daughter cell (Figs. 5 and 6). This asymmetric division accounts for the appearance of uninfected cells in populations that previously were 100% infected. The generation of uninfected cells from infected parent cells is apparently the key to the maintenance of persistent infection. These uninfected cells must also be susceptible to infection because periodically all the cells of a population contained rickettsiae (see Results); this observation excludes the possibility that some cells are resistant to infection.

Fig. 7. J774 cells infected with phase I *C. burnetii* at 42 d postinfection. The centrally located cell in telophase is giving rise to both a heavily infected daughter cell and a lightly infected one. Arrows point to heavily infected vacuoles (more than 100 rickettsiae per vacuole). Bar, 3 μ m.

The ultimate destiny of heavily infected cells is unknown; presumably they eventually die and lyse, because many extracellular *C. burnetii* can be seen in the growth medium. Such presumed death and lysis is apparently not a frequent event because the doubling times of normal and infected cell populations are approximately the same (Baca *et al.*, 1985).

Another prokaryotic organism that can also cause persistent infection and that proliferates in vacuoles is *Ehrlichia sennetsu*, the aetiological agent of Kagami fever in humans (Minamishima, 1965) and the recently described Potomac horse fever (Holland *et al.*, 1985). Minamishima (1965) showed that cultured cells heavily infected with *E. sennetsu* could divide, giving rise to an infected daughter cell and a normal uninfected daughter cell.

Several studies have suggested that impaired cellular immunity may account, in part, for chronic *C. burnetii* infection in humans and animals (Hinrichs & Jerrells, 1976; Koster *et al.*, 1985). Such persistent infection may also be due, in part, to asymmetric division of infected cells.

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