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# Interaction of *Chlamydia psittaci* Reticulate Bodies with Mouse Peritoneal Macrophages

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Noninfectious reticulate bodies of *Chlamydia psittaci* are readily phagocytized by thioglycolate-elicited mouse peritoneal macrophages in monolayer culture. The internalized reticulate bodies are rapidly destroyed as indicated by a 60 to 70% decrease in trichloroacetic acid-precipitable radioisotopic counts in the macrophage pellet by 10 h and a concomitant increase of the trichloroacetic acid-soluble radiolabeled chlamydial nucleic acid in the cytoplasm. This intracellular destruction of reticulate bodies in macrophages is independent of the multiplicity of infection. Reticulate bodies at a high multiplicity of infection, up to 1,000:1, are also incapable of inducing immediate cytotoxicity in macrophages as evidenced by the lack of early release of the host cell-soluble cytoplasmic enzyme lactic dehydrogenase. Thus, it appears that the virulence factors for (i) initiation or maintenance of intracellular survival via circumvention of phagolysosome formation and (ii) host cell damage are either missing or not expressed by the RB form of this bacterium.

In the process of survival and adaptation the chlamydiae have developed a highly specialized development cycle which is unique among the procaryotes. The elementary body (EB) is adapted for extracellular survival and is the infectious form of the organism which makes contact with the target epithelial cells of the mucous membranes. Because of the ease with which these obligate intracellular parasites enter host cells, which are not normally actively phagocytic, Byrne and Moulder (2) have suggested that the chlamydiae play a positive role in ensuring their uptake. The EB is internalized in the host phagosome, and the whole of the developmental cycle takes place in this membrane-bound microenvironment. Soon after uptake the EB is reorganized into the reticulate body (RB) form. This form is suited only for intracellular survival: it is metabolically active, highly permeable, and fragile. The RB divide by binary fission and fill the expanding phagosome with as many as 200 to 1,000 progeny. Before release from the host cell, the RB return to the more compact, resistant EB form.

Thus, there are two forms of the organism, each with apparently its own biological functions. The virulence factors identified so far for the EB include (i) attachment and parasite-specified phagocytosis (2), (ii) initiation of intracellular survival via circumvention of phagolysosome formation (4, 14, 15), and (iii) induction of cytotoxicity both in vivo (3, 9) and in vitro (5, 8, 13-15). Are these same virulence factors expressed in RB? Evidence to date suggests that the RB are not taken up by the non-professionally phagocytic cells (12) and that RB are unable to induce toxic death in mice (3).

The purpose of this study was to expand our knowledge of the virulence factors of RB particularly in regard to the critical early events of host-parasite interaction. Is the circumvention of phagolysosome formation initiated by the EB a permanent event and essential prior to differentiation into RB or must RB participate in the maintenance of this protected state? Do the RB participate in immediate cytotoxicity? A prerequisite to answering these two questions depended on internalization of the RB in the host cells. Since the RB are noninfectious and are not normally taken up by the non-professionally phagocytic cells, these studies were performed by using the professionally phagocytic macrophage as the host cell.

## MATERIALS AND METHODS

**Propagation of chlamydiae.** EB of *Chlamydia psittaci* (Cal 10 strain) were grown in L-929 suspension cells according to the method of Tamura and Higashi (11). Harvest and purification of the EB were essentially as described before (15). The RB were grown and purified essentially according to Tamura et al. (12). Eighteen-hour-infected L-cells were harvested by centrifugation at 480 × *g* for 10 min and resuspended in a 0.25 M sucrose solution containing 0.033 M tris(hydroxymethyl)aminomethane (Tris) buffer. The

L-cells were disrupted by three cycles of Omni-mix (Sorvall) homogenization, 45 s each at a no. 3.5 setting, followed by centrifugation. The homogenates were centrifuged at 482 × *g* for 10 min and the collected supernatant fractions were then placed on a 30% (wt/wt) sucrose solution prepared in 0.033 M Tris buffer. After centrifugation at 5,875 × *g* for 60 min, the pellet was resuspended in a 10% (wt/wt) potassium tartrate solution containing 0.033 M Tris buffer and layered onto a 20 to 40% (wt/wt) potassium tartrate–0.033 M Tris gradient. The gradient was centrifuged at 50,000 × *g* for 1 h. The middle band (density, ca. 1.18), containing the purified RB, was washed once in the 0.033 M Tris buffer and examined in the electron microscope.

**$^3$H-labeling of the RB.** For radiolabeling the RB, [5-$^3$H]uridine (1 mCi/liter) was added at the time of inoculation of the L-cells. The infected cells were then harvested at 18 h and purified as described.

**Titration of chlamydiae.** For titration, the purified EB preparation was examined by direct particle count in the electron microscope via the method of Sharp (10), and the infectivity was assayed for inclusion-forming unit titer (15).

RB were also titrated by direct particle count, but the inclusion-forming unit assay could not be performed in L-cells as the RB are not infectious for L-cells. However, since RB are phagocytized by macrophages, these host cells were substituted in the infectivity assay. Thioglycolate-elicited mouse peritoneal macrophages, 24 h old, were inoculated with RB at a multiplicity of infection (MOI) of 1:1. After centrifugation for 30 min at 280 × *g*, the infected macrophages were incubated for 24, 48, and 72 h and then examined for inclusions after May-Grunwald-Giemsa staining. The rare inclusions found, less than 1%, could be accounted for by EB contamination of the RB preparation.

**Harvest and infection of mouse peritoneal macrophages.** Peritoneal macrophages were obtained from 8- to 10-week-old Swiss-Webster randombred female mice which had been injected intraperitoneally with 3.0 ml of thioglycolate medium 5 days before harvest. The macrophages were harvested and infected as previously described (14).

**Assay for macrophage cytotoxicity.** The leakage of lactic dehydrogenase from macrophages has been found to be a sensitive and reliable indicator of damage to these host cells. Our methods were described previously (15), and the assay for lactic dehydrogenase was performed according to Bergmeyer (1). The results are expressed in terms of enzyme units per milliliter of supernatant; 1 enzyme unit = 1 μmol of β-reduced nicotinamide adenine dinucleotide oxidized per min at 25°C.

**Determination of the uptake and persistence of RB in macrophages.** Determinations of uptake and persistence of RB in macrophages were performed as described for EB (15). Since the percentage of trichloroacetic acid-precipitable $^3$H-labeled EB counts in the zero-time inoculum almost always correlated with the inclusion-forming unit titer, it was tempting to assume the percentage of trichloroacetic acid-precipitable, $^3$H-labeled counts could be equated with viability. At best this can only be considered as a measure of "intactness" of the EB. However, in lieu of a reliable method for determining the viability of RB, this same measure was used for RB determinations. Therefore, the percent ingestion of $^3$H-labeled RB by macrophages was designated by the ratio of the trichloroacetic acid-precipitable counts in the macrophage pellet at 45 min to those in the zero-time inoculum, multiplied by 100. The percent persistence of intact $^3$H-labeled RB in macrophages was designated as the ratio of the trichloroacetic acid-precipitable counts in the macrophage pellet at 2, 6, and 10 h to those in the macrophage pellet at 45 min, multiplied by 100.

## RESULTS

**Uptake and intracellular fate of $^3$H-labeled RB in macrophages.** The uptake of *C. psittaci* RB by thioglycolate-elicited mouse peritoneal macrophages was determined to be 35.1% at a low MOI (1:1) and 50.1% at a high MOI (100:1). These numbers represent the average percentage obtained from eight separate experiments. In general, the calculated percent uptake of RB by macrophages was slightly less than that reported for EB, 51.4 and 64.4%, respectively (15).

Internalization of the noninfectious form of chlamydiae in the professionally phagocytic cells resulted in an average loss of 60 to 70% of the trichloroacetic acid-precipitable radioactive counts in the macrophage pellet at 10 h regardless of the MOI (Fig. 1A). In keeping with intra-

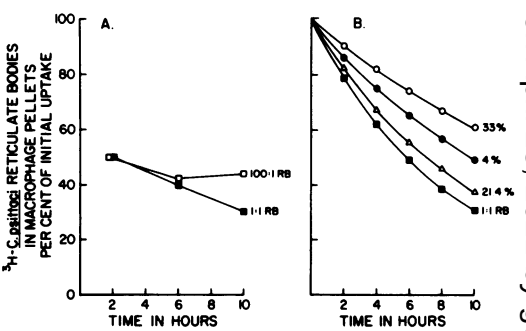


FIG. 1. *$^3$H-labeled C. psittaci RB remaining associated with thioglycolate-elicited macrophage pellets after ingestion. The percent survival of $^3$H-labeled chlamydiae in macrophages is designated: (A) as the ratio of trichloroacetic acid-precipitable counts in the macrophage pellet at 2, 6, and 10 h to those in the macrophage pellet at 45 min, multiplied by 100; and (B) as the curves found by linear regression of ln percent trichloroacetic acid-precipitable counts in the macrophage pellet to those in the macrophage pellet at 45 min, multiplied by 100, versus time. Symbols: (■) RB, MOI = 1:1; (□) RB, MOI = 100:1; (○) RB, MOI = 1:1, + EB (33% inclusion-forming units [IFU]), MOI = 5:1; (△) RB, MOI = 1:1, + EB (21.4% IFU), MOI = 5:1; (●) RB, MOI = 1:1, + EB (4% IFU) MOI = 5:1.*

cellular destruction, there was a concomitant increase in the trichloroacetic acid-soluble counts first in the cytoplasm of the host cells, followed later by release of the trichloroacetic acid-soluble counts into the extracellular environment. By comparison, the loss of radiolabel from EB at an MOI of 1:1 was only 3%, but if the EB were pretreated with heat or opsonized with homologous antibody, there was destruction of the EB (45 and 23%) in phagolysosomes (15). Thus, it appears that RB alone are unable to provide for their intracellular survival.

**Protection of RB from intracellular destruction in macrophages by EB.** In an attempt to determine if the presence of EB is required for the intracellular survival of RB in the host cell, purified nonlabeled EB of increasing infectivity titers (particle count/inclusion-forming unit ratios of 25:1, 5:1, and 3:1, respectively) were mixed with a suspension of purified, radiolabeled RB at a ratio of 5 EB to 1 RB. By providing an inoculum with excess EB, at a concentration below that required for initiating immediate host cell cytotoxicity (M. Gardner, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, D6, p. 70), it was reasoned that some viable EB might be taken up with RB in the macrophage phagosomes or, more likely, the presence of EB in nearby phagosomes might influence the surrounding microenvironment. Under these circumstances uptake of the radiolabeled RB was essentially the same as reported above, averaging 45%. The rate of destruction of RB by macrophages, when combined with the EB of low inclusion-forming units (4 and 21.4%), was not significantly different from that of RB alone at an MOI of 1:1 (Fig. 1B). However, in the presence of EB of higher infectivity titer (33%), there appeared to be less destruction of the intracellular RB in macrophages. This difference in the rates of RB destruction was verified by Student's $t$ test and found to be statistically significant at a 90% confidence level ($\alpha = 0.10$).

**Can RB induce immediate cytotoxicity in macrophages?** When macrophages were infected in vitro with EB at a low MOI (1:1), there was no release of lactic dehydrogenase until about 40 h after inoculation. This host cell damage is thought to be independent of the multiplication of the obligate intracellular parasite. When the macrophages were infected with purified EB at a high MOI (100:1), release of lactic dehydrogenase was apparent about 2 h after inoculation. The amount of lactic dehydrogenase release at 6 h was equal to that released at 40 h with a low MOI (15). This damage is multiplication independent and has been termed "immediate cytotoxicity" (8). When thioglycolate-elicited mouse peritoneal macrophages were infected with purified *C. psittaci* RB at MOIs of up to 1,000:1, there was no release of lactic dehydrogenase as late as 10 h after inoculation. These results are compared in Fig. 2.

## DISCUSSION

RB of *C. psittaci* are rapidly phagocytized by mouse peritoneal macrophages. Whereas EB at optimal conditions for survival are able to provide for their intracellular protection by circumvention of phagolysosome formation, this virulence factor is either absent or not expressed in RB. The loss of radiolabel from RB under conditions predicted for survival (1:1 and 100:1) averages 60 to 70%. It therefore appears from these studies that RB can neither initiate nor maintain the protected state of their microenvironment. An electron microscopy study by Lawn et al. (6) revealed uptake by macrophages of RB contaminating the EB inoculum and rapid destruction of the RB in phagolysosomes.

Some additional support for the premise that this protective function is the sole property of EB was suggested in the experiments where mixtures of EB and RB were added to macrophage monolayers (Fig. 1B). With populations of EB of high infectivity titer, there appeared to be some protection of the RB from intracellular destruction.

Circumvention of phagolysosome formation could mean (i) lack of contact of the lysosomes with the parasite-laden phagosome or (ii) inhibition of fusion of juxtaposed lysosome and phagosome membranes. It has been suggested that lysosomes do not come in contact with

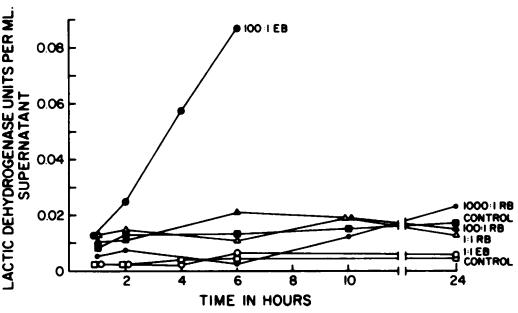


FIG. 2. *Release of lactic dehydrogenase from thioglycolate-elicited macrophages inoculated in vitro with RB of C. psittaci at MOIs of 1,000:1, 100:1, and 1:1 compared to release of lactic dehydrogenase from thioglycolate-elicited macrophages inoculated with EB at MOIs of 100:1 and 1:1. Symbols: (●) EB, MOI = 100:1; (○) EB, MOI = 1:1, (□) control run with EB samples; (×) RB, MOI = 1,000:1; (▲) RB, MOI = 100:1; (△) RB, MOI = 1:1, (■) control run with RB samples.*

chlamydiae-laden phagosomes in the early stages of normal infection even though there may be an accumulation of the chlamydiae-containing phagosomes near the nuclear hoff and the Golgi apparatus where primary lysosomes are being released (6). Conversely, Friis (4) reported that lysosomes were invariably found adjacent to the chlamydial vacuole but that fusion did not occur. There was no host lysosomal response noted up to 24 h in chloramphenicol-treated, infected L-cells, suggesting there was no active synthesis of a protein lysosomal inhibitor required for protection of the infecting *C. psittaci*. He therefore postulated that the ability of the chlamydial cell to prevent the host lysosomal response was the property of some intrinsic architectural feature of the parasite already present at the time of entry. This probable cell wall component is inactivated by heat and masked by antibody, resulting in destruction of the pretreated EB in phagolysosomes (14, 15).

Large quantities of infectious EB induce a toxic death in animals. This effect is neutralized by anti-EB antisera, and isolated EB cell walls will absorb neutralizing antitoxin. RB do not induce this in vivo toxicity nor are RB cell walls capable of absorbing neutralizing antibody (3). The in vitro equivalent is believed to be the immediate cytotoxicity phenomenon first characterized by Moulder et al. (8) in L-cells and subsequently reported by others in macrophages (5, 14, 15). This investigation has clearly demonstrated that RB are not capable of eliciting the immediate cytotoxic response in macrophages. This is not surprising since a cytotoxic response is not revealed in normally infected host cells at a time when large numbers of RB are present in the developing chlamydial phagosome.

The EB and the RB are two forms of the same organism with identical hereditary material, yet they can be distinguished from one another morphologically, biochemically, physiologically, and biologically. We believe that the cell wall of the EB plays a major role in parasite-specified phagocytosis, prevention of phagolysosome formation, and induction of host cytotoxicity. It is probable that the inability of RB to participate in these biological functions is a phenotypic phenomenon based on differences in cell wall composition and organization (7). The situation could be analogous to the relationship between a bacterium and its derived unstable L-phase variant.

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