

## Cell Cycle Distribution Patterns and Generation Times of L929 Fibroblast Cells Persistently Infected with *Coxiella burnetii*

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***Coxiella burnetii* established a persistent infection of various cell lines including L929 mouse fibroblasts. Although the basis for such persistence is unknown, the phenomenon does require continual growth, proliferation, and maintenance of viability of the host cells. We examined the effect of short- and long-term infection on the host cell's generation time and cell cycle. Flow cytometric studies of actively growing normal and infected cells stained with mithramycin or propidium iodide revealed no significant difference in cell cycle distribution patterns or changes in ploidy level associated with persistent infection with either phase I or phase II *C. burnetii*. The population doubling times of infected and normal cells were similar.**

*Coxiella burnetii* is an obligate intracellular procaryotic parasite that occasionally establishes persistent infection in its natural hosts and in cells maintained in culture. This rickettsial agent of Q fever in humans has been detected in placentas from women who had experienced Q fever 2 to 3 years previously and who had apparently recovered (16). Latent infections in experimental animals have been reactivated by X-irradiation (14), multiple cortisone injections (15), or as a result of parturition (13). In 3 to 20 years after a bout of Q fever, endocarditis may occur, although rarely, in individuals with a history of previously damaged heart valves (see reference 3). In these in vivo infections, the types of cells that sequester the rickettsiae are unidentified.

Several cell lines have been persistently infected with the Q fever agent, including the L929 mouse fibroblast line and several macrophage-like cell lines (2, 5). Some of these infected cell lines (L929, J774, P388D1) have been maintained in our laboratory in an actively growing state for over a year and without the addition of normal cells. *C. burnetii* exhibits antigenic phase variation (phases I and II) and both phases can persistently infect cells. The parasites proliferate within vacuoles that arise from the fusion of phagocytic vacuoles with lysosomal granules (1, 5).

Because chronic infection of the cell lines may mimic or reflect what occurs in humans and animals, we have chosen to study these infected cell lines as model systems for the study of persistent infection. In the present report we demonstrate that, despite an enormous number of intracellular parasites, some of the host cell's growth properties (e.g., cell cycle and population doubling time) are unaffected.

Detailed cell cycle analysis has not been performed on rickettsia-infected cells and, to our knowledge, only on a few other intracellular procaryote-infected cells. Some studies have indicated that cell cycle changes do occur subsequent to infection with some viruses and intracellular procaryotes (7, 11; S. R. Pelc and T. T. Crocker, *Biochem. J.* 78:20p, 1961). It was of interest to determine whether changes in host cell growth characteristics (i.e., cell cycle, generation

time) occur after infection with *C. burnetii* that might provide clues regarding the basis of persistent infection.

### MATERIALS AND METHODS

***C. burnetii* propagation and infection of L929 cells.** *C. burnetii* (phases I and II) organisms were originally obtained from the Rocky Mountain Laboratory, U.S. Public Health Service, Hamilton, Mont. Organisms were propagated in embryonated eggs, and heavily infected 10% yolk sac homogenates were added to normal L929 cells maintained in suspension culture. L929 cells, originally obtained from R. Erickson, University of Colorado Medical School, Denver, were grown in antibiotic-free Eagle essential medium, spinner modified, with L-glutamine, NaHCO<sub>3</sub> (0.22%), and 5% heat-inactivated calf serum. Approximately 0.1 ml of the infected yolk sac homogenate was added to 5 ml of L929 cells (initial cell density,  $2.5 \times 10^5$  per ml). Eventually the cell suspension volume was increased to 25 ml and maintained in 125-ml screw-cap Erlenmeyer flasks held at 35°C in a New Brunswick G24 Incubator Shaker (100 rpm) (New Brunswick Scientific Co., Inc., Edison, N.J.).

In some experiments Colcemid (GIBCO Laboratories, Grand Island, N.Y.) was added to cell cultures at a final concentration of 0.10 µg/ml for periods of up to 8 h to arrest L929 cells in mitosis (metaphase).

Intracellular rickettsiae were enumerated by examining Gimenez-stained (9) cells. Cells containing 50 or more rickettsiae were considered heavily infected.

The proliferation kinetics of infected and normal cells maintained in culture were determined by performing cell counts during a 72-h period at 6-h intervals. Viable cell counts were determined by dye exclusion (erythrosin B), using a hemacytometer (12). Routinely, duplicate samples were counted from replicate flasks.

Cell cultures were routinely tested for bacterial and mycoplasma contamination by standard procedures.

**Cytometric analysis of normal and infected L929 cells.** Infected and normal cells in asynchronous growth were prepared for flow cytometry as follows. The cells were centrifuged ( $160 \times g$ , 5 min, room temperature) and the pellets were suspended in a small volume of ice-cold Hanks balanced salt solution. After suspension, ice-cold ethanol was added, while mixing, to a final ethanol concentration of

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TABLE 1. Population doubling times of normal and *C. burnetii*-infected L929 cells<sup>a</sup>

Expt	<i>C. burnetii</i> antigenic phase	% of cells infected <sup>b</sup>	% Viable cells <sup>c</sup>	Population doubling time (h)
1		0 (uninfected)	90	36
		0 (uninfected)	82	33
	I	95 (70)	79	36
	I	83 (63)	45	39
	II	61 (43)	95	36
2		79 (42)	93	36
		0 (uninfected)	91	33
		0 (uninfected)	88	42
	I	44 (26)	88	36
	I	40 (16)	87	33
	II	46 (20)	87	34
	II	56 (26)	87	27

<sup>a</sup> L929 cells were exposed to *C. burnetii*, and 37 to 40 (experiment 1) and 84 to 87 (experiment 2) days postexposure, the population viability and degree of infection were determined as described in the text. During the 3-day interval, population doubling time, degree of infection, and viability were determined. Duplicate, independently prepared cultures of infected and normal cells were prepared at the beginning of the experiment (day 0) and monitored throughout the duration of the experiment (87 days).

<sup>b</sup> Gimenez-stained (9) cells were examined and the degree of infection was determined as described in the text. A minimum of 100 cells was examined for each sample. A cell was designated infected if at least one rickettsia was observed associated with the cell. The numbers in parentheses indicate the percentage of cells with 50 or more cell-associated rickettsiae.

<sup>c</sup> At least 100 cells were examined for viability by the dye exclusion technique described in the text.

70%. Suspensions of fixed cells were adjusted to approximately  $1.5 \times 10^6$  per ml.

Three DNA-specific dyes with different binding properties were used in these studies: Hoechst 33342, which binds preferentially to adenine-thymine regions; mithramycin, which binds to guanine-cytosine regions; and propidium iodide which, used in combination with RNase, intercalates into double-stranded DNA without base specificity. After ethanol fixation for a minimum of 24 h, the cells were centrifuged to remove the ethanol and the cell pellets were suspended in mithramycin (Pfizer Inc., New York, N.Y.), propidium iodide, or Hoechst 33342 (Calbiochem-Behring, San Diego, Calif.) solution. Mithramycin-staining solution (100  $\mu$ g/ml) was prepared by dissolving 2.5 mg of mithramycin in 25 ml of 0.85% NaCl-15 mM MgCl<sub>2</sub>, pH 7.0 to 7.4 (6). Cell clumps were removed by filtration through a 62- $\mu$ m nylon filter. Alternatively, fixed cells were stained in phosphate-buffered saline solution containing propidium iodide (10  $\mu$ g/ml) and RNase A (50  $\mu$ g/ml; code R; Worthington Diagnostics, Freehold, N.J.) and incubated for 30 min at 37°C before analysis. Mithramycin-stained cells were examined in the Los Alamos National Laboratory argon-ion laser-equipped flow cytometer (10) at 457.8 nm; propidium iodide-stained cells were examined at 488 nm.

Ethanol-fixed cells were also stained with the DNA-specific Hoechst 33342 dye (1.0  $\mu$ g/ml in phosphate-buffered saline) and examined with UV excitation in an epifluorescence microscope.

## RESULTS

At various days after exposure of L929 cells to phase I and II *C. burnetii*, the population doubling times of the exponentially growing cell populations were determined. Simultaneously, cell viability and degree of infection were noted. Table 1 shows that, regardless of the length of time or degree

of infection, the population doubling times were approximately the same as in uninfected controls (ca. 36 h). It should be noted that the majority of the cells were infected with at least one or more rickettsiae. Cell populations infected for 40 days had at least 43% of the cells containing 50 or more parasites. After 87 days of infection the doubling times of infected cells were essentially the same as those of uninfected cells. The viability was at least 87% even after almost 3 months of infection.

Flow cytometric analyses of mithramycin- and propidium iodide-stained infected and normal L929 cells were performed at 1 and 24 h after staining. Regardless of the duration of infection (10 to 59 days) or degree of infection (Fig. 1 and 2), the populations of normal and infected cells showed no differences in their DNA distribution patterns (Fig. 2; Table 2). Flow cytometric-derived histograms provided no indication that rickettsial infection induced polyploidy in L929 host cells (data not shown). DNA content distribution patterns obtained for infected cell populations treated for 8 h with Colcemid showed an expected increase in the area within the G<sub>2</sub> + M (4C DNA content) region; however, no peak was observed at the 8C DNA content position where Colcemid-arrested polyploid mitotic cells

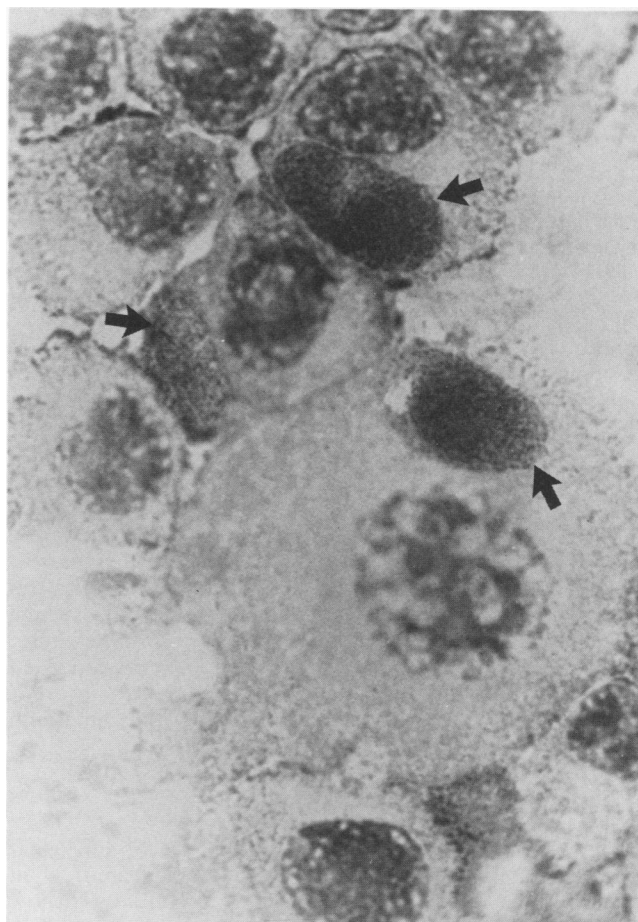


FIG. 1. Photomicrograph of phase I *C. burnetii*-infected L929 cells. The cells had been infected for 10 days and, before photography, were stained by the Gimenez method (9). The population viability was 89%, with 75% of the cells infected and 25% heavily infected. Arrows point to vacuoles engorged with *C. burnetii* (several hundred per vacuole). Magnification,  $\times 5,000$ .

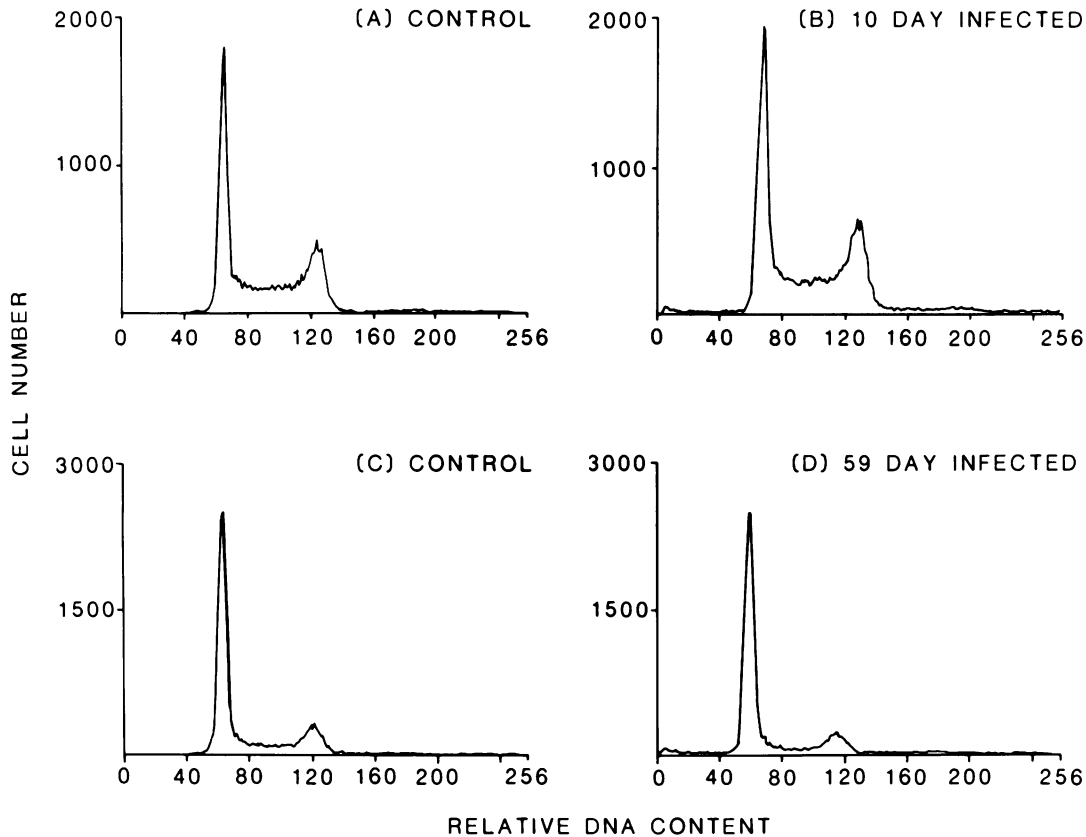


FIG. 2. Relative DNA content frequency distribution histograms for mitramycin-stained control (untreated) and phase II *C. burnetii*-infected mouse L929 cells. Differences in the two control histograms (A and C) reflect the degree of confluency inhibition in the two populations at the time of cell harvest. Similar DNA distribution profiles were obtained with propidium iodide-stained cells. Computer-fit analysis for the histograms are shown in Table 2. The population viabilities for the control cells were 95% (A) and 92% (C). Viability of the 10-day-infected cells (B) was 100%, with 33% of the cells infected with at least one rickettsia and 10% infected with 50 or more parasites. The 59-day-infected cells were 94% viable; 46% of the cells were infected, with 27% heavily infected.

would accumulate. The small areas in the DNA histogram extending beyond the  $G_2 + M$  peaks are attributable to cell clumps (i.e., cells and possibly some fluorescent cellular debris). These results are similar to those obtained in five repeated experiments.

Direct observation with an epifluorescence microscope of infected and normal cells stained for as long as 24 h with mitramycin, propidium iodide, or Hoechst 33342 showed that the rickettsial fluorescence was very faint whereas host nuclear fluorescence was intense. This observation correlated with the flow cytometric DNA profile data, which indicated that the rickettsial DNA content contributed little, if any, to the fluorescence distribution patterns since no major shift in the DNA histogram was detected.

The results obtained were approximately the same with both phase I and II *C. burnetii*-infected cells.

#### DISCUSSION

Changes in the cell cycle after entry of a procaryotic agent have been reported; however, these reports are few in number. For example, psittacosis-infected HeLa cells show a doubling in duration of S phase and generation time (Pelc and Crocker, *Biochem. J.* 78:20p, 1961), whereas ornithosis-infected HeLa cells exhibit extended  $G_1$ ,  $G_2$ , and possibly M phases (7). It should be noted, however, that persistent infection does not result with either agent and that the infected cell population eventually lyses.

In the case of virus-infected cells, there have been a few reports on the effect of viral infection on the cell cycle. Human adenoviruses cause alteration of the cell cycle of some growing rodent cells and induce chromosome damage in a cell cycle-dependent fashion, requiring the expression of one or more early viral genes (11).

Our results show that, despite an enormous parasite burden, the L929 population remains essentially unperturbed with respect to cell cycle progression, generation time, and genome stability. That the parasite does not affect these growth characteristics is consistent with the establishment of long-term persistent infection.

During the course of these studies we have found that under the conditions used the rickettsiae do not acquire

TABLE 2. Percentages of cells in phases of the cell cycle<sup>a</sup>

Cells	% in phase:		
	$G_1$	S	$G_2 + M$
Control (uninfected)	37.8	48.0	14.2
Infected (10 days)	34.5	48.7	16.8
Control (uninfected)	60.5	28.0	11.5
Infected (59 days)	68.7	23.9	7.4

<sup>a</sup> Derived by computer-fit analysis of the DNA histograms (Fig. 2), using the Dean and Jett program (8). Differences in values for the two control populations reflect the degree of confluency inhibition at the time of cell harvest.

sufficient dye to interfere with analysis of the host cell cycle. Reasons for weak rickettsial staining by each of the three DNA-specific dyes with differing binding properties are unclear at this time; however, it is suspected that the rickettsial envelope limits entry of the stains.

The consequences of persistent procaryotic intracellular parasitism on the eucaryotic cell cycle or generation time are relatively unexplored. This would appear to be an important area of study because any growth behavior changes (or lack of such changes) that result from parasitism may indicate and reflect the pathophysiological status of the cell.

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