INFECTION AND IMMUNITY, Oct. 1989, p. 3189–3193
0019-9567/89/103189-05$02.00/0

Vol. 57, No. 10

Fusion of Inclusions following Superinfection of HeLa Cells by Two Serovars of *Chlamydia trachomatis*

JOHN C. RIDDERHOF[1]† AND ROBERT C. BARNES[2]‡*

Department of Parasitology and Laboratory Practice, School of Public Health, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514,[1] and Sexually Transmitted Diseases Laboratory Program, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333[2]

Received 23 May 1989/Accepted 26 June 1989

We used a double-label immunofluorescence assay to examine the ability of *Chlamydia trachomatis* serovar F to infect and develop within HeLa 229 cells previously infected with serovar E. No exclusion to superinfection occurred for up to 24 h following infection by serovar E. The percentage of HeLa cells infected in cultures inoculated with both strains was identical to that of cells in cultures inoculated with one strain as a control. Organisms of both serovars were located within the same intracellular inclusion in 88 to 95% of HeLa cells infected with both serovars. The proportion of superinfected HeLa cells containing both strains in separate inclusions increased when there was exposure to inhibitors of cytoskeletal structure and transport. We used this inhibition to demonstrate that fusion of *C. trachomatis* phagosomes occurs throughout the developmental cycle.

The intracellular parasites of the genus *Chlamydia* have a distinctive intracellular developmental cycle. Following attachment and ingestion by the host cell, the infectious elementary body (EB) differentiates to the metabolically active reticulate body (6, 16). The reticulate body undergoes replication by binary fission within an intracellular phagosome, which is bounded by membrane initially derived from the host cell. After numerous replicative divisions of the reticulate bodies, the phagosome becomes visible as the chlamydial inclusion. By an unknown mechanism, perhaps mediated through the chlamydial outer membrane, the chlamydial phagosome avoids fusion with host cell lysosomes (5). Thus, avoidance of phagosome-lysosome fusion may be critical to the intracellular survival of chlamydiae.

In contrast to cells infected in vitro by *Chlamydia psittaci* organisms (11, 16), mammalian cells infected by *C. trachomatis* usually contain only one chlamydial inclusion, even under high multiplicities of infection (2, 3). This suggests that, for *C. trachomatis*, either multiple infecting EBs are excluded from infection following initial infection by one organism (superinfection exclusion) or phagosomes from multiple infecting organisms may coalesce during the development of the organism. Prior microscopic studies of *C. trachomatis* suggested that concurrent infection of a single host cell by multiple EBs of the same serovar could occur and that coalescence of phagosomes probably accounted for the failure to observe multiple inclusions late in the infective cycle (2, 3).

C. trachomatis has been classified with monoclonal antibodies into more than 15 serovars (22). The epitopes determining serovar specificity reside predominantly on the major outer membrane protein (20). Recently, we reported instances of clinical infection by multiple serovars of *C. trachomatis* (1). Coinfection of a single epithelial cell by two strains of *C. trachomatis* with subsequent coalescence of endosomes could bring chlamydial organisms into close contact; this might then allow genetic exchange leading to serological diversity. Thus, studies of the fate of different *C. trachomatis* strains infecting the same host cell can provide information regarding possible mechanisms of phagosomal membrane fusion and potential mechanisms for genetic exchange. We employed serogroup-specific monoclonal antibodies with double-label immunofluorescence microscopy to study the intracellular development of superinfected HeLa 229 cells. We found that prior infection does not influence superinfection with another serovar of *C. trachomatis* during the first 24 h following the initial infection and that fusion of chlamydial phagosomes occurs throughout the intracellular developmental cycle.

(This work was presented in part at the 87th Annual Meeting of the American Society for Microbiology, Atlanta, Ga., 1 to 6 March 1987 [D-156, p. 98].)

* Corresponding author.

† Present address: Division of Public Health, Office of Laboratories, Dover, DE 19903.

‡ Present address: Consultants in Medicine, 800 E. Chestnut St., Bellingham, WA 98225.

MATERIALS AND METHODS

HeLa 229 cells. The HeLa 229 cells and *C. trachomatis* strains were kindly provided by C.-C. Kuo, Department of Pathobiology, University of Washington. Monolayers of HeLa cells were grown in 150-cm^2 tissue culture flasks at 37°C and were transferred two to three times weekly. The growth medium was Eagle minimal essential medium supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah), 2 mM L-glutamine, 2% nonessential amino acids, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (all from GIBCO Laboratories, Grand Island, N.Y.). Cells were maintained without antibiotics until they were inoculated with chlamydiae. Cells were tested for mycoplasma contamination periodically by 4-6-diamidino-2-phenylindole staining (Sigma Chemical Co., St. Louis, Mo.) (4) and culture (Flow Laboratories, Inc., McLean, Va.).

C. trachomatis. The genital isolates E/UW-5/Cx and F/UW-6/Cx were passed at least 10 times in HeLa 229 cells before being used in these experiments. *C. trachomatis* strains were cultivated in HeLa 229 cells treated with DEAE-dextran (14) in HSC buffer (10 mM HEPES, 0.2 M sucrose, 0.09 M NaCl, 1×10^{-3} M $MgCl_2$, 1.5×10^{-4} M $CaCl_2$). EBs were harvested 72 h after inoculation, partially purified by differential centrifugation in HSC, and pelleted

through a 35% Renografin-76 (Squibb Diagnostics, New Brunswick, N.J.) cushion (13). The pelleted EBs were then washed in HSC, repelleted as described above, suspended in HSC, divided into aliquots of 0.5 ml, and stored in vapor-phase liquid nitrogen.

Monoclonal antibody reagents. Monoclonal antibodies to *C. trachomatis* KB8 (serovars E/L1 specific) and FC2 (serovar F specific) have been described previously (22). Antibodies were purified from murine ascites by protein A-Sepharose affinity chromatography (Pharmacia, Uppsala, Sweden). Antibody KB8 was conjugated to sulforhodamine (Texas Red; Molecular Probes, Inc., Junction City, Oreg.) according to the instructions of the manufacturer. Antibody FC2 was conjugated to fluorescein (FITC-FC2), using fluorescein isothiocyanate in basic aqueous solution by standard methods (8).

Superinfection. Monolayers of HeLa cells were inoculated with chlamydial strains 24 to 36 h after plating. *C. trachomatis* serovar E was inoculated onto host cell monolayers in 96-well microdilution plates that were rocked for 1 h at 35°C. The inoculum was then removed, the monolayer was rinsed with phosphate-buffered saline, and growth medium was added. The infected HeLa cells were incubated at 35°C for various times before superinfection. To superinfect, growth medium was aspirated from the infected HeLa cell monolayer. The monolayer was then washed as before, and the inoculum of serovar F in HSC buffer was added. Infection with serovar F was allowed to proceed for 1 h at 37°C at 1 × *g*; then monolayers were washed twice with phosphate-buffered saline. The HeLa cells were then detached with 0.02% trypsin in phosphate-buffered saline and replated onto 12-mm glass cover slips in shell vials containing growth medium such that individual cells could be distinguished microscopically (12). Single-infection controls for both serovars were treated as superinfected cells with HSC buffer added in place of the primary or secondary inoculum. Shell vials were incubated at 37°C for 24 or 48 h following superinfection. Cover slips were fixed with methanol, stained with a mixture of FITC-FC2 and antibody KB8 conjugated to sulforhodamine for 1 h, rinsed in phosphate-buffered saline and distilled water, and mounted on slides with 10% glycerol and *N*-propyl gallate (7). Slides were examined with a Zeiss standard fluorescence microscope equipped with a ×63 Neofluor oil immersion objective and a sliding mount containing Texas Red and FITC filter cubes. In randomly selected fields, 200 cells were counted on each cover slip and classified as uninfected, E infected, F infected, or E and F infected.

Microtubule and microfilament inhibitors. Cytochalasins B and D, colchicine, vincristine, and vinblastine were dissolved in dimethyl sulfoxide and added to growth medium at final concentrations of 1.0 and 2.5 μg/ml for cytochalasins B and D, 2.5 and 5.0 μg/ml for vincristine and vinblastine, and 1.0 and 2.0 μg/ml for colchicine. In experiments to test the effect of inhibitors on HeLa cell infection, the inhibitors were added to HeLa cells 1 h before superinfection and maintained in the growth medium until fixation and staining of the cell layer. All cells were superinfected with serovar F 4 h after infection with serovar E, then incubated 48 h before staining. Single-infected and superinfected untreated cells were included as controls. Duplicates of each control were superinfected in growth medium without cycloheximide to measure the effect of cycloheximide on superinfection. Duplicate wells were tested for cell viability with trypan blue (Sigma) staining. More than 95% of the cells were viable in each microtubule or microfilament inhibitor control vial after 48 h of incubation.

In experiments to examine the kinetics of phagosome fusion, HeLa cells were infected with serovar E and superinfected with serovar F 4 h later. At various times thereafter, cytochalasin D (2.5 μg/ml) was added to the growth medium to inhibit further fusion of phagosomes. At 48 h after superinfection, cells were fixed, stained with monoclonal antibody mixture, and counted.

TABLE 1. Percentage of HeLa cells containing inclusions following infection by *C. trachomatis* serovars E and F, with serovar F added at various times following infection with serovar E

Serovar observed (serovar added)	% Cells infected[a] for indicated h of superinfection					
	0	1	2	4	8	24
E (E and F added)	71.2	66.9	68.9	70.8	70.8	62.8
F (E and F added)	30.8	27.9	32.6	25.3	24.8	30.2
Both (E and F added)	24.6	22.8	25.8	21.1	20.7	22.1
E (only E added)	70.6	70.2	72.0	77.7	72.3	69.3
F (only F added)	33.0	31.7	28.4	22.5	26.2	32.7

[a] Percentages are the averages of three experiments, with four replicate wells with 200 cells counted per well for each experiment. The average coefficient of variation is 10.8%.

RESULTS

Superinfection. Infection of HeLa 229 cells by *C. trachomatis* serovar F occurred in similar proportions for cells infected by serovar E as well as controls (Table 1). The proportions of cells infected by each serovar were also similar for superinfected cells and single-infection controls. By using our standard EB preparations, serovar E infected 69% ± 3% of all cells counted versus 71% ± 3% for controls, while serovar F infected 29% ± 4% for both superinfected and single-infected cells (mean plus standard deviation for three separate experiments). The proportion of cells infected by each serovar following superinfection was independent of time of superinfection for at least 24 h. The addition of cycloheximide to the growth medium increased the number of cells infected by either serovar but did not change the distribution of phagosome morphologies following superin-

TABLE 2. Effect of cycloheximide on superinfection of HeLa cells

Serovar in single or double infection	Mean % cells infected ± SD[a]	
	Cycloheximide added	No cycloheximide added
Single infection		
E	77.3 ± 4.9	39.4 ± 4.5
F	23.0 ± 1.4	9.2 ± 3.4
Double infection		
E	74.7 ± 3.2	38.0 ± 3.5
F	26.4 ± 3.7	10.4 ± 2.2
E + F	23.0 ± 2.9	8.2 ± 2.5
E + F/F[b]	87.5 ± 4.0	78.1 ± 9.2
E + F separate[c]	1.2 ± 1.4	3.9 ± 4.7

[a] Mean plus or minus standard deviation of four replicate wells with 200 cells counted per well. Cells were incubated in growth medium with or without 1 μg of cycloheximide per ml added after initial infection and superinfection.

[b] F-infected cells that also contained serovar E.

[c] Coinfected cells with the superinfecting F serovar in a separate inclusion.

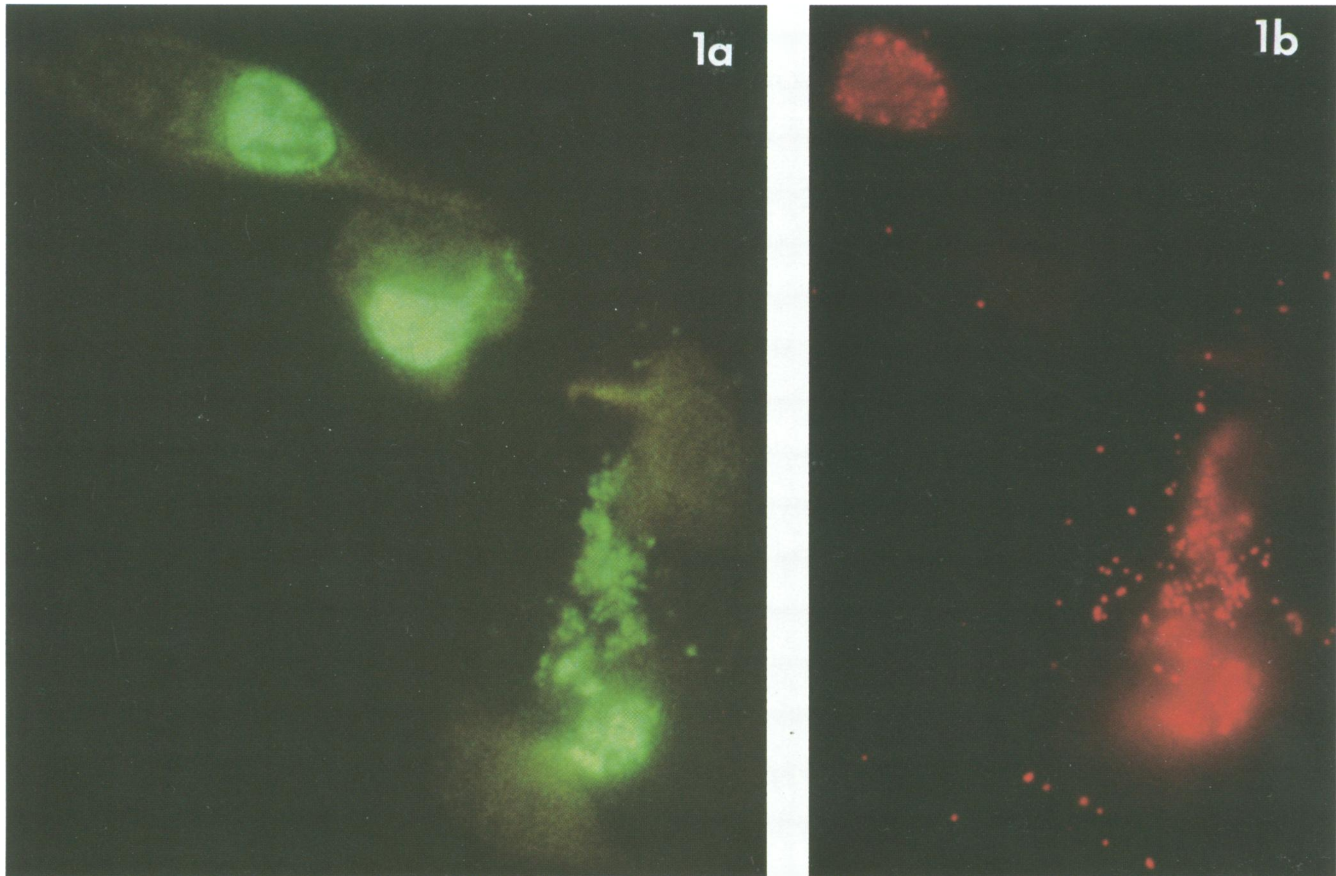

FIG. 1. (a and b) Superimposable photomicrographs of the same microscopic field of several HeLa cells which were stained with antibodies to both serovars E and F. (a) This photomicrograph was taken using FITC optics, which visualizes inclusions of serovar F; (b) this was taken with Texas Red optics, which visualizes inclusions of serovar E. Two of the inclusions seen contain both serovars E and F, and one (middle) contains only serovar F.

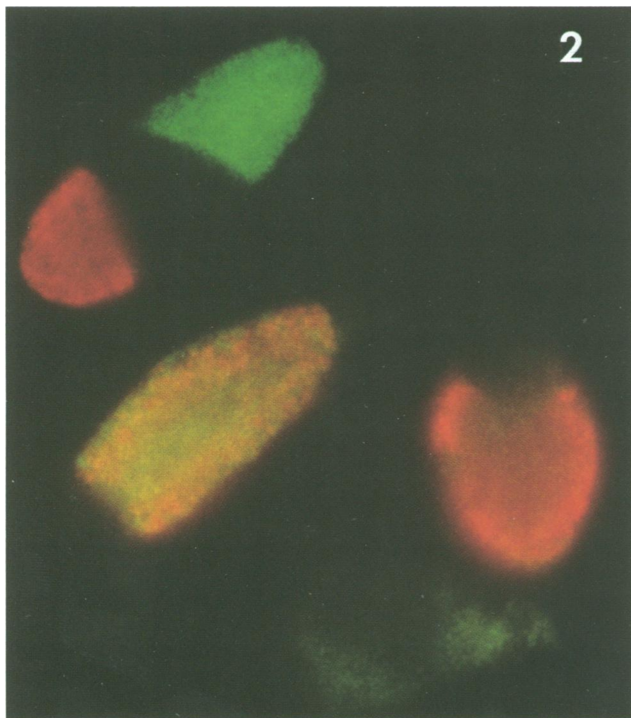

FIG. 2. Photomicrograph of a microscopic field of HeLa cells stained with antibodies to both serovars E and F and photographed with double exposure using first FITC and then Texas Red optics. The cell at the top contains both serovars, at opposite poles of the host cell, and the cell just below it contains both serovars in the same inclusion.

fection (Table 2). The proportion of superinfected cells (E + F) reflected a high percentage of all serovar F-infected cells (E + F/F) with (87.5%) or without (78.1%) cycloheximide.

Two morphologic forms of chlamydial phagosomes were observed within superinfected HeLa 229 cells at 48 h. The predominant form (>95% of cells infected by both serovars) consisted of a single inclusion containing EBs of both serovars (Fig. 1 and 2). Less frequently, two separated inclusions, each containing EBs of one serovar, were seen. The proportion of cells with a single inclusion containing both serovars was greater if superinfected cells were stained at 48 h following superinfection (>95%) than if they were stained 24 h following superinfection (87%).

Effect of cytoskeletal inhibitors on superinfection. The microfilament inhibitors cytochalasins B and D and the microtubule inhibitors colchicine, vincristine, and vinblastine had different effects on the efficiency of superinfection and on the proportion of superinfected cells exhibiting phagosomes that contained both serovars (Table 3). When inhibitors were added 1 h before superinfection, the infection by serovar F was relatively unaffected for infections in the presence of colchicine, vincristine, and cytochalasin B but

TABLE 3. Effect of microtubule or microfilament inhibitors on the proportion of cells infected with *C. trachomatis* serovars E and F

Inhibitor	Mean % cells[a] infected by:			
	E	F	E + F	E + F (separate)[b]
Control	74.7	26.4	23.1	1.2
Colchicine	79.2	21.1	17.9	12.3
Vincristine	76.8	21.5	17.3	14.2
Vinblastine	79.9	16.7	15.7	21.5
Cytochalasin B	74.0	21.2	17.0	8.8
Cytochalasin D	70.1	4.7	3.0	50.0

[a] Mean of duplicate wells with 200 cells counted per well.
[b] Percentage of coinfected cells (E + F) that contained each serovar in a separate phagosome.

was substantially reduced in the presence of vinblastine or cytochalasin D at 2.5 μg/ml. All inhibitors increased the proportion of coinfected cells in which the serovar E and F phagosomes were separated; the greatest effect was noted with cytochalasin D.

Figure 3 illustrates the effect of adding cytochalasin D at various times following superinfection. The proportion of cells with single phagosomes containing both serovars increased continuously throughout the developmental cycle. A decrease in infectivity as measured by total numbers of cells infected was not detected when cytochalasin D was added after superinfection (data not shown).

DISCUSSION

Moulder and co-workers (17) found that mouse L cells persistently infected with *C. psittaci* organisms were resistant to superinfection with the same strain. Demonstrating such latent, persistent infection and superinfection exclusion has been more difficult with *C. trachomatis* (15). Under conditions of infection dramatically different from those used to establish persistence in the previously cited studies, we have found that prior productive infection of HeLa cells by a strain of one *C. trachomatis* serovar does not exclude superinfection by a strain of another serovar. This observation confirms and extends earlier findings suggesting that multiple *C. trachomatis* phagosomes fuse at some time following infection (2, 3). In our studies, superinfection of HeLa cells resulted in single inclusions containing EBs of both serovars. Previous studies have noted single inclusions in cells infected with a single serovar, even when high multiplicities of infection favored the ingestion of more than one organism (2, 3). Our findings suggest either that multiple-infecting chlamydial EBs are transported to the same phagosome at the time of ingestion, or shortly thereafter, or that multiple-infecting organisms could reside within individual phagosomes which fuse when approximated by cytoplasmic flow throughout the chlamydial developmental cycle.

The inhibition of phagosomal fusion, but not infectivity, by adding cytochalasin D after superinfection demonstrated that fusion of chlamydial phagosomes is a microfilament-dependent process distinct from ingestion of the agent. The ability of cytochalasin D to inhibit inclusion formation by the F serovar when added before superinfection agrees with the findings of Ward and Murray (23) that this drug affects the initial steps of attachment and endocytosis.

Our observation that cultured mammalian cells can be coinfected by different chlamydial serovars has theoretical relevance to the ontogeny of oculogenital infection in humans and to the evolution of serovars of the organism. The ability of organisms of different antigenic structure to infect the same host cell may provide a mechanism for exchange of structural determinants, explaining the serologic diversity of the major outer membrane protein. Until genetic markers are identified for *C. trachomatis*, this hypothesis remains purely speculative.

Ultrastructural studies (10, 23) suggest that the fusion of *C. trachomatis* phagosomes occurs very shortly after infection. These previous studies, however, used high multiplicities of infection (ca. 1,000:1) to bring individual endocytosed EBs into close proximity for better observation in electron microscopy sections. Our study, using lower multiplicities of infection (ca. 10:1), indicates that phagosomal fusion occurs throughout the intracellular developmental cycle and may reflect a phenomenon separate from the intracellular sorting associated with receptor-mediated endocytosis. Our results do not indicate whether the fusion of endocytic vesicles containing *C. trachomatis* is a result of random collisions or is directed by cellular mechanisms of cytoplasmic flow.

There are conflicting studies on the mechanism of attachment and ingestion of chlamydial organisms. Some studies support a classic receptor-mediated attachment and ingestion model (10, 11, 18, 19), whereas a zipper mechanism of ingestion has been suggested by some ultrastructural studies (23). In other experimental models of receptor-mediated endocytosis, an attached ligand is lateralized to clathrin-coated pits and ingested in multiple endosomes which fuse within several minutes in metabolically active cells at 37°C (9, 21). Thus, observation of prompt coalescence of infecting particles in this study would have indirectly supported the hypothesis of receptor-mediated endocytosis in chlamydial ingestion. Our experimental system cannot accurately identify individual EBs at very early times following superinfection; thus, our results provide no direct information regarding chlamydial attachment and ingestion.

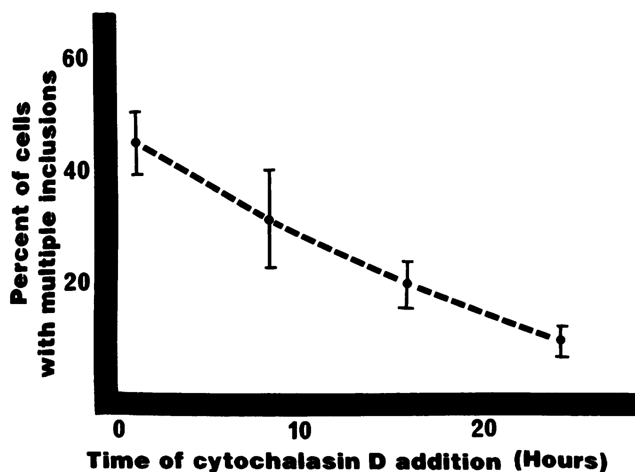

FIG. 3. Chart showing proportion of coinfected cells containing two phagosomes with differing serovars. At various times after superinfection (horizontal axis), cytochalasin D was added to replicate cultures to prevent further fusion of chlamydial phagosomes.

ACKNOWLEDGMENT

The authors thank Priscilla Wyrick for critical review of the manuscript.

LITERATURE CITED

1. **Barnes, R. C., R. J. Suchland, S. P. Wang, C.-C. Kuo, and W. E. Stamm.** 1985. Detection of multiple serovars of *Chlamydia trachomatis* in genital infections. J. Infect. Dis. **152:** 985–989.
2. **Bernkopf, H., P. Mashiah, and Y. Becker.** 1962. Correlation between morphological and biochemical changes and the appearance of infectivity in FL cell cultures infected with trachoma agent. Ann. N.Y. Acad. Sci. **98:**62–81.
3. **Blyth, W. A., and J. Taverne.** 1972. Some consequences of the multiple infection of cell cultures by TRIC organisms. J. Hyg. **70:**33–37.
4. **Del Guidice, R. A., and H. E. Hopps.** 1977. Detection of mycoplasma contamination in cell cultures by direct fluorescent staining. *In* G. J. McGarrity, D. G. Murphy, and W. W. Nichols (ed.), Mycoplasma infection of cell cultures. Plenum Publishing Corp., New York.
5. **Eissenberg, L. G., P. B. Wyrick, C. H. Davis, and J. W. Rumpp.** 1983. *Chlamydia psittaci* elementary body envelopes: ingestion and inhibition of phagolysosome fusion. Infect. Immun. **40:** 741–751.
6. **Friis, R. R.** 1972. Interaction of L cells and *Chlamydia psittaci*: entry of the parasite and host responses to its development. J. Bacteriol. **110:**706–721.
7. **Giloh, H., and S. W. Sedat.** 1982. Fluorescence microscopy: reduced photobleaching of rhodoamine and fluorescein protein conjugates by n-propyl gallate. Science **217:**1252–1255.
8. **Goding, J. W.** 1983. Monoclonal antibodies: principles and practice. Academic Press, Inc., New York.
9. **Goldstein, J. L., M. S. Brown, G. W. Anderson, D. W. Russell, and W. J. Sneider.** 1985. Receptor mediated endocytosis: concepts emerging from the LDL receptor system. Annu. Rev. Cell Biol. **1:**1–39.
10. **Hodinka, R. L., C. H. Davis, J. Choong, and P. B. Wyrick.** 1988. Ultrastructural study of endocytosis of *Chlamydia trachomatis* by McCoy cells. Infect. Immun. **56:**1456–1463.
11. **Hodinka, R. L., and P. B. Wyrick.** 1986. Ultrastructural study of mode of entry of *Chlamydia psittaci* into L-929 cells. Infect. Immun. **54:**855–863.
12. **Horoschak, K., and J. W. Moulder.** 1978. Division of single host cells after infection with chlamydiae. Infect. Immun. **19:**281–286.
13. **Howard, L., N. S. Orenstein, and N. W. King.** 1974. Purification on renograffin density gradients of *Chlamydia trachomatis* grown in the yolk sac of eggs. Appl. Microbiol. **27:**102–106.
14. **Kuo, C.-C., S. P. Wang, B. B. Wentworth, and J. T. Grayston.** 1972. Primary isolation of TRIC organisms in HeLa 229 cells treated with DEAE-dextran. J. Infect. Dis. **125:**665–668.
15. **Lee, C. K., and J. W. Moulder.** 1981. Persistent infection of mouse fibroblasts (McCoy cells) with a trachoma strain of *Chlamydia trachomatis*. Infect. Immun. **32:**822–829.
16. **Moulder, J. W.** 1984. Family I. *Chlamydiaceae* Rake 1957, 957^{AL}, p. 729–739. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
17. **Moulder, J. W., N. J. Levy, and L. P. Schulman.** 1980. Persistent infection of mouse fibroblasts (L cells) with *Chlamydia psittaci*: evidence for a cryptic chlamydial form. Infect. Immun. **30:**874–883.
18. **Söderlund, G., and E. Kilhström.** 1983. Effect of methylamine and monodansylcadaverine on the susceptibility of McCoy cells to *Chlamydia trachomatis* infections. Infect. Immun. **40:**534–541.
19. **Söderlund, G., and E. Kihlström.** 1983. Attachment and internalization of a *Chlamydia trachomatis* lymphogranuloma venereum strain by McCoy cells: kinetics of infectivity and effect of lectins and carbohydrates. Infect. Immun. **42:**930–935.
20. **Stephens, R. S., M. R. Tam, C.-C. Kuo, and R. C. Nowinski.** 1982. Monoclonal antibodies to *Chlamydia trachomatis*: antibody specificities and antigen characterizations. J. Immunol. **128:**1083–1089.
21. **Stoorvogel, W., H. J. Geuze, and G. J. Strous.** 1987. Sorting of endocytosed transferrin and asiaglycoprotein occurs immediately after internalization in HepG2 cells. J. Cell Biol. **104:** 1261–1268.
22. **Wang, S. P., C.-C. Kuo, R. C. Barnes, R. S. Stephens, and J. T. Grayston.** 1985. Immunotyping of *Chlamydia trachomatis* with monoclonal antibodies. J. Infect. Dis. **152:**985–989.
23. **Ward, M. E., and A. Murray.** 1984. Control mechanisms governing the infectivity of *Chlamydia trachomatis* for HeLa cells: mechanisms of endocytosis. J. Gen. Microbiol. **130:** 1765–1780.