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Persistent Infection of Mouse Fibroblasts (L Cells) with *Chlamydia psittaci*: Evidence for a Cryptic Chlamydial Form

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When monolayers of mouse fibroblasts (L cells) were infected with enough *Chlamydia psittaci* (strain 6BC) to destroy most of the host cells, 1 in every 10^5 to 10^6 originally infected cells gave rise to a colony of L cells persistently infected with strain 6BC. In these populations, the density of L cells and 6BC fluctuated periodically and reciprocally as periods of host cell increase were followed by periods of parasite multiplication. Successive cycles of L-cell and 6BC reproduction were sustained indefinitely by periodic transfer to fresh medium. Isolation of L cells and 6BC from persistent infections provided no evidence that there had been any selection of variants better suited for coexistence. Persistently infected populations consisting mainly of inclusion-free L cells yielded only persistently infected clones, grew more slowly, and cloned less efficiently. They were also almost completely resistant to superinfection with high multiplicities of either 6BC or the lymphogranuloma venereum strain 440L of *Chlamydia trachomatis*. These properties of persistently infected L cells may be accounted for by assuming that all of the individuals in these populations are cryptically infected with 6BC and that cryptic infection slows the growth of the host cell and makes it immune to infection with exogenous chlamydiae. According to this hypothesis, the fluctuations in host and parasite density occur because some factor periodically sets off the conversion of cryptic chlamydial forms into reticulate bodies that multiply and differentiate into infectious elementary bodies in a conventional chlamydial developmental cycle.

Almost 50 years ago, the pioneering studies of Meyer and Eddie (13) and Burnet (2) established the importance of persistent infection of birds with *Chlamydia psittaci*. Recent expansion of our knowledge of the natural history of *Chlamydia trachomatis* infections in humans (18) has emphasized the importance of persistent infection in these diseases as well, thus bearing out Meyer's (11) prediction that the pathogenic proclivities of *C. psittaci* in birds and mammals would find their parallel in *C. trachomatis* and humans. Despite these indications of the importance of persistent infections in chlamydial disease, most cell culture models mimic instead the rare fulminating case—the entire host cell population is infected and killed in a short time. Only a few investigators have established and studied persistent chlamydial infections in cell cultures. Morgan (14), Bader and Morgan (1), and Hatch (5) obtained latent infections of *C. psittaci* (strain 6BC) in chicken embryo cells and in L cells by depriving infected cultures of metabolites needed for reproduction of both host and parasite and restored chlamydial multiplication by supplying the missing metabolites. Pollard and Sharon (17) produced latent infection in McCoy cells with a turkey strain of *C. psittaci* by adding aminopterin and revived multiplication by supplying folinic acid. Manire and Galasso (9) established cultures of HeLa cells that stayed infected with the meningopneumonitis strain of *C. psittaci* for many months without detectable change in either host or parasite. Officer and Brown (16) similarly maintained a poulation of Chang human liver cells persistently infected with *C. psittaci* 6BC for over a year and observed the selection of a more virulent chlamydial variant and a more resistant host cell. In all of these experiments, chlamydiae and host cells probably were able to coexist because the host cells, by virtue of either nutritional or genetic blocks, were comparatively resistant to parasitism by *C. psittaci*. In experiments more closely resembling those reported here, Schoenholz (19) infected rabbit corneal cell cultures with enough *C. psittaci* 6BC to destroy most of the host cells and demonstrated the existence of persistent infection in the survivors by recovering infectious chlamydiae after raising the temperature of incubation or lowering the pH of the cultures.

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We describe here an indefinitely persisting infection of L cells with *C. psittaci* 6BC in which initial contact of host and parasite resulted in destruction of almost all of the L cells. From the few survivors emerged a persistent infection characterized by large, periodic, and reciprocal fluctuations in host and parasite density. L cells and *C. psittaci* isolated from persistent infections showed no detectable changes, but a previously undescribed cryptic form of *C. psittaci* may have been essential for maintenance of the persistent infection.

MATERIALS AND METHODS

Maintenance of wild L cells and wild *C. psittaci* 6BC. Stocks of wild L cells and wild *C. psittaci* 6BC were maintained as described by Hatch (5). When wild L cells were to be compared with persistently infected L cells, they were first grown for several generations under the conditions used for propagating persistently infected cultures. Periodic cultures for mycoplasma were negative (Flow Laboratories, Rockville, Md.).

L cells were counted in a Coulter Counter. Cell viability was estimated by trypan blue exclusion. *C. psittaci* was titrated by a modification of the 50% infectious dose (ID_{50}) unit method of Hatch (5) in which 5×10^5 instead of 5×10^6 L cells were at risk of infection with the sample being titrated. One ID_{50} unit per L cell is equivalent to 0.7 infectious chlamydial cell per host cell (7).

Maintenance of L cells persistently infected with *C. psittaci* 6BC. When colonies of persistently infected L cells had become established in a 25-cm^2 cell culture flask as described in Results, the colonies (usually one to three per flask) were allowed to grow until each contained several thousand cells. Then the flask was washed once with 5 ml of medium 199 containing 10% heat-inactivated fetal calf serum, 0.1% sodium bicarbonate, and 0.25 mg of streptomycin sulfate per ml. A second 5 ml of medium was added, and the colonies were dispersed by shaking with 30 glass beads (3 mm in diameter). The dispersed cells were transferred to fresh 25-cm^2 flasks containing 5 ml of medium equilibrated with 5% CO_2–95% air and incubated at 37°C. Once the initial persistently infected colonies had been dispersed, the cultures were maintained in either of two ways.

First, the persistently infected population stayed in a single flask throughout the period of propagation. When the L cells were destroyed by chlamydial multiplication, the flask was washed once with medium to remove dead and detached cells, 5 ml of fresh medium was added, and incubation was continued until the L-cell population had regrown and had again been destroyed. The flask was washed, fresh medium was added, and incubation was continued. This procession of events could be maintained indefinitely.

In the second method, the persistently infected monolayer was dispersed with glass beads when the inclusion-bearing L cells made up 5 to 50% of the population and then was transferred to fresh 25-cm^2 flasks at initial densities of 10^4 to 10^5 cells per flask. The interval between transfers was usually 2 to 3 weeks. This method also allowed indefinite maintenance of persistently infected cultures.

L-cell populations persistently infected with *C. psittaci* have been frozen in liquid nitrogen for over 1 year and then thawed to resume multiplication as persistently infected cultures with all of the properties of the unfrozen populations.

Enumeration of L cells and *C. psittaci* in persistently infected cultures. The number of adherent L cells in a persistently infected population was defined as the number of L cells that remained after the monolayer had been washed vigorously three times with 5-ml portions of phosphate-buffered saline. The adherent cells were dispersed into 5 ml of phosphate-buffered saline with glass beads and counted in a Coulter Counter.

C. psittaci suspensions for ID_{50} titration were prepared from persistently infected cultures by adding glass beads, dispersing the monolayer cells into the supernatant medium, and diluting the dispersed cells with an equal volume of sucrose-phosphate buffer. The infected cells were disrupted in a sonic cleaning bath, and the infectivity of the sample (the sum of free and L-cell-associated infectivity) was titrated as for wild *C. psittaci*.

Determination of the percentage of inclusion-bearing cells in persistently infected L-cell populations. Persistently infected cultures were examined several times a week with phase-contrast microscopy. When phase-contrast observation indicated that a determination of percentage of inclusion-bearing cells should be made, a monolayer was washed three times with 5-ml portions of phosphate-buffered saline, fixed with 5 ml of absolute ethanol, and stained with Giemsa for 10 min. Excess stain was removed by washing in tap water, and the flask was allowed to dry. The bottom of the 25-cm^2 flask, on which the stained cells lay, was cut away from the rest of the flask with the tip of an electric soldering iron and handled as an ordinary glass microscope slide. The percentage of inclusion-bearing cells was determined by examining 100 to 500 cells at a magnification of ×300.

Cloning of persistently infected L cells. Clones of L cells from persistently infected cultures were obtained by dispersing the L cells with glass beads and diluting them in growth medium until inoculation of 1 ml of the diluted L-cell suspension into 4 ml of medium in a 25-cm^2 flask equilibrated in 5% CO_2 produced L-cell colonies in only 20 to 30% of the flasks inoculated. After 15 to 25 days of incubation, flasks with only one colony were washed once with medium, and the clone of L cells was dispersed with glass beads and transferred to three or more fresh 25-cm^2 flasks containing 5 ml of equilibrated medium. The resulting persistently infected culture was then propagated and maintained as already described.

Efficiency of cloning was measured by adding 100 to 1,000 L cells to each of three 25-cm^2 flasks and observing the appearance of L-cell colonies as just described. At 10 to 20 days after plating out of the L cells, the flasks were stained with Giemsa, and the colonies were counted with a bacterial colony counter.

Growth rate of persistently infected L cells.

Growth rates of L cells persistently infected with *C. psittaci* were measured by modification of the method of Horoschak and Moulder (6) for use in 25-cm^2 cell culture flasks. Persistent infections were plated out in a number of 25-cm^2 flasks at a density of 10^4 cells per flask. For 5 to 8 days thereafter, one flask was stained with Giemsa each day, and the mean number of L cells in 25 randomly selected colonies was determined by microscopic examination at ×300.

Superinfection of persistently infected L cells. Persistently infected cultures were transferred to fresh 25-cm^2 flasks at a density of 10^4 cells per flask. When the number of L cells in each flask had reached 10^5 and when 1 to 50% of them contained inclusions, the cultures were challenged with an inoculum of *C. psittaci* 6BC or *C. trachomatis* (strain 440L). The growth medium was removed, the monolayer was washed once with 5 ml of phosphate-buffered saline, and then 10, 100, or 1,000 ID_{50} of chlamydiae per L cell in the persistently infected population was added in a volume of 0.5 ml of phosphate-buffered saline. The flasks were shaken at 100 strokes per min for 90 min at 37°C. The inoculum was poured off, the monolayer was washed once with 5 ml of medium, 5 ml of fresh medium was added, and the challenged monolayers were incubated for 24 h, after which they were stained with Giemsa and the percentage of inclusion-bearing cells was determined as usual. An unchallenged persistently infected monolayer was also stained at the time of superinfection, and another was stained 24 h later to give a measure of the endogenous increase in inclusion-bearing cells that occurred in the absence of exogenous challenge. Wild L-cell monolayers were similarly challenged at the same time as the persistent infections. To get comparable monolayers, 10^4 wild L cells were seeded into 25-cm^2 flasks and incubated for 5 to 6 days, at which time they had increased to a density of approximately 10^5.

RESULTS

When L cells were infected in suspension with 0.01 to 10 ID_{50} of *C. psittaci* per host cell and then plated out in 25-cm^2 plastic cell culture flasks at a density of 4,000 cells per cm^2 (100,000 cells per flask), the resulting monolayers appeared to be totally destroyed by infection in 4 to 14 days, depending on the multiplicity of infection. However, if these monolayers were washed to remove detached cells, covered with fresh medium, and incubated for another 2 to 3 weeks, a few colonies of L cells appeared in some of the flasks. Some of these colonies had L cells with chlamydial inclusions in their cytoplasm, and others showed no sign of chlamydial infection. However, on continued propagation, all of the colonies produced inclusion-bearing L cells.

In these persistently infected cultures, the densities of L cells and *C. psittaci* fluctuated wildly in a periodic and reciprocal manner (Fig. 1 and 2). When a persistently infected population was plated out in a new flask, L cells without inclusions dominated the early stages of host cell growth (Fig. 2A). As the L-cell population increased (cf. Fig. 1A and B), almost all host cells exhibited chlamydial inclusions (Fig. 2B), and soon thereafter the L-cell monolayer was apparently destroyed, an event we shall call wipeout (cf. Fig. 1B and C). However, some L cells survived and grew out to form a new population of largely inclusion-negative host cells (Fig. 2C). We shall call this event regrowth. Again, as the L-cell density increased (cf. Fig. 1C and D), most of the host cells developed chlamydial inclusions (Fig. 2D) and were destroyed, and again a few survivors regrew.

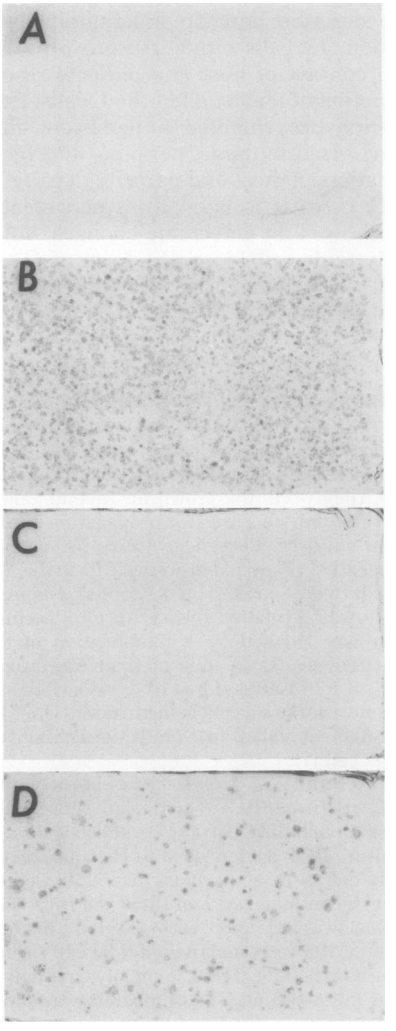
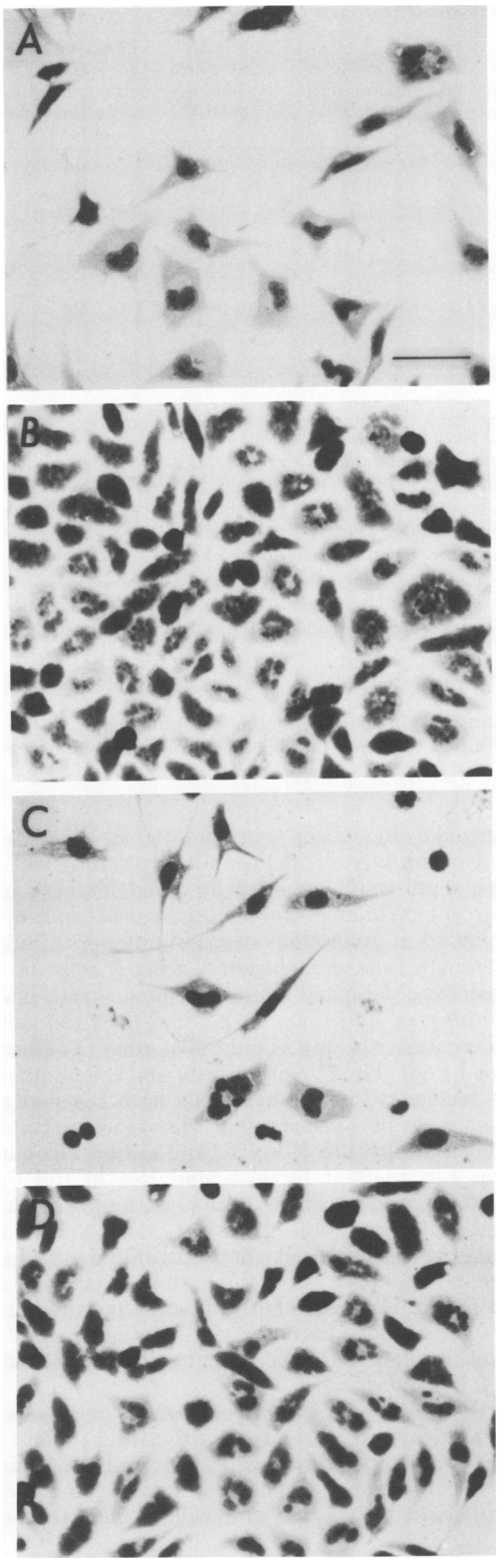


FIG. 1. *Wipeout and regrowth of an L-cell population persistently infected with C. psittaci. (A to D) Photographs of monolayers stained with Giemsa at various times after the persistently infected population was transferred into fresh flasks. (A) 3 days; (B) 12 days; (C) 27 days; (D) 34 days.*

We have held L-cell-*C. psittaci* populations in a single flask, with feeding only after each



wipeout, for over 7 months, during which time we observed seven cycles of wipeout and regrowth. However, persistent infections were routinely (and indefinitely) maintained by replating the cultures at low density (10^4 to 10^5 cells per 25-cm^2 flask) at intervals of 10 to 20 days, when 5 to 50% of the L cells had chlamydial inclusions. All of the experiments described here were done with two independently derived, persistently infected L-cell series which appeared to behave identically. Once a persistent infection had become established, differences in behavior within a single series were not observed at different passage levels.

Frequency of appearance of persistently infected L-cell colonies. When each of a number of 25-cm^2 flasks was seeded with 10^5 wild L cells that had been infected in suspension with wild *C. psittaci*, somewhat less than half of them developed one or more persistently infected L-cell colonies 2 to 3 weeks after most of the L cells had been destroyed by infection. This observation was the basis of a simple estimate of the frequency with which the interaction of different populations of L cells and *C. psittaci* gave rise to persistently infected host cell colonies (Table 1). Raising the multiplicity of infection gave a modest increase in frequency. Freshly cloned populations of host and parasite produced about as many persistently infected colonies as did stock populations that had not been cloned for many generations. L cells and *C. psittaci* reisolated from persistent infection were not more consistently productive of new persistently infected L-cell colonies than were wild populations. None of the variables tested probably had any real effect on frequency, because the observed differences were not significantly greater than those expected by chance variations in the relatively small populations used. Therefore, all of the frequencies recorded in Table 1 were combined to estimate that the probability of any one flask of 10^5 L cells giving rise to one or more persistently infected colonies was 0.33. If it is assumed that all of the L cells were infected either by the inoculum itself or by its progeny, then the first term of the Poisson distribution (which gives the expected frequency of flasks with no colonies) predicts that this probability is the equivalent of one persistently in-

FIG. 2. *Fluctuation of host and parasite density in a population of L cells persistently infected with C. psittaci. (A through D) photomicrographs of the Giemsa-stained monolayers shown in Fig. 1. Note the changes in both the total number of L cells and the proportion of L cells with visible inclusions. (A) 3 days; (B) 12 days; (C) 27 days; (D) 34 days. Bar in (A), 50 μm. ×200.*

TABLE 1. *Frequency of appearance of persistently infected L-cell colonies*[a]

Source of L cells	Source of *C. psittaci*	Multiplicity of infection (ID_{50} per L cell)	Day visible colonies appeared[b]	Flasks with one or more persistently infected L-cell colonies (% of total)[c]
Wild	Wild	0.01	22	14
Wild	Wild	0.1	14	36
Wild	Wild	1.0	11	43
Wild	Wild	10.0	11	57
Wild, freshly cloned[d]	Wild	1.0	14	14
Wild	Wild, freshly cloned[d]	10.0	25	22
Wild	Persistent infection, L cell-free[e]	0.5	20	22
Persistent infection, drug cured[f]	Wild	0.1	17	71
Persistent infection, drug cured[f]	Persistent infection, L cell-free[e]	0.5	27	22

[a] Suspensions of L cells at a density of 5×10^5 cells per ml were infected by shaking at 100 strokes per min for 90 min at 37°C with the indicated chlamydial inoculum, and 10^5 infected cells were plated out in 25-cm^2 flasks containing 5 ml of medium. They were incubated at 37°C until the monolayers had been destroyed by infection. The flasks were washed once with medium; 5 ml of fresh medium was added, and the flasks were incubated until no new colony had appeared in a previously colony-free flask for at least 5 days.

[b] The day on which visible colonies first appeared was calculated with the day on which the L-cell monolayer appeared completely destroyed counted as day 0.

[c] Fourteen flasks were plated out with each L cell-chlamydia pair. The true number of persistently infected original colonies could not be determined with certainty because satellite colonies frequently arose around the original colonies.

[d] Clones of wild L cells were obtained as described in the text. Clones of wild *C. psittaci* were isolated from plaques on wild L cells.

[e] *C. psittaci* free of L cells was recovered from the medium above a persistently infected monolayer.

[f] L cells were recovered from a persistent infection in which *C. psittaci* had been eliminated by treatment with rifampin.

fected colony for every 2.5×10^5 L cells originally infected.

Periodic fluctuation in host and parasite density in persistently infected L-cell populations. The cycles of wipeout and regrowth of persistently infected cultures illustrated visually in Fig. 1 and 2 were defined quantitatively by frequent measurement of four variables. The first was the number of adherent L cells as counted with a Coulter Counter. The second variable was the percentage of adherent L cells that contained visible inclusions of *C. psittaci* as determined by examination of Giemsa-stained monolayers. The third variable was a function of the first two, the number of inclusion-containing adherent L cells. The final variable was the total *C. psittaci* infectious titer (extracellular plus intracellular) expressed in terms of L-cell ID_{50} units.

When a long-established persistently infected culture containing 20% inclusion-bearing host cells was dispersed and replated in 25-cm^2 flasks at a density of 10^5 cells per flask, the curves of Fig. 3 were obtained by making these four observations at intervals of 3 and 4 days. These curves plainly delineate the cycles of wipeout and regrowth. Periods of regrowth were characterized by increasing L-cell number and decreasing percentage of inclusion-positive host cells. Wipeouts were preceded by rapid increase in percentage of inclusion-containing cells and culminated in precipitous drops in numbers of adherent L cells as inclusion-bearing L cells died and were detached from the monolayers. The number of infectious *C. psittaci* cells did not fluctuate as sharply as did the number of adherent L cells. The *C. psittaci* titer and the number of inclusion-positive host cells both reached a maximum between the first (day 5) and second (day 21) wipeouts. However, the second wipeout was not marked by a second rise in the *C. psittaci* titer, possibly because continuing extracellular inactivation of the previously released chlamydiae obscured the contribution of the second wipeout. Throughout the period shown in Fig. 3, the persistently infected cultures always had more than enough infectious *C. psittaci* to infect all of the L cells in them, at least 100 ID_{50} per L cell.

Properties of *C. psittaci* and L cells separated out of persistent infections. Persistently infected cultures were separated into their two components, which were then compared with wild *C. psittaci* and wild L cells to see

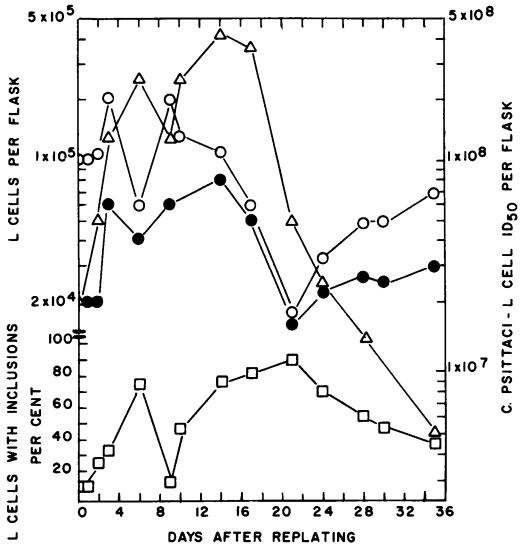


FIG. 3. *Changes in L-cell number and C. psittaci titer in a persistently infected culture. The experiment was performed as described in the text. Wipeouts occurred on days 5 and 21. Symbols: ○, total number of adherent L cells per flask; □, percentage of inclusion-bearing L cells; ●, number of inclusion-bearing L cells; △, number of L-cell ID_{50} units of C. psittaci per flask.*

whether establishment of the persistently infected state had been accompanied by the selection of variants better suited for coexistence of host and parasite.

Populations of *C. psittaci* free of L cells were obtained by the usual method of harvesting chlamydiae from cell cultures. When wild L cells were infected with *C. psittaci* derived from a persistent infection (persistent-infection *C. psittaci*), inclusion morphology as observed in Giemsa-stained monolayers, growth rate as measured by increase in ID_{50} units with time, and burst size (yield of ID_{50} units per infected L cell) were the same for wild and persistent-infection chlamydiae.

L cells were freed of detectable *C. psittaci* by holding persistently infected monolayers for 1 week in 50 μg of either rifampin or chlortetracycline per ml. An L-cell population was considered cured of persistent chlamydial infection when it had been transferred at weekly intervals for 10 weeks without observing chlamydial inclusions by Giemsa staining. On one occasion a persistently infected L-cell population became spontaneously cured of infection with *C. psittaci*. Both drug-cured and spontaneously cured L-cell cultures had the same growth rates and cloning efficiencies as wild L cells.

To see whether there had been changes in the ability of the parasite to infect or of the host to resist, the interaction between all four possible pairings of wild and persistent-infection *C. psittaci* with wild and persistent-infection L cells were examined. The ID_{50} of both chlamydial inocula was first determined for wild L cells. Then both wild and persistent-infection L cells were inoculated with approximately 0.5, 1.0, and 2.0 wild L-cell ID_{50} per host cell of either wild or persistent-infection *C. psittaci*. The infected host cells were incubated and the percentage of infected L cells was determined as in a routine ID_{50} titration. When the logarithm of the percentage of uninfected host cells was plotted against inoculum size (5), a set of four nearly identical, nearly linear curves was obtained (Fig. 4). Although small changes in host and parasite may have escaped detection, it is unlikely that shifts in capacity to infect or not to be infected big enough to account for establishment of the persistently infected state would have gone unnoticed.

Comparison of the multiplication of inclusion-free and inclusion-containing L cells in persistent and acute infections of L cells with *C. psittaci*. When we found that separating persistent infections into their two components yielded L cells and chlamydiae indistinguishable from their wild counterparts, we next looked at the persistently infected host cell itself to see whether it behaved in a way that would account for establishment and maintenance of the persistently infected state.

Figure 5 shows what happened when three different L-cell populations were plated out in

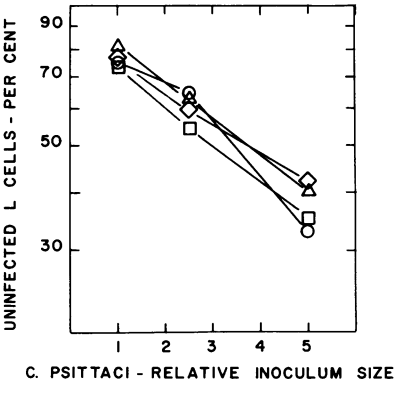


FIG. 4. *Titration of wild and persistent-infection C. psittaci in wild and persistent-infection L cells. The experiment was performed as described in the text. Symbols: △, wild C. psittaci titrated in wild L cells; ◇, persistent-infection (L-cell-free) C. psittaci in wild L cells; □, wild C. psittaci in persistent-infection (rifampin-cured) L cells; ○, persistent-infection C. psittaci in persistent-infection L cells.*

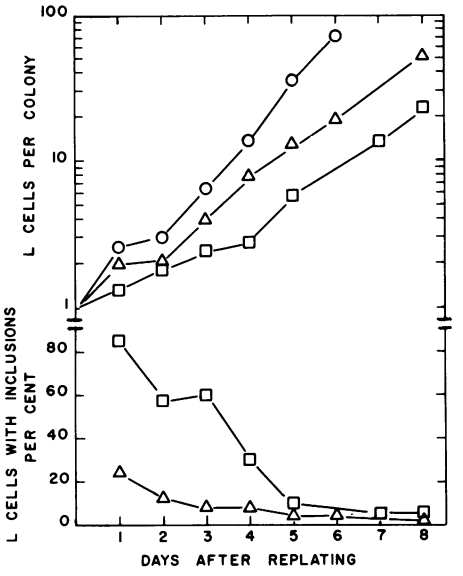


FIG. 5. *Multiplication of inclusion-free and inclusion-containing L cells in a population persistently infected with C. psittaci. The experiment was done as described in the text. Symbols: ○, spontaneously cured persistent infection; □, persistent infection with 89% inclusion-bearing host cells on day 1; △, persistent infection with 27% inclusion-bearing host cells on day 1.*

25-cm^2 flasks at a density of 10^4 cells per flask, a density so low that cell growth occurred in discrete colonies. Growth rate was determined by measuring mean colony size (6), and the percentage of inclusion-bearing L cells was measured in Giemsa-stained monolayers. One population was a spontaneously cured persistent infection. It grew at the same rate as the uninfected wild L cells of Fig. 6. The other two were persistent infections with approximately 30 and 90% inclusion-bearing cells on the first day after replating. Both grew more slowly than the spontaneously cured population, and as multiplication progressed, the percentage of inclusion-bearing cells dropped to low levels, indicating that almost all of the dividing cells were ones without visible evidence of infection. When observations were continued beyond the period shown in Fig. 5, the proportion of inclusion-containing L cells began to increase; the 30% population wiped out on day 15, and the 90% one wiped out on day 20.

Figure 6 describes the behavior of three comparable populations of wild L cells, one uninfected and the other two acutely infected with wild *C. psittaci* to the extent of about 30 and 90% infected cells to match the persistent infections of Fig. 5. The infected populations started to multiply, and the proportion of inclusion-bearing L cells momentarily decreased as had already been described by Horoschak and Moulder (6). However, when the first brood of infectious progeny was released on day 2, the percentage of infected L cells rose rapidly as a second round of infection took place, and the infected L-cell populations were quickly wiped out.

Properties of clones isolated from persistent infections. Comparison of the data of Fig. 5 and 6 revealed an important difference between persistent and acute infections of L cells with *C. psittaci*. In persistent infections, there was a prolonged period of multiplication of inclusion-free L cells without a corresponding increase in the number of visibly infected host cells. We therefore examined more closely the behavior of these multiplying, inclusion-free L cells. We obtained nine clones of L cells from persistently infected populations in which 23 to 25% of the cells showed visible inclusions. Persistently infected populations cloned with only 5 to 10% of the efficiency of wild L cells, and

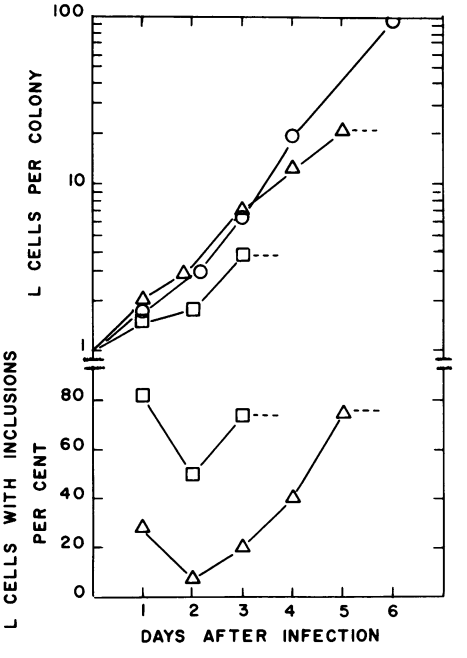


FIG. 6. *Multiplication of inclusion-free and inclusion-containing host cells in a population of wild L cells acutely infected with wild C. psittaci. The experiment was done as described in the text. Symbols: ○, uninfected L cells; □, acutely infected L cells with 83% inclusion-bearing cells on day 1; △, acutely infected L cells with 29% inclusion-containing cells on day 1. Broken lines indicate the time at which the acutely infected populations were destoyed.*

visible colonies appeared more slowly. When colonies finally appeared, they contained only inclusion-free cells. However, when the nine original clonal colonies were dispersed and grown as ordinary monolayers, all nine clonal populations eventually contained inclusion-bearing cells, which first appeared from day 27 to 47 of propagation of the clones.

Resistance of persistently infected L cells to superinfection with exogenous chlamydiae. The finding that all of the clones gave rise to persistent infections was unexpected because, by chance alone, only one in four should have come from an inclusion-bearing L cell. It suggested that single inclusion-free L cells in a persistent infection may be the progenitors of persistently infected clones because they are really infected with *C. psittaci*, but in a form that cannot be identified by microscopic examination of Giemsa-stained cells. In search of independent support for the idea of a cryptic chlamydial form, we examined persistently infected populations for resistance to superinfection with wild *C. psittaci* on the chance that the presence of cryptic *C. psittaci* in inclusion-free L cells might make them resist the intrusion of exogenous chlamydiae. Long-established persistent infections were plated out in 25-cm^2 flasks at a density of 10^4 cells per flask and allowed to multiply until each flask contained approximately 10^5 cells of which 1 to 50% contained visible inclusions. Other flasks were seeded with wild L cells and allowed to grow to comparable density. Then 10 to 1,000 ID_{50} of wild *C. psittaci* were added to the wild and persistently infected monolayers. The flasks were shaken for 90 min, fresh medium was added, and the monolayers were stained with Giemsa after 24 h of incubation. Table 2 shows that higher multiplicities of *C. psittaci* were required to achieve a given level of infection of wild L cells when the 25-cm^2 flasks contained only 10^5 L cells instead of 10^6 cells as in the monolayer assay system usually used in this laboratory (3). However, 100 ID_{50} of *C. psittaci* per L cell consistently infected most of the cells in a wild monolayer. In contrast, when a persistently infected monolayer was comparably exposed to as much as 1,000 ID_{50} per L cell, the number of inclusion-bearing cells seen 24 h later was no more than in an unchallenged control population. Table 2 also shows that the spontaneously cured L cells had regained their original susceptibility and that persistently infected L cells were resistant to *C. psittaci* from a persistent infection. Persistently infected L cells were also resistant to challenge with a

TABLE 2. *Resistance of persistently infected L cells to superinfection with C. psittaci and C. trachomatis*

L-cell population challenged	Challenging chlamydial inoculum		L cells with inclusions		
	Strain	ID_{50} per cell	Time after challenge (h)	*C. psittaci* inclusions (%)	*C. trachomatis* inclusions (%)
Wild	Wild *C. psittaci*	10	24	28	
		100[a]	24	99	
Persistent infection, spontaneously cured	Wild *C. psittaci*	10	24	30	
		100[a]	24	91	
Persistent infection	Wild *C. psittaci*	0	0	6	
		0	24	14	
		10	24	21	
		100	24	22	
		1,000	24	21	
Persistent infection	Persistent-infection *C. psittaci*, L cell-free	0	0	21	
		0	24	35	
		10	24	5	
		100	24	28	
Wild	Wild *C. trachomatis*[c]	10	24		15
		100[a]	24		64
Persistent infection	Wild *C. trachomatis*[c]	0	0	1	
		0	24	7	
		10	24	6	<0.2[b]
		100	24	2	<0.2[b]
		1,000	24	7	<0.2[b]

[a] An inoculum of 1,000 ID_{50} per L cell was immediately toxic (15).
[b] No *C. trachomatis* inclusions seen in 500 L cells.
[c] Lymphogranuloma venereum strain 440L.

lymphogranuloma venereum strain (440L) of *C. trachomatis*. Replicate flasks of all challenged cultures were kept until wipeout to be sure that the resistant L cells eventually supported overt chlamydial multiplication. Challenge with exogenous *C. psittaci* did not change the time at which wipeout occurred.

DISCUSSION

The event that initiated persistent infection of L cells with *C. psittaci* appears to have happened on the average once in every 10^5 to 10^6 host cells infected. We assume that each persistently infected colony that appeared after the destruction of an acutely infected population consisted of the descendents of a single infected L cell in which one of these rare events took place. Detection and isolation of such a persistently infected colony depended on infection of a relatively large number of L cells with enough *C. psittaci* to destroy all of the L cells in which persistent infection was not established.

The behavior of persistently infected L-cell populations may be explained by invoking the presence of a cryptic state of chlamydial infection—cryptic in the sense that no evidence of infection is seen when the persistently infected L cell is stained with Giemsa and looked at under the microscope. To account for the behavior of persistently infected cultures, the putative cryptic chlamydial form, which we will call the cryptic body, must be endowed with a number of properties in addition to "invisibility." The observations that virtually all of the inclusion-free L cells in a persistently infected population are immune to infection with exogenous chlamydiae and give rise to persistently infected clones are most simply explained by assuming that all of the cells in a persistent infection are either overtly or cryptically infected with *C. psittaci* and that in some yet to be explained way cryptic infection confers immunity to superinfection. The slower multiplication rate and lessened cloning efficiency of persistently infected L-cell populations, even those with very low levels of inclusion-bearing cells, are probably also manifestations of cryptic infection. For every cell in a persistently infected culture to be cryptically infected, the cryptic bodies must multiply such that, at each L-cell division, there is a high probability that each daughter cell will get at least one of them. The effectiveness of rifampin and chlortetracycline in curing L cells of cryptic infection means that reproduction of the cryptic body requires the transcription of chlamydial deoxyribonucleic acid by procaryotic ribonucleic acid polymerases and the translation of the resulting messenger ribonucleic acid on procaryotic ribosomes, thus ruling out any explanation of the cryptically infected state that depends on integration of the chlamydial genome with that of its host. Aside from these requirements, we have no idea what the physical nature of the cryptic body might be.

The cryptic body hypothesis will explain periodic fluctuations in host and parasite density in persistently infected cultures if it is assumed that some factor periodically initiates the conversion of cryptic bodies into reticulate bodies that multiply and differentiate into infections elementary bodies in a conventional chlamydial development cycle. When a persistently infected culture containing both inclusion-free and inclusion-containing L cells is transferred to a new culture vessel, the inclusion-free (yet cryptically infected) cells multiply more rapidly than the inclusion-containing ones so that the L-cell population becomes largely inclusion-free. During this period of increase in proportion of inclusion-free host cells, a few of the cryptically infected L cells convert to overt infection and release infectious *C. psittaci*, but the inclusion-free cells are resistant to infection with exogenous chlamydiae. Then something initiates a mass conversion of cryptic bodies into reticulate bodies, and most of the host cells are destroyed by overt chlamydial multiplication. However, a few cryptically infected L cells do not convert to overt infection, and these cells survive to initiate a new cycle of regrowth.

In the early stages of our investigation, we thought that persistently infected cultures might constitute simple models of the predator-prey relationship in which host and parasite populations show inverse fluctuations in density, because prey density varies inversely with predator density whereas predator density varies directly with that of the prey (10). Although the interaction of populations of intracellular parasites with populations of their host cells does resemble in some respects the predator-prey relation, in particular the relation between the parasitoid arthropods and their hosts (4), predator-prey interaction does not explain several crucial properties of the persistently infected L-cell system such as the phenomenon of superinfection, the failure to isolate clones of uninfected host cells from predominantly inclusion-free populations, and the weeks of inclusion-free growth of clonally derived persistent infections.

Another way in which the inclusion-free L cells in a persistent infection might become resistant to challenge with exogenous chlamydiae would be for the inclusion-containing cells in that population to release a diffusible factor that renders the inclusion-free host cells immune to superinfection. However, this supposition offers

no ready explanation for the second and third properties of persistent infections given in the preceding paragraph.

The cryptic body hypothesis is only a guide to continued investigation. It asks many questions and answers none of them. What kind of a host-parasite interaction is it that produces a cryptically infected L cell? What is the physical nature of the cryptic body? What at first keeps the cryptic body from differentiating into a reticulate body and what eventually stimulates this conversion? What is the mechanism of superinfection immunity, and is it the same mechanism that blocks conversion of cryptic bodies into reticulate bodies?

Another question that is always asked of every persistent infection of cells in culture is, What is its relation to persistent infection of the natural host? The question is not answered easily. An intact animal with its multiple mechanisms of constitutive and inducible resistance to infection is an infinitely more complicated environment for parasite persistence than a cell culture. Demonstration of a mechanism whereby parasites and host cells may coexist indefinitely in vitro means only that such a mechanism might also operate in vivo. However, we think it unlikely that *C. psittaci*, with one of the smallest of all procaryotic genomes (8), would evolve and then conserve such a complicated way of reacting with host cells if that reaction did not have some adaptive value. It has been known for almost 50 years that hosts latently infected with chlamydiae resist challenge with exogenous chlamydiae (12). Although this resistance is probably due at least in part to cell-mediated immunity (20), the phenomena described here may also make a contribution.

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