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Persistent Infection of L Cells with an Ovine Abortion Strain of *Chlamydia psittaci*

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L cells inoculated at multiplicities of infection ≥ 1 inclusion-forming unit of the abortigenic chlamydial strain B577 were destroyed within 10 to 15 days. Upon continued incubation in fresh medium, a few surviving cells repopulated the flasks, and the reemerging cultures remained persistently infected. The persistent state was characterized by cycles of repopulation with a low ratio of infected cells and cycles of extensive cytopathic changes in which >90% of the cells had chlamydial inclusions and which could be delayed or even terminated by penicillin treatment. Immunofluorescence and superinfection during the period of repopulation revealed that the persistently infected cells could adsorb chlamydiae but their multiplication was arrested. This nonpermissive state could be terminated by the specific action of cycloheximide. L cells spontaneously cured from a persistent infection exhibited no change in susceptibility to chlamydiae when compared with normal L cells. However, chlamydiae derived from L cells after 7.5 months of persistence destroyed L-cell monolayers more rapidly and at lower multiplicities of infection than the wild type. This state of chlamydia-host cell interaction could not be established with the arthropathogenic strain LW613 because chlamydial infectivity was lost after the first cytolytic burst of infection in the cell cultures. The persistence described for the strain B577-L-cell system appears to differ from previously described models involving other chlamydial strains.

Chlamydial agents that infect domestic mammals can induce a large variety of clinical conditions such as abortion and other genital infections, enteritis, polyarthritis, conjunctivitis, encephalitis, and pneumonia (24). An important state of the host-parasite interaction in some of these chlamydial infections is the persistent, clinically inapparent intestinal infection, which plays a significant role in spreading the organism within animal populations (5, 13, 14, 16, 20, 23). Very little is known about the mechanisms of persistence at the cellular level in the infected animals, and simpler models are desirable for detailed study.

A few cell culture models of *Chlamydia psittaci* persistence have been described previously (1, 2, 4, 6, 12). However, in most instances, the cultures were deprived of important metabolites for chlamydial multiplication, and the persistent state was lost after correction of the nutrient deficiency (1, 2, 6). Recently, Moulder et al. (10) described a persistent infection of L cells with the 6BC strain of *C. psittaci* in which a hypothetical cryptic body played a key role in perpetuating the infection for indefinite periods of time. Because different strains of *C. psittaci* behave differently in cultured cells (18, 19), chlamydiae of different pathogenic properties may establish persistent infections by unique mechanisms. The objective of this investigation was to develop in vitro models of persistence with abortigenic and arthropathogenic chlamydial strains of ovine and bovine origin, using the approach of Moulder et al. (10).

MATERIALS AND METHODS

Growth of normal L cells. L cell clone 5b (mouse fibroblasts) was kindly provided by J. Moulder of the University of Chicago. The cells were grown and maintained as suspension cultures as described by Hatch (6) in medium 199 supplemented with 5% fetal calf serum and containing 12 mM sodium bicarbonate, 200 μg of streptomycin sulfate per ml, and 75 μg of vancomycin per ml (growth medium).

Propagation of chlamydiae. The B577 strain of *C. psittaci*, originally isolated from an aborted lamb (20), was used in these investigations. This strain had been passaged 33 times in the yolk sacs of chicken embryos before it was adapted to multiply in suspension cultures of L cells as described by Perez-Martinez and Storz (J. Vet. Med., in press). L-cell-propagated chlamydial stocks were obtained by harvesting infected suspension cultures 48 h postinoculation as described by Hatch (6). The LW613 chlamydial strain, originally isolated from a calf suffering from polyarthritis (21), was used in limited trials. This strain was in the 18th chicken embryo passage, and we did not succeed in adapting it to multiply in suspension L-cell cultures.

Titration of chlamydial infectivity. Infectivity titers of *C. psittaci* B577 were determined as described by Perez-Martinez and Storz (in press). Infectivity titers of the LW613 strain in L cells were not determined.

Establishment and maintenance of L cells persistently infected with *C. psittaci* B577. L cells persistently infected with *C. psittaci* B577 were obtained by the method described by Moulder et al. (10). Briefly, suspension cultures were infected with a multiplicity of infection (MOI) of 5 to 10 chlamydial inclusion-forming units (IFU) per L cell, seeded into 25-cm^2 flasks (2×10^6 cells per flask) to allow the formation of monolayers, and incubated at 37°C in 5% CO_2 until >90% of the cells were destroyed through cytocidal chlamydial multiplication (wipe out). At this point, the flasks were rinsed twice with Earle balanced salt solution and reincubated in fresh growth medium to allow regrowth of surviving L cells. Developing colonies were dispersed with 0.5 ml of a solution of 0.1% trypsin (1:250 trypsin; Difco Laboratories, Detroit, Mich.) and 0.4% EDTA in Earle balanced salt solution and reincubated in the same flask. Upon regrowth to confluency, the cells were trypsinized as described above and seeded at a 1:5 ratio in new flasks.

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Daughter flasks were divided in a similar manner at intervals of 5 to 7 days until the cells were wiped out again and a new repopulation cycle began.

Attempts to establish persistent infections with the LW613 strain were done by infecting monolayers of L cells with suspensions of freshly harvested yolk sacs of infected chicken embryos. Infection of the monolayers was enhanced by centrifugation and cycloheximide treatments (19).

Determination of rates of infection in L cells persistently infected with *C. psittaci* B577. The percentage of persistently infected cells bearing chlamydial inclusions at any given time was determined by Giemsa staining small samples of cells. Alternatively, the percentage of persistently infected cells containing chlamydial antigens was determined by an indirect fluorescent-antibody (FA) reaction. Samples of cells seeded onto four-chambered slides (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) were treated with a hyperimmune rabbit serum (anti-*C. psittaci* B577) and a commercial fluorescein isothiocyanate-labeled goat anti-rabbit gamma globulin conjugate (U.S. Biochemical Corp., Cleveland, Ohio).

Treatment of persistently infected L cells with penicillin. L cells persistently infected with B577 chlamydiae were seeded into six 25-cm^2 flasks. One flask served as an untreated control, and the remaining flasks received 100 IU of penicillin G per ml of medium for 1, 2, 4, 8, and 16 days, respectively. The cells were maintained as described above until the monolayer was wiped out or for a maximum of 2 months.

Treatment of persistently infected L cells with cycloheximide and centrifugation. L cells persistently infected with B577 chlamydiae were seeded into 24 vials with cover slips and incubated for 24 h at 37°C in 5% CO_2. The vials were divided into four groups. Group 1 served as an untreated control. Group 2 was centrifuged at 2,000 × *g* for 30 min at 37°C. Vials from group 3 each received 1 ml of growth medium with 4 μg of cycloheximide per ml, and group 4 was centrifuged and treated with cycloheximide as described above. The cover slips in two vials of each group were Giemsa stained at 24, 48, and 72 h posttreatment, and the percentage of cells with inclusions was determined.

Stationary and centrifuge-assisted superinfection of L cells persistently infected with *C. psittaci* B577. Persistently infected L cells were seeded into four 25-mm petri dishes at a density of 2×10^5 cells per dish. They were incubated for 24 h at 37°C in 5% CO_2 and superinfected with an MOI of 3 to 5 IFU of wild-type B577 chlamydiae per L cell. For stationary superinfection, the inoculum was adsorbed for 1.5 h at 37°C, while in the centrifuge-assisted superinfection adsorption took place at 2,000 × *g* for 30 min at 37°C. After adsorption, the cultures were washed once with Dulbecco phosphate buffer solution, and fresh growth medium was added. The number of chlamydial inclusions per 100 cells was determined 48 h postsuperinfection.

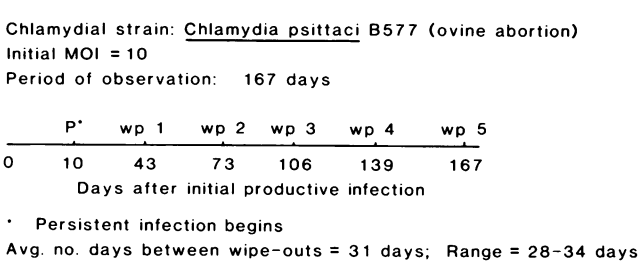

FIG. 1. Frequency of cytolytic episodes or wipe outs in L cells persistently infected with *C. psittaci* B577.

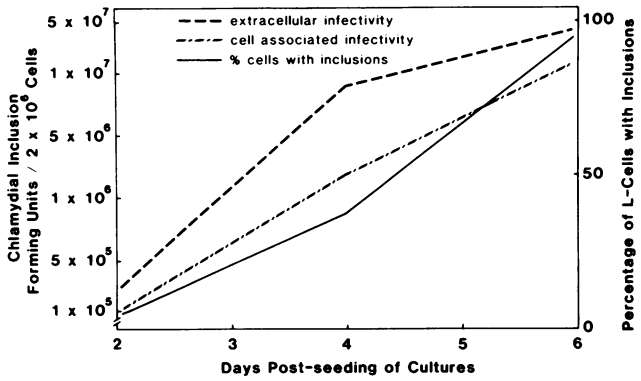

FIG. 2. Kinetics of inclusion formation and chlamydial infectivity titers during 6 days before a wipe out in L cells persistently infected with *C. psittaci* B577.

Cycloheximide treatment of persistently infected L cells after stationary and centrifuge-assisted superinfection. Petri dishes were seeded with persistently infected L cells as described in the previous section. The cells were superinfected with an MOI of 0.25 to 0.5 IFU of wild-type *C. psittaci* B577 per L cell, and 2 μg of cycloheximide per ml was added in the growth medium. The percentage of cells with inclusions was measured 48 h later.

Assay for interferon activity in supernatants from cell cultures persistently infected with *C. psittaci* B577. The fibroblast interferon activity of pooled cell culture fluids collected from persistently infected L cells during the repopulation stage was assayed with normal L cells and vesicular stomatitis virus as described by Havell and Vilcek (7).

Statistical analysis. The statistical significance of the infectivity levels obtained in persistently infected and normal L cells after different treatments was assessed with a two-tailed Student's *t* test (17).

RESULTS

Establishment of persistent infection of L cells with *C. psittaci* B577. About 10 days after the initial infection, L-cell monolayers were destroyed as a result of a typical chlamydial cytopathic expression. However, a few surviving cells repopulated the flasks upon reincubation in fresh medium and remained persistently infected (Fig. 1). Periods of cellular repopulation alternated with periods of extensive cell destruction, a process which Moulder et al. (10) referred to as a wipe out.

L-cell monolayers infected with the LW613 strain of *C. psittaci* developed extensive cytopathic changes after a first round of centrifuge- and cycloheximide-enhanced chlamydial replication. However, after removal of the cycloheximide 5 days postinfection, the cells regrew to confluency, and the infection disappeared soon thereafter. Consequently, experiments with the LW613 strain were discontinued at this point.

The conditions which determine the maintenance of the B577 persistent state were further defined through the kinetics of chlamydial inclusion formation and infectivity before a wipe out. At the beginning of a repopulation cycle, only a small percentage of cells had inclusions as determined in Giemsa-stained preparations. The percentage of cells with inclusions increased with time to practically 100%. Simulta-

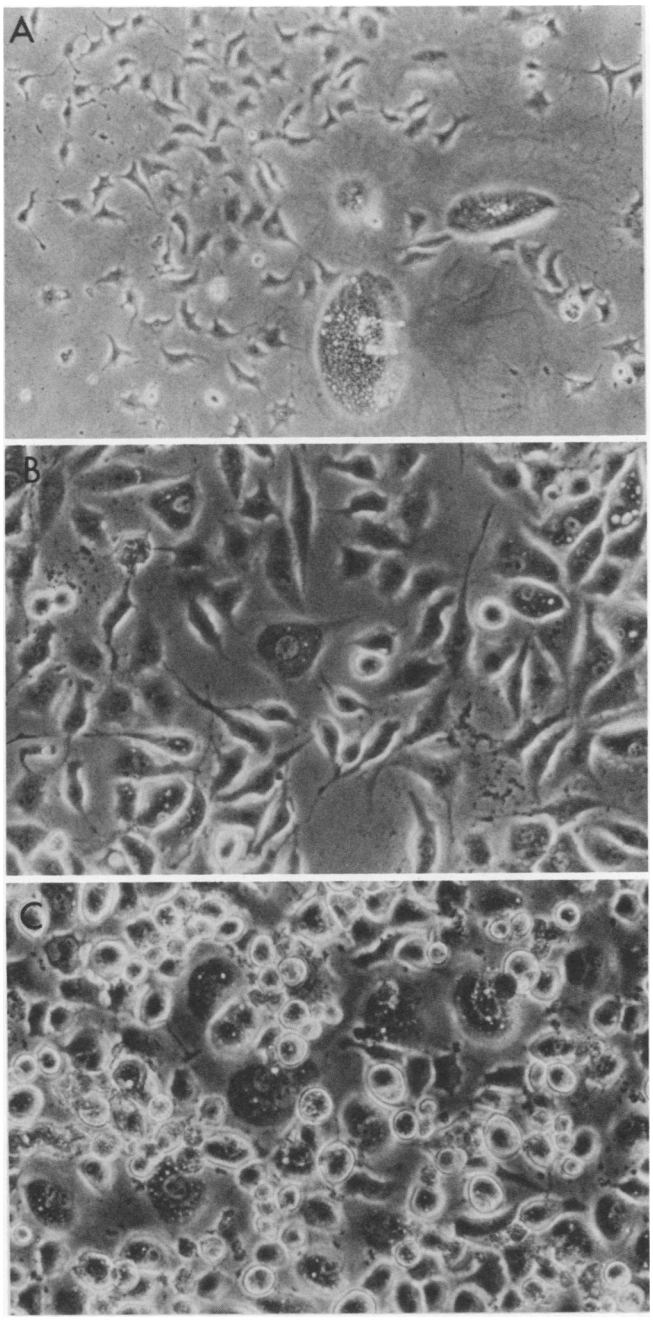

FIG. 3. Phase-contrast photomicrographs of sequential changes in L cells persistently infected with *C. psittaci* B577. (A) Cells at the beginning of a repopulation cycle (day 10). (B) Same population of cells 10 days later. The cells have continued growing, and the number of cells with chlamydial inclusions has increased. (C) Confluent monolayer of L cells showing extensive cytopathic changes as a result of a dramatic increase in the number of cells with chlamydial inclusions (day 28, 2 days before a new wipe out). Magnification, ×250.

neously, both the cell-associated and the extracellular chlamydial infectivity increased and the wipe out occurred (Fig. 2). The steady increase in the number of persistently infected cells bearing chlamydial inclusions was also monitored by phase-contrast microscopy in unstained, live cultures (Fig. 3A to C).

Effect of penicillin G on the onset of a wipe out in L cells persistently infected with *C. psittaci* B577. To determine how long the wipe out could be delayed before the persistent state was lost, we took advantage of the chlamydiostatic effect of penicillin (Fig. 4). One day of penicillin treatment had no effect on the onset of the wipe out when compared with that of the untreated controls. Treatment for 2 days delayed the wipe out for 5 days, while treatments of 4 and 8 days delayed it approximately 12 days. When penicillin was present for 16 days the persistent state was lost, and the cultures were cured of the chlamydial infection.

To explain the results obtained after 2, 4, and particularly 8 days of penicillin treatment, at which time the number of cells with inclusions declined to barely detectable levels and reappeared soon after removal of the penicillin, we hypothesized that a chlamydial variant better suited for coexistence with the L cells had been selected during establishment of the persistent infection. This hypothesis was tested by studying the adsorbing properties of the chlamydial strain after 7.5 months of persistence (Table 1). Wild-type chlamydiae gave higher infectivity titers when the adsorbing medium was sucrose-phosphate buffer (3) rather than growth medium, but significantly higher titers were observed in presence of the growth medium when the persistent B577 strain was used. Accordingly, the persistent mutant strain could spread out and kill L-cell monolayers more efficiently than the wild type could (Table 2).

Immunofluorescent reaction of L cells persistently infected with *C. psittaci* B577. To study the repopulation stage of the persistent infection, we used an indirect FA test capable of detecting all developmental stages of chlamydiae. In some instances, only typical chlamydial inclusions were observed (Fig. 5A), and the percentage of infected cells correlated with that obtained with the Giemsa stain. On other occasions, cells with typical chlamydial inclusions were found, but in addition, various numbers of cells with particulated chlamydial antigens (presumably elementary bodies) on the cell surface were also present (Fig. 5B). This pattern of fluorescence did not correlate with the Giemsa stain and could be observed for several days, long before the wipe out occurred.

Effect of cycloheximide and centrifugation on L cells persistently infected with *C. psittaci* B577. The observations made

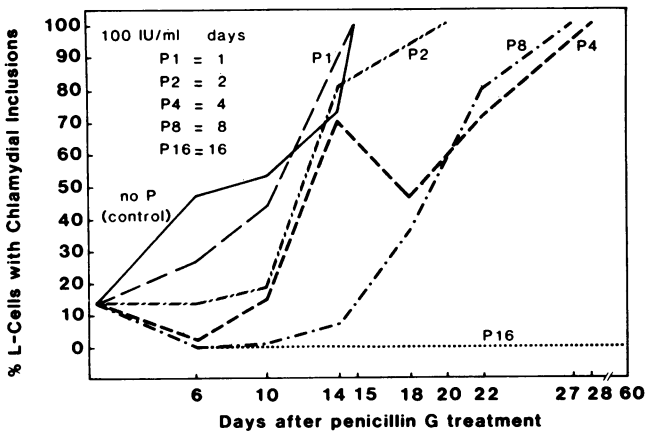

FIG. 4. Effect of penicillin G (P) treatment (100 IU/ml) on the onset of a wipe out in L cells persistently infected with *C. psittaci* B577. Wipe outs are represented as the time points at which virtually 100% of the L cells have chlamydial inclusions.

TABLE 1. Infectivity of wild-type (WB577) and persistent (PB577) *C. psittaci* for L cells suspended either in medium 199 or sucrose-phosphate buffer (SPB)

Strain	Passage no.	Relative inoculum size (ml)	No. of inclusions/100 cells[a]		Statistical significance (*P*) (*t* test)
			SPB	Medium 199	
WB577	2	1.0	106 ± 5.8	41 ± 2.3	<0.005
		0.2	58 ± 1.2	8 ± 1.3	<0.001
PB577	7.5 mo[b]	1.0	69 ± 3.9	126 ± 9.0	<0.005
		0.2	23 ± 1.0	60 ± 0.75	<0.001

[a] Mean ± standard error for four experiments with 100 cells each.
[b] Months of persistence.

with the FA test suggested that during repopulation, the L cells can adsorb elementary bodies but do not permit chlamydial multiplication and formation of inclusions.

This hypothesis was tested by studying the effect of cycloheximide and centrifugation on the percentage of cells with inclusions. In the experiment illustrated in Fig. 6, about 15% of the cells had inclusions as determined by the Giemsa stain, but 80% of the cells were FA positive when both cells with inclusions and cells with surface fluorescence were accounted for. No significant differences ($P > 0.05$) were found between the control cells and the cells that were centrifuged. However, when the cells were treated with cycloheximide alone or in combination with centrifugation, statistically significant differences ($P < 0.05$ and $P < 0.005$, respectively) were observed (Fig. 6). In particular, the combined treatment boosted the percentage of cells with inclusions to about 80%, which is the percentage of L cells that were positive by immunofluorescence at the beginning of the experiment. Therefore, the persistently infected cells appear to go through a period of partial nonpermissiveness which can be altered by mainpulation with cycloheximide and centrifugation.

Superinfection of L cells persistently infected with *C. psittaci* B577. To confirm and to further assess the extent of the nonpermissive state, monolayers of persistently infected cells were superinfected with known MOIs of wild-type *C. psittaci* B577. Persistently infected cells superinfected in the absence of cycloheximide (Table 3) were significantly less susceptible when compared with the normal L-cell population. When cycloheximide was added to the growth medium, differences in susceptibility were not observed (Table 4).

Interferon activity in supernatants of cell cultures persistently infected with *C. psittaci* B577. Pooled samples of cell culture fluids collected from persistently infected cells in which a small percentage of cells had chlamydial inclusions were devoid of antiviral activity for vesicular stomatitis virus propagated in normal L-5b cells.

TABLE 2. Comparative efficiency of secondary cycles of infection (in L cells) between wild-type (WB577) and persistent (PB577) *C. psittaci*

Strain	Days to lyse at an MOI (IFU/L cell) of[a]:				
	3	1	0.5	0.1	0.01
WG577	9	9	13	19	ND[b]
PB577	6	8	10	12	14

[a] Days needed to lyse >95% of the cells.
[b] ND, Not determined.

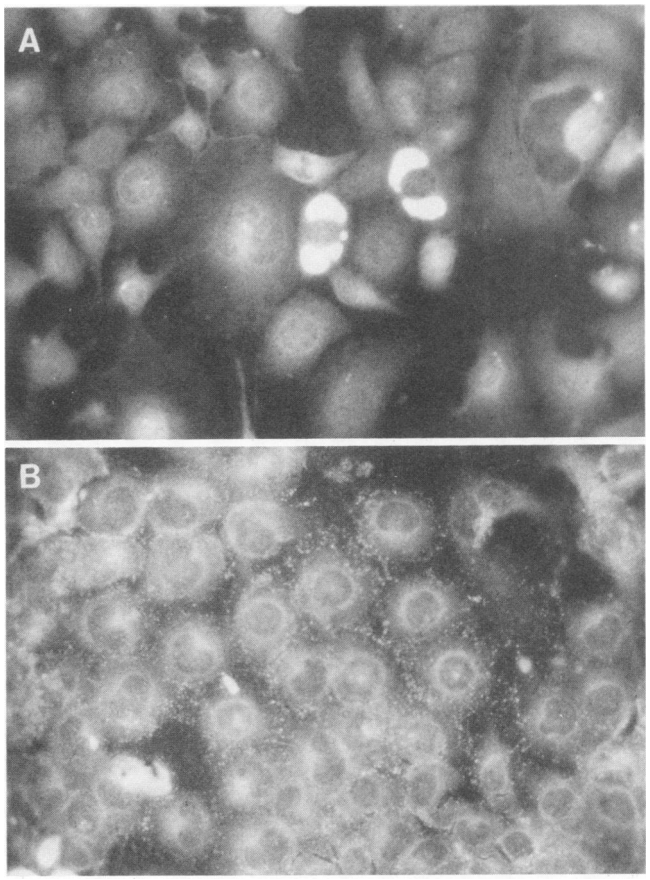

FIG. 5. Differential immunofluorescent reaction of L cells persistently infected with *C. psittaci* B577. (A) Cells with typical intracytoplasmic chlamydial inclusions exhibit specific fluorescence. (B) Large numbers of cells with a pattern of fluorescence suggestive of chlamydial elementary bodies attached to the cell surface are evident. Magnification, ×400.

DISCUSSION

Persistent infections were established in vitro with the abortigenic chlamydial strain B577 but not with the arthropathogenic strain LW613. This observation may be relevant from the point of view of chlamydia-host interac-

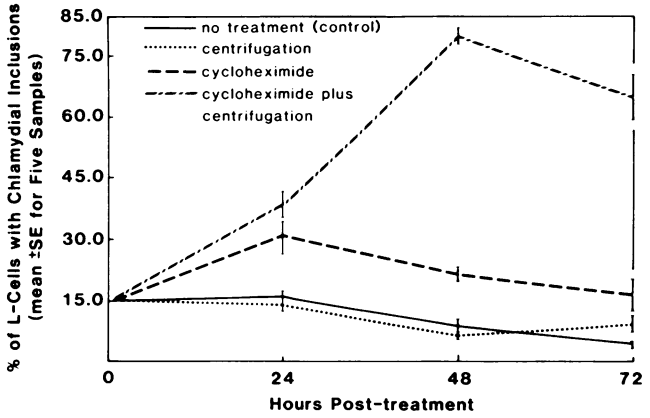

FIG. 6. Effect of centrifugation or cycloheximide treatments or both on the number of persistently infected L cells showing chlamydial inclusions. SE, Standard error.

TABLE 3. Stationary and centrifuge-assisted superinfection of L cells persistently infected with *C. psittaci*

Treatment	No. of inclusions/100 cells at 48 h			Statistical significance (*P*) (*t* test)
	No superinfection	Superinfection[a]		
		PL-5b	NL-5b	
No treatment	51			
Centrifugation only	ND[b]			
Stationary infection		55 ± 4.0	90 ± 3.5	<0.005
Centrifuge-assisted infection		96 ± 2.9	128 ± 5.0[c]	<0.025

[a] Mean ± standard error for four experiments with 100 cells each. MOI of 3 to 5 IFU per cell. PL-5b, Persistently infected L-cells; NL-5b, normal L-5b cells (MOI controls).
[b] ND, Not determined.
[c] 50 to 70% of the cells lysed at 48 h.

tions in naturally occurring infections of animals. The B577 strain is classified as a biotype 1 of *C. psittaci* and is indistinguishable from other strains commonly isolated from cattle and sheep with clinically inapparent persistent intestinal infections (18, 20). In contrast, the LW613 strain is a biotype 2 of *C. psittaci* and has only been isolated from animals suffering from polyarthritis, conjunctivitis, or meningoencephalomyelitis (18, 21). Naturally occurring persistent infections with biotype 2 have not been documented.

The B577 persistent state is characterized by cycles of low cytopathic expression and cycles of high cytopathic expression. These cycles seem to be determined, at least in part, by temporary changes in the susceptibility of L cells to chlamydial infection and by the selection of a chlamydial mutant better adapted to the culture conditions.

Immunofluorescence studies, together with centrifugation and cycloheximide treatments, were instrumental in showing that the persistently infected cultures go through periods of resistance in which infectious elementary bodies may adsorb but productive infection does not occur. These changes in susceptibility are not absolute but are relative to the susceptibility of normal L cells and can be abolished by cycloheximide treatment, a situation similar to that described for L cells treated with interferon and infected with *Chlamydia trachomatis* (15). Efforts to detect an antiviral activity indicative of fibroblast interferon in supernatants collected from persistently infected cell cultures were unsuccessful. Consequently, unlike the previously described model of persistence with the 6BC strain of *C. psittaci* in which resistance to superinfection is mediated by an attachment defect (11), the factors responsible for changing the susceptibility of cultures persistently infected with the B577 strain remain unknown at this point. The susceptibility of L cells spontaneously cured from the persistent infection was the same as that of normal L cells when infected in parallel with wild-type *C. psittaci* B577 (data not shown).

In contrast to the observations of Moulder (9), in which the onset of a wipe out is suppressed by replacing the medium 199 by Earle minimal essential medium, the behavior of cultures persistently infected with the B577 strain was not affected by Earle minimal essential medium (data not shown).

In our study, morphological evidence for the presence of a chlamydial "cryptic body" was not obtained with the FA test used. Additional evidence against the presence of a cryptic body in the B577-L-cell system was provided by the fact that penicillin-sensitive chlamydial infectivity was required for maintenance of the persistent state. Also, persistently infected cultures could be cured from the chlamydial infection simply by repeated subculture of the cells at very low cell densities (100 cells per 60-mm petri dish) (data not shown), indicating that not every cell in the population is infected.

The lack of cryptic bodies in the B577-L-cell model of persistence resembles the *C. trachomatis* G17-McCoy cell model described by Lee and Moulder (8). However, in that system, 100 to 150 days of chlamydia-host cell coexistence are required to establish persistence, maintenance of persistence involves direct cell-to-cell transfer of chlamydiae, the efficiency of infection of the persistent G-17 strain does not change, and the persistently infected McCoy cells are fully susceptible to superinfection.

L cells persistently infected with the B577 strain are useful for the production of large quantities of chlamydial antigens required for seroepidemiological studies using inclusion immunofluorescence and enzyme-linked immunosorbent assay tests (J. A. Perez-Martinez, N. Schmeer, and J. Storz, Am. J. Vet. Res., in press), they have potential as a possible tool for attenuating chlamydial virulence, and they are useful in vitro models for studying chlamydia-host cell interactions.

TABLE 4. Stationary and centrifuge-assisted superinfection of L-cells persistently infected with *C. psittaci*, followed by cycloheximide treatment

Treatment	% Cells with inclusions at 48 h			Statistical significance (*P*) (*t* test)
	No superinfection	Superinfection[a]		
		PL-5b	NL-5b	
No treatment	3			
Cycloheximide only	6	19 ± 2.9	22 ± 2.2	<0.5
Cycloheximide plus centrifugation	23	80 ± 1.8	89 ± 2.0	<0.05

[a] Mean ± standard error for four experiments with 100 cells each. MOI, 0.25 to 0.5 IFU per cell. PL-5b, Persistently infected L cells; NL-5b, normal L-5b cells (MOI controls).

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