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Inhibition of Phagolysosome Fusion is Localized to *Chlamydia psittaci*-laden Vacuoles

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Intracellular survival of *Chlamydia psittaci* is in part dependent on the ability of the organism to thwart phagolysosome formation. Circumvention of phagolysosome fusion could be either localized to chlamydia-laden vacuoles or generalized to all phagosomes in the host cell. To determine which of these modes is in operation the ability of chlamydia elementary and reticulate bodies to protect *Saccharomyces cerevisiae* from degradation in macrophage phagolysosomes was examined via acridine orange and Giemsa staining. No statistically significant difference was evident between the amount of fusion observed in coinfected macrophages and those infected with yeast cells alone. This was not dependent on some unique interaction between the chlamydia and the yeast cells since viable count studies to determine the protection of a second organism, *Escherichia coli*, also failed to show significantly different amounts of inactivation of the bacteria by macrophages in the presence of *C. psittaci*. Therefore, the inhibition of phagolysosome fusion is localized to chlamydia-laden phagosomes.

Chlamydia species are obligate intracellular parasites which enter host cells via phagocytosis (5). Intracellular survival of any organism is contingent on its ability to avoid destruction by the lysosomal enzymes of the host. Ultrastructural studies indicate that ferritin-labeled lysosomes fail to fuse with chlamydia-laden phagosomes, thus allowing the organism to effectively evade the bactericidal enzymes (19). Because noninfectious, opsonized, or damaged chlamydiae are rapidly destroyed in phagolysosomes (19), circumvention of phagolysosome fusion (P-LF) must be crucial to the survival of the bacterium.

Upon phagocytosis of the nonmetabolizing extracellular form of the organism, the elementary body (EB), two things happen: inhibition of P-LF is initiated, and the organism begins a transformation to the metabolically active reproductive form, the reticulate body (RB). Circumvention of P-LF is maintained throughout most or all of the bacterial developmental cycle (18). This suggests that either the EB effects a permanent blockage of P-LF or that the RB must subsequently maintain this inhibition. In previous studies it has been demonstrated that phagocytized RB are rapidly destroyed in macrophage phagolysosomes, indicating that RB are unable to inhibit P-LF (1). However, due to the difficulties involved in purifying these fragile forms, the viability of the RB in this situation is questionable, and the results are nonconclusive.

In an attempt to elucidate the mechanism(s) involved in circumvention of P-LF by *Chlamydia* species, the following question was asked: "Do all phagosomes in a host cell fail to fuse with lysosomes or is this limited specifically to chlamydia-laden vacuoles?" Failure of lysosomes to fuse with any phagosomes would indicate a generalized shutdown in the normal phagolysosome-forming process. To answer this question, the ability of chlamydiae to protect coinfecting yeast cells or *Escherichia coli* from destruction in phagolysosomes was assessed. Our data reveal that neither the EB nor the RB form of the bacterium can protect coinfecting organisms from degradation, thus indicating that inhibition of P-LF is localized to chlamydia-laden vacuoles. (A preliminary report of these findings was presented at the 80th Annual Meeting of the American Society for Microbiology, Miami Beach, Fla., May, 1980.)

MATERIALS AND METHODS

Propagation of *Chlamydia*. The Cal 10 meningopneumonitis strain of *Chlamydia psittaci* was routinely grown in 929-L cell suspension cultures (17). Harvest and purification of EB was performed by the method of Tamura and Higashi (17), except that trypsin treatment was not used.

Titration of IFU of *Chlamydia*. The concentration of infectious units per milliliter (IFU/ml) was determined by counting the cytoplasmic inclusions stained with May-Grunwald-Giemsa stain as described previously (21).

Harvest of mouse macrophages. Unelicited peritoneal macrophages from 6- to 10-week-old female Swiss Webster mice were harvested by the method of Cohn and Benson (4). The viability and concentration

of macrophages were determined via a hemacytometer count of macrophages which excluded trypan blue. Macrophages were suspended in TC199 medium supplemented with 15% heat-inactivated fetal calf serum, sodium bicarbonate, 0.02% streptomycin, and 0.1% kanamycin and plated at a concentration of 3.5×10^6 to 4.0×10^6 per petri plate (10 by 35 mm) containing five 12-mm cover slips. After a 1-h adsorption at 37°C in an atmosphere of 5% CO_2, monolayers were washed with phosphate-buffered saline to remove the nonadherent cells and replenished with fresh medium.

Propagation of yeast cells. *Saccharomyces cerevisiae* was subcultured on Sabaroud dextrose agar slants at least once every week. Viability and cell numbers were determined by trypan blue exclusion in a hemacytometer. Yeast cells used in experiments were from 2-day-old cultures.

Propagation of *E. coli*. *E. coli* K-12 MC4100 was grown on Trypticase soy agar or in Trypticase soy broth (BBL Microbiology Systems) at 37°C and subcultured on solid medium every 4 to 5 days.

Monitor for EB protection of yeast cells with acridine orange. All procedures were performed in the dark, and all incubations were carried out in a light, tight box to prevent photodynamic damage to cells and possible quenching of fluorescence (16). Macrophage monolayers were pretreated with acridine orange (1 μg/ml of TC199 medium) at 37°C in an atmosphere of 5% CO_2 as described by Hart and Young (11) to concentrate the dye into lysosomes. One of the following inocula was then added: 0.5 ml of TC199 medium, 0.5 ml of a yeast cell suspension at a multiplicity of infection of three yeast cells per macrophage; 0.5 ml of an EB suspension at a multiplicity of infection of 10 IFU/macrophage; or 0.5 ml of an EB suspension plus yeast cells at a multiplicity of infection of 10 EB per 3 yeasts per macrophage (simultaneous infection). The plates were centrifuged at $413 \times g$ at room temperature for 15 min and then placed in a candle jar at 37°C for 30 min. After three washes with PBS, fresh medium was added, and the plates were returned to the candle jar. At 45 min, 2 h, and 3.5 h, wet-mount preparations of the cover slips were examined by fluorescent microscopy with a fluorescein filter and photographed under ultraviolet light. After development of the film, the yeast phagosomes were monitored for evidence of P-LF. For sequential infections, monolayers were first exposed to 0.5 ml of EB at 10 IFU/macrophage, centrifuged as described above, and incubated for 15 min. Then, 0.5 ml of a yeast cell suspension (three yeasts per macrophage) was added. These plates were not centrifuged, but the yeast cells readily settled onto the monolayer and were phagocytized before the first 45-min time point.

Inactivation of *E. coli* in EB-infected macrophages. Macrophages plated at 3.5×10^6 to 4.0×10^6 cells per plate (10 by 35 mm) containing five cover slips were incubated overnight in TC199 medium devoid of antibiotics. Either 0.5 ml of medium or 0.5 ml of EB (10 IFU per macrophage) was added as an inoculum, and the plates were centrifuged at $413 \times g$ for 15 min at room temperature. Monolayers were then incubated at 37°C in 5% CO_2 for 15 min to permit uptake. Cover slips were subsequently removed and placed in individual dram vials, fresh medium was added, and the vials were reincubated for 1 h.

A 50-μl sample of a late-log phase suspension of *E. coli* (3×10^7 colony-forming units per ml) was added to EB-infected macrophages or to uninfected controls. The vials were reincubated at 37°C in a candle jar. After 25 min the supernatant was collected, the monolayers were washed four times with PBS, and the washes were combined with the supernatant. Then 0.5 ml of a bacteriophage suspension (7×10^9/ml each of φK3 and φ80-vir) was added to the dram vials, and the vials were reincubated. A sample of the supernatant-wash mixture was plated to determine the number of uningested *E. coli*. The number of ingested *E. coli* was calculated by subtracting the number uningested from the initial number added as an inoculum as determined by a plate count.

At 1 h after addition of *E. coli*, the monolayers were again washed twice with PBS. A final 1.0 ml wash of PBS was saved and plated to determine the number of extracellular *E. coli* remaining.

Two 2- to 4-mm glass beads were added to each vial along with 1.0 ml of PBS. The vials were Vortexed three times in 10-s bursts to rupture the macrophages, and the homogenate was plated. The number of *E. coli* maintaining colony-forming activity was determined from the difference in the number of colony-forming units in the homogenate and those remaining extracellular. This number was converted to a percentage and subtracted from 100 to calculate the percentage inactivated by the macrophages.

Degradation of yeast cells in the presence of EB or RB. For EB studies macrophage monolayers were simultaneously infected with EB and yeast cells as described above, and the infected cultures were incubated at 37°C in a candle jar, washed four times with PBS at 30 min, and fixed at 45 min and 3.5 h with methanol for 5 min. Giemsa-stained cover slips (10) were then examined for yeast "ghosts" and compared with yeast cell-infected control samples.

Alternately, EB were centrifuged onto monolayers as described above, and the petri plates were returned to the incubator for 24 h to allow the transition of EB into RB. Yeast cells were then added at a multiplicity of infection of three yeast cells per macrophage and allowed to settle onto the monolayers at 37°C in a candle jar. After 45 min the monolayers were washed as described above, replenished with fresh medium, and reincubated. At 2 and 6 h postinoculation with yeast cells, the cover slips were methanol fixed, Giemsa stained, and examined as previously described.

Transmission electron microscopy. For transmission electron microscopy, control and infected macrophage monolayers were washed thoroughly and prefixed in 1.5% glutaraldehyde prepared in Sorenson buffer (pH 7.5) for 60 min at 25°C. After extensive rinsing in buffer, primary fixation of the monolayers was accomplished by 1% osmium tetroxide in Sorenson buffer for 90 min at 25°C. The monolayers were then harvested with a rubber policeman and enrobed in agar. Samples were dehydrated in alcohol and embedded in Spurr low-viscosity resin. Thin sections were poststained with 5% alcoholic uranyl acetate for 15 min, followed by lead citrate for 4 min. All specimens

were examined with an AEI electron microscope accelerated at 60 kV with a 30-μm objective aperture.

RESULTS

Monitor for EB Protection of *S. cerevisiae* in macrophages by the acridine orange technique. Macrophages were preincubated with acridine orange and allowed to concentrate the dye into secondary lysosomes. Examination by fluorescent microscopy revealed autofluorescing green macrophages with numerous red-orange lysosomes dispersed throughout the cytoplasm (Fig. 1A). The appearance of these cells did not alter throughout the 3.5-h observation period. Macrophages infected with EB were indistinguishable from the macrophage controls at all time points (Fig. 2A). Due to the size of the EB (0.25 μm), it cannot be seen at this magnification. However, duplicate 45-h Giemsa-stained samples indicated that 50 to 80% of the macrophages were infected.

After phagocytosis of *S. cerevisiae*, the migration of lysosomes to yeast phagosomes was evidenced by the appearance of satellite lysosomes encircling the dark yeast phagosomes by 45-min postinfection (Fig. 1B). Subsequent fusion was initially detectable as a red rim outlining the yeast cells. The organisms became red as they absorbed the dye, and as the yeast cells were digested they subsequently turned yellow and then green by 2 and 3.5 h after inoculation (Fig. 1C).

Simultaneous inoculation of EB and *S. cerevisiae* onto the macrophage monolayers could conceivably result in sequestration of the two organisms within the same phagosome. Although transmission electron microscopy provided evidence that the organisms entered macrophages in separate phagosomes during simultaneous infections (Fig. 3), sequential infections, exposing macrophages first to EB and then to yeast cells, were done to enhance the probability that this would occur.

Both simultaneous and sequential infections of macrophages with EB and yeast cells were similar in appearance to yeast cell control samples. Lysosomes continued to migrate to (Fig. 2B) and to fuse with (Fig. 2C) yeast phagosomes. Statistical analysis by Student's *t* test indicated that there was an initial decrease in the proportion of yeast phagosomes which fused with lysosomes by 45 min in the simultaneously infected samples (Table 1). However, by 2 and 3.5 h the extent of fusion was the same in the control and test samples. The level of P-LF observed when EB were added before yeast cells was statistically equivalent to that seen in yeast cell control samples at all times.

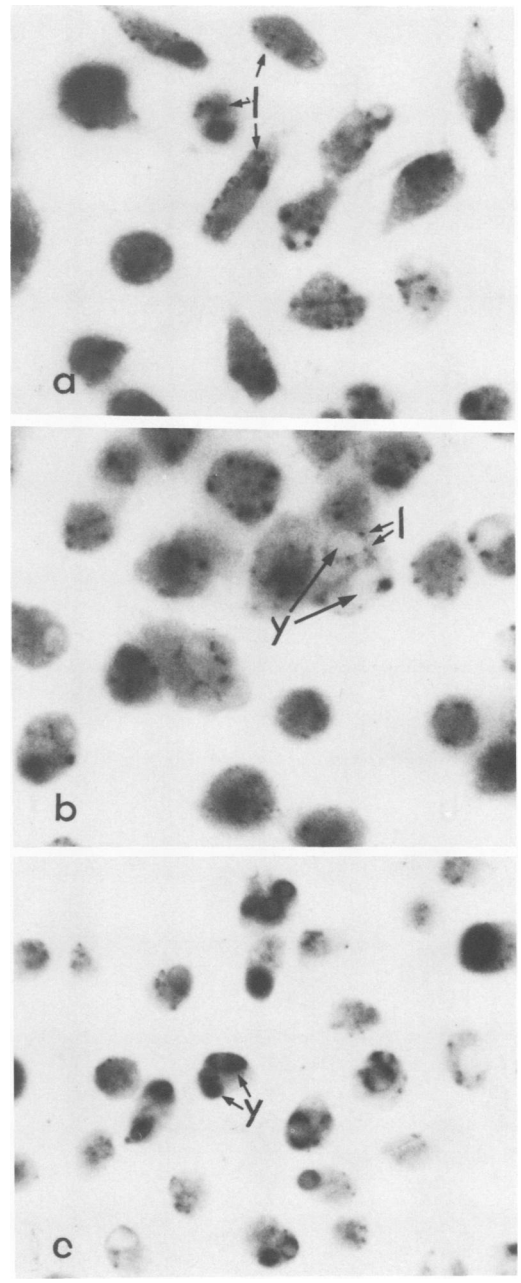

FIG. 1. *Fusion of acridine orange-labeled lysosomes with yeast phagosomes in macrophages. (a) Acridine orange-labeled lysosomes (l) were scattered throughout the cytoplasm of uninfected macrophages; (b) lysosomes rimmed yeast phagosomes (y) 45 min after addition of yeast cells to monolayers; (c) by 3.5 h, extensive fusion of yeast phagosomes with lysosomes had occurred. Magnifications, (a) and (c) ×1,250, (b) ×750.*

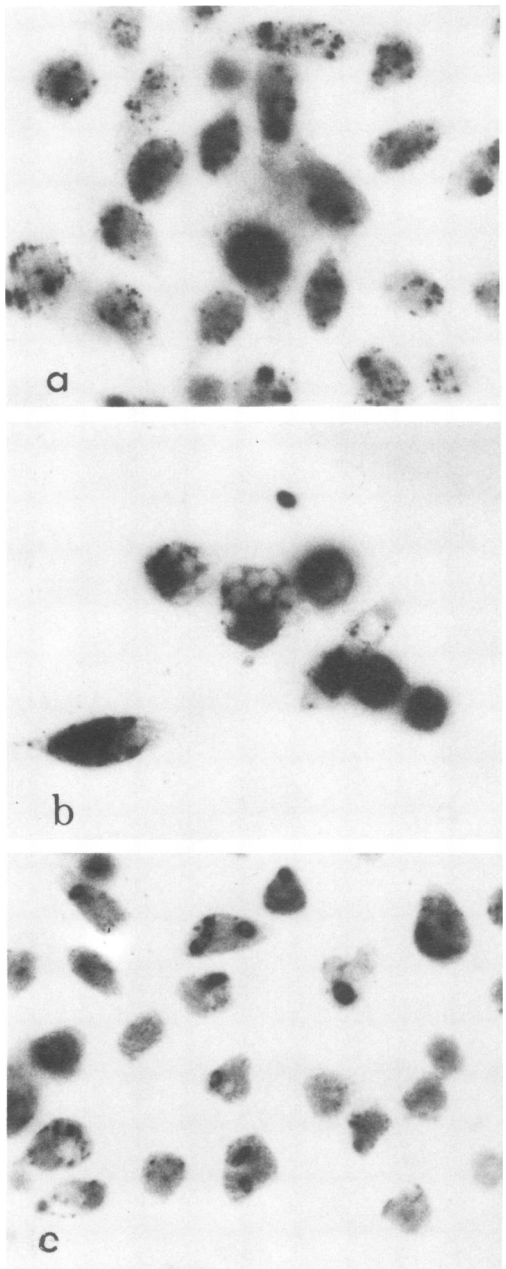

FIG. 2. *(a) Macrophages infected with EB. (b) Macrophages coinfected with yeast cells and EB 45 min and (c) 3.5 h after addition of yeast cells. For both Fig. 1 and 2 negative black and white prints were made from color slides for clarity, and thus phagosomes which were dark under ultraviolet light appear white. Magnification, ×1,250.*

Ingested yeast cells are inactivated, i.e., lose the ability to multiply soon after P-LF has occurred. To substantiate the data obtained through the acridine orange technique and to verify the possibility of an initial lag in yeast P-LF in simultaneous infections, viable count studies were attempted. Unfortunately, it proved virtually impossible to remove extracellular yeast cells by extensive washing of the monolayers, and antibiotics could not be used without killing intracellular yeast cells in addition to extracellular ones.

An alternate method was chosen based on reports that various phagocytized fungi stain differentially with Giemsa stain depending on viability; live fungi stain deep blue, whereas fungi undergoing degradation stain pale blue, pink, or white (15). Preparations of our strain of *S. cerevisiae* which were killed by heating to 100°C for 20 min contained 99% (361/365) pale blue, pink, or white ghosts, whereas unheated samples contained only 2% (7/414). Thus the presence of ghosts in Giemsa-stained, yeast-infected macrophages was used to monitor the degradation of fungi, presumably in phagolysosomes. There was no statistically significant difference in the number of degraded yeast cells in yeast cell control samples and in macrophages coinfected with EB (Fig. 4) at 45 min or 3.5 h

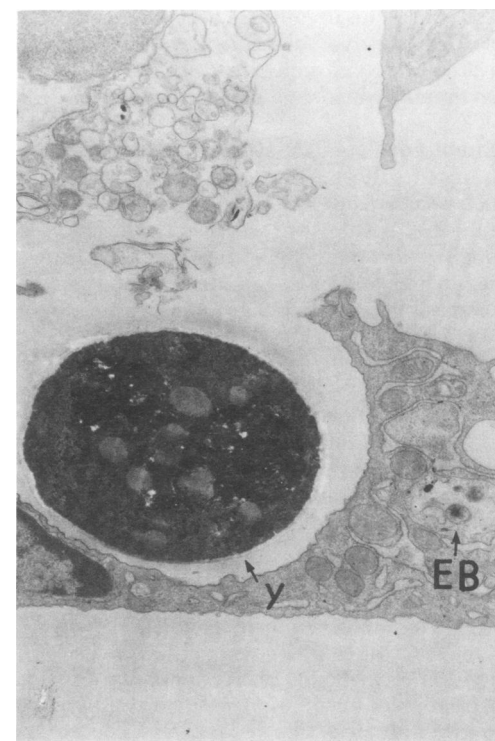

FIG. 3. *Transmission electron micrograph of a mouse peritoneal macrophage soon after simultaneous inoculation with S. cerevisiae (y) and C. psittaci EB. The two microorganisms are found in separate phagosomes. Magnification, ×10,300.*

TABLE 1. *Fusion of acridine orange-labeled lysosomes with S. cerevisiae phagosomes in EB-infected macrophages*

Type of infection	Fraction of yeast phagosomes that fused with lysosomes at[a]:		
	45 min	2 h	3.5 h
Macrophages plus yeast cells	24/99 (24)	24/37 (65)	31/ 77 (40)
	22/74 (30)	35/64 (55)	25/32 (78)
	17/90 (18)		91/113 (80)
	53/146 (36)		51/73 (70)
Macrophages plus yeast cells plus EB (simultaneous)	4/32 (12)	65/109 (60)	22/40 (55)
	7/77 (9)	24/56 (43)	56/120 (47)
	27/227 (12)	89/158 (56)	83/157 (53)
Macrophages plus yeast cells plus EB (sequential)	21/93 (23)	35/69 (51)	96/120 (80)
	50/150 (33)	69/126 (55)	74/115 (64)

[a] Data were pooled from experiments performed on separate days. There was a significant difference between control samples and EB-infected samples only for the 45-min simultaneous infection data ($P = 0.05$). Percentages are given within parentheses.

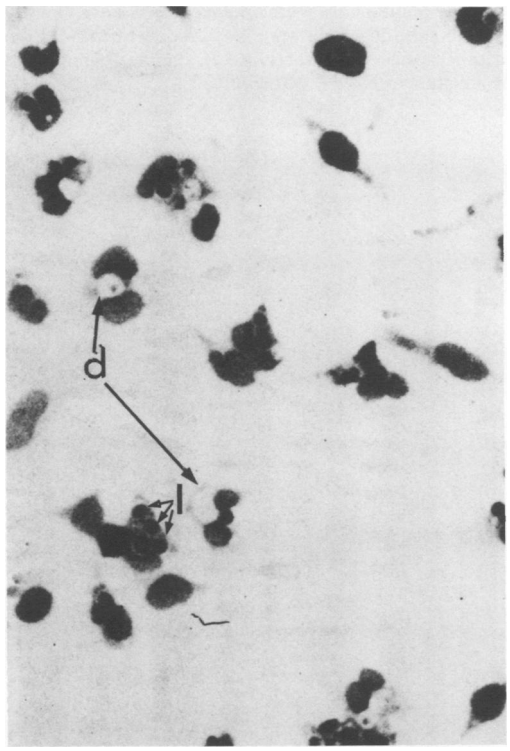

FIG. 4. *Giemsa stain of macrophages simultaneously infected with EB and S. cerevisiae 3.5 h postinoculation. Monolayers infected with yeast cells only were similar at 3.5 h. (d) Degraded yeast cells; (l) live or undegraded yeast cells.*

(Table 2). Substantiation of the light microscopy data regarding the degradation of yeasts in EB-infected macrophages was provided by transmission electron microscopy (Fig. 5).

Inactivation of *E. coli* in EB-infected macrophages. To ascertain that the failure of the parasite to protect yeast phagosomes was not due to a unique interaction between chlamydia and yeast cells, the ability of EB to protect a second organism, *E. coli*, was studied. Inoculation of *E. coli* onto macrophage monolayers by centrifugation resulted in only 4% ingestion of *E. coli* by 1 h postinoculation, regardless of the presence of EB. This may have been due to centrifugation, although ingestion of EB and yeast cells was unimpaired by this technique. Thus, rather than simultaneously infecting the macrophages with EB and *E. coli*, we allowed the *E. coli* to settle onto the macrophage monolayers at 1.5 h postinoculation of the macrophages with EB by centrifugation. The samples were monitored for internalization of the *E. coli* 25 min later. By this method, an average of 50% of the *E. coli* was ingested in both control and

TABLE 2. *Degradation of yeast cells in macrophages in the presence of EB*

Macrophages	Fraction of yeast cells visible as ghosts (degraded) at[a]:	
	45 min	3.5 h
Yeast cell-infected	74/330 (22)	51/177 (29)
	67/374 (18)	104/349 (30)
	57/302 (19)	115/383 (30)
	63/290 (22)	143/462 (31)
	122/585 (21)	112/576 (19)
	98/513 (19)	144/486 (30)
Coinfected with EB and yeast cells	114/732 (16)	208/570 (36)
	74/333 (22)	188/461 (41)
	132/444 (30)	134/369 (36)
	133/888 (15)	156/540 (29)
	129/617 (21)	70/417 (17)
		193/778 (25)

[a] Pooled data were analyzed by the Student's t test, and no significant difference between control samples and EB-infected samples for either time point at $P = 0.05$ was evident.

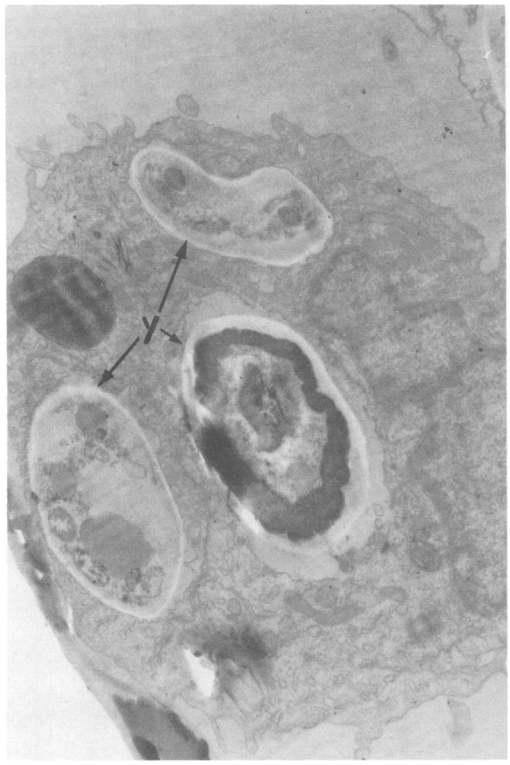

FIG. 5. *Transmission electron micrograph of yeast cells (y) undergoing degradation (morphological) in an EB-infected mouse peritoneal macrophage. Magnification, ×10,300.*

EB-infected macrophages after 1 h. After lysis of the extracellular bacteria by the specific coliphages φK-3 and φ80-vir, the macrophages were mechanically ruptured 1 h postinoculation, and the number of internalized colony-forming units was determined (Table 3). There was no statistical difference in the percentage of ingested *E. coli* which was inactivated in the EB-infected macrophages as compared with the control macrophages. This indicated that the ability of macrophages to inactivate *E. coli* was not impaired by the presence of EB.

Degradation of yeast cells in the presence of RB. It was of interest to determine whether the presence of RB in macrophages could alter the ability of lysosomes to fuse with yeast phagosomes. *S. cerevisiae* were added to macrophages 24 h after infection with EB, by which time the transition for EB to RB had occurred (Fig. 6). Acridine orange-labeled lysosomes, however, could not be consistently seen in the 24-h-old chlamydiae-infected macrophages. Therefore, the Giemsa stain method was employed for monitoring yeast cell viability. The percentage of yeast ghosts within macrophages

TABLE 3. *Inactivation of E. coli in macrophages coinfected with EB*

Type of infection	CFU released per 1 × 10^7 ingested organisms[a]	% *E. coli* inactivated[a]
Macrophages plus *E. coli*	0	100
	0	100
	1×10^5	99
	0	100
	6×10^4	99
	2×10^5	98
Macrophages plus EB plus *E. coli*	0	100
	4×10^5	98
	0	100
	2×10^6	90
	2×10^3	100
	4×10^5	96

[a] The pooled data were analyzed by Student's *t* test, and no significant difference was found in the ability of macrophages to inactivate *E. coli* in the presence or absence of EB ($P = 0.05$).

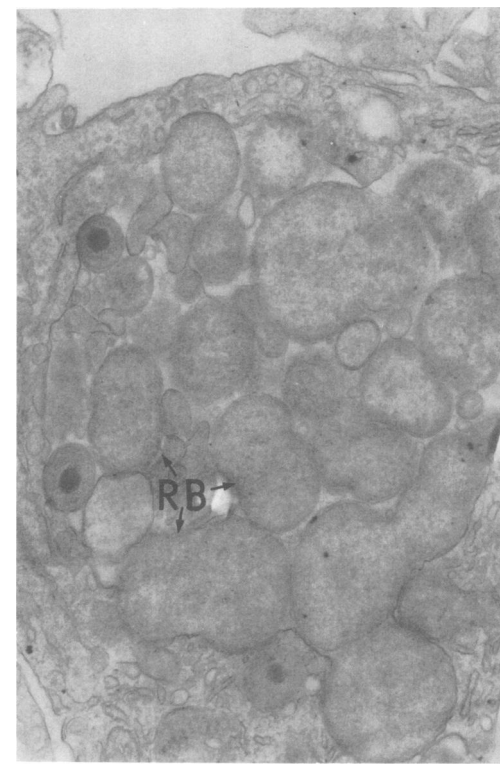

FIG. 6. *Transmission electron micrograph of a mouse peritoneal macrophage inoculated with C. psittaci EB and incubated for 30 h. By this time EB developed into RB and binary fission of RB was underway. Magnification, ×21,000.*

increased over 6 h (Table 4), and no statistically significant difference was evident in the amount of degradation in RB-infected macrophages in comparison with yeast cell control samples.

DISCUSSION

Our data provide evidence that circumvention of P-LF by *C. psittaci* is a localized phenomenon confined to the chlamydia-laden phagosome. It further implies that neither EB nor RB release a soluble factor or metabolic product which diffuses into the macrophage cytoplasm to protect coinfecting microorganisms from destruction. These studies are, however, unable to clarify whether the interaction of EB with the phagosome effects a permanent block of P-LF or whether RB must subsequently maintain this inhibition.

Circumvention of P-LF by *C. psittaci* is not due to lysosomal dysfunction; lysosomes maintain mobility and continue to recognize and fuse with other phagosomes. This inhibition of P-LF without obvious involvement of the lysosomes contrasts with what is known about other well-studied agents. *Mycobacterium tuberculosis* cell wall sulfatides (8), poly D-glutamic acid, dextran sulfate (11), and ammonia (7) all accumulate in lysosomes, presumably rendering them incapable of fusion, although it is possible that there is some effect on the phagosomal membrane as well. Yet like *Chlamydia* species, *Toxoplasma gondii* vacuoles may also escape P-LF without disturbing normal lysosomal functions (14). A published electron photomicrograph by Jones and Hirsch (13) shows a macrophage containing two toxoplasmas in separate phagosomes. Thorotrast-containing lysosomes fused with the phagosome surrounding the dead protozoan even though the adjacent phagosome enclosing the living organism was protected from fusion. Although this suggests localized inhibition of P-LF, entry of the dead organism may have preceded entry of the live one, thus obliterating the detection of any generalized protective activity.

TABLE 4. *Degradation of S. cerevisiae in macrophages in the presence of RB*

Type of infection	Fraction of yeast cells visible as ghosts (degraded) at[a]:	
	2 h	6 h
Macrophages plus yeast cells	40/163 (24)	330/925 (36)
	215/875 (25)	358/764 (47)
	179/712 (25)	245/609 (40)
Macrophages plus RB plus yeast cells	35/132 (26)	297/833 (36)
	133/495 (27)	150/381 (41)
	36/148 (24)	216/602 (36)

[a] Statistical analysis of the pooled data by Student's *t* test indicated that there was no significant difference in the level of yeast degradation in the presence or absence of RB for either time point ($P = 0.05$).

Many hypotheses can be advanced to explain how *Chlamydia* species implement localized inhibition of P-LF. An obvious possibility is that membrane signals or recognition sites on the phagosomal membrane are altered, perhaps during the initial intimate interaction of the host and parasite surfaces preparatory to phagocytosis. Additionally, the organism may attach to and enter at an unusual site on the host cell, resulting in a failure to trigger a lysosomal response. There are several pieces of current evidence to substantiate these ideas. Cytochalasin B, an inhibitor of phagocytosis, is without effect on the uptake of *Chlamydia* species by L-cells or macrophages (9). Both *C. psittaci* and *T. gondii* enter host cells by parasite-induced phagocytosis (2, 12). Yet when the surface integrity of *C. psittaci* is altered by heat or antibody treatment or when *T. gondii* is prefixed with glutaraldehyde, the organisms lose the ability to prevent P-LF. In these latter instances, it may be that the host cell, rather than the parasite, directs entry as in the case of the Fc receptor of macrophages for opsonized bacteria. It follows that a specific ligand-receptor interaction is required for parasite-specified phagocytosis with concurrent lack of P-LF. In receptor-mediated endocytosis (6), certain requisite nutritional and regulatory proteins attach at receptor-coated pits on the eucaryotic cell surface. The resultant coated vesicles have various specific intracellular destinations: some fuse with lysosomes and others do not. Coated vesicles containing nutritional proteins, for example, are programmed to fuse with lysosomes, resulting in reduction of the proteins to amino acids for cell utilization. It seems likely that obligate intracellular parasites such as *Chlamydia* species and *T. gondii* may take advantage of such a specialized region on host cells.

Differences in opportunistic receptor-dependent endocytic events may also occur between nonprofessional and professional phagocytic cells. *Leishmania braziliensis* promastigotes enter human skin fibroblasts in an oriented manner, and P-LF does not occur. Promastigote entry into macrophages is not oriented, and P-LF does occur. Chang (3) postulated that two distinct modes of entry may be responsible for the intracellular fate of the organism and that the survival of the promastigotes in the fibroblasts may allow transformation of the promastigote into the amastigote, which could then infect macrophages and be resistant to lysosomal enzymes.

Receptors on the host cell surface can influence the final destination of endocytosed mate-

rial (6). Whereas obligate extracellular organisms apparently attach only to receptors which trigger P-LF or fail to induce phagocytosis at all, certain obligate intracellular parasites, such as those discussed here, probably avail themselves of receptors which fail to trigger a lysosomal response. Thus, circumvention of P-LF may not necessarily be an act of aggression by the microbe against the host, but an exploitation of host cell receptors intended for regulatory molecules with a physiological function.

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