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Interaction of *Chlamydia psittaci* with Mouse Peritoneal Macrophages

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L-cell-grown *Chlamydia psittaci* elementary bodies (EB) were rapidly phagocytized by mouse peritoneal macrophages in vitro. However, the intracellular fate of chlamydiae in macrophages appeared to be dependent on the multiplicity of infection (MOI), i.e., the EB-to-macrophage ratio, and the treatment of the EB. At an MOI of 1:1 or less, survival is maximal, and growth and multiplication of live, untreated chlamydiae did occur. In contrast, at a high MOI (100:1), survival of chlamydiae is reduced, as confirmed by release of ^{3}H-labeled nucleic acid into the supernatant. At the high MOI, macrophage damage occurred that resulted in significant release of lactic dehydrogenase, beginning 2 h postinfection. This immediate macrophage cytotoxicity was abolished by pretreatment of EB with heat (5 min at 56°C) and was reduced about 50% by coating EB with homologous antibody. Pretreatment of the chlamydiae with heat or opsonizing antibody provides increased uptake of EB by macrophages but may contribute to increased destruction of these obligate intracellular pathogens in professional phagocytic cells.

Previous studies performed in our laboratory demonstrated that elementary bodies (EB) of *Chlamydia psittaci,* harvested from suspension L-cells, could survive and grow in macrophages under the appropriate conditions (18). Maximum survival and multiplication required infection of the professional phagocytic cells with live, untreated EB at a multiplicity of infection (MOI) of 1:1 or less. Phagolysosome formation was circumvented, the EB transformed into reticulate bodies, which in turn underwent binary fission, and mature, infectious EB was released between 72 to 96 h postinoculation. Conversely, pretreatment of the EB with heat or homologous antibody resulted in some intracellular destruction of the chlamydiae via fusion of lysosomes with the chlamydiae-laden phagosome. Infection of thioglycolate-stimulated or unstimulated mouse peritoneal macrophages with live EB at an MOI of 100:1, however, resulted in early damage to the host cells. This latter phenomenon has been termed "immediate cytotoxicity" (10).

The above studies were monitored morphologically by means of transmission electron microscopy. This report examines by means of biochemical assays the uptake and intracellular fate of elementary bodies of *C. psittaci* in mouse peritoneal macrophages cultured in vitro.

MATERIALS AND METHODS

Strain and growth of chlamydiae. The Cal 10 meningopneumonitis strain of *C. psittaci* was grown in 929 L-cell suspension cultures according to the method of Tamura and Higashi (15). L-cells in suspension culture were routinely propagated on rotary shakers or Spinner flasks. The medium consisted of 0.01% yeast extract-0.5% lactalbumin hydrolysate-Earle balanced salt solution (YLE) containing 10% calf serum, streptomycin (0.02%), and kanamycin (0.01%). The medium for infected L-cells was the same except that the concentration of calf serum was reduced to 3%. Infected cultures were incubated at 37°C for 44 to 48 h. Growth of the chlamydiae in L-cells was monitored by Giemsa- or Macchiavello-stained smears.

Harvest and purification of EB. The routine procedure for harvest and purification of EB of *C. psittaci* was as previously described (15), with the following modifications: (i) trypsin treatment of EB was not used; (ii) crude EB preparations were centrifuged through 30 rather than 25% sucrose cushions; (iii) the linear sucrose gradient was replaced by a discontinuous gradient of 38, 44, and 59% Renografin-76 (E. R. Squibb & Sons) (vol/vol). With the present purification procedure, there is no detectable loss of infectivity of the purified EB.

Titration of chlamydiae. For titration, the purified EB preparation was examined by direct particle count in the electron microscope via the method of Sharp (14), and the infectivity was assayed by a modification of the inclusion-forming unit (IFU) titer described by Kuo and Grayston (9). Appropriate dilutions of EB (0.2 ml) were centrifuged (280 × *g* for 15 min onto L-cell monolayers, 1.5×10^5 to 2×10^5 cells per chamber) in tissue culture four-chamber slides (Lab-Tek Products). May-Grünwald-Giemsa-stained inclusions were counted at 30 h. The infectivity titer of purified preparations of EB used for these studies

averaged 50%, or 1 IFU per two particles.

Preparation of ^{3}H-labeled *C. psittaci*. Numerous attempts to adapt the 929 suspension L-cells, originally obtained from Higashi, Kyoto University, Japan, to grow in minimal essential medium were unsuccessful. Thus, the isotope labeling of chlamydiae in infected L-cells was performed in YLE medium.

Since *C. psittaci* do not take up thymidine very efficiently (6), [^{3}H]uridine was used routinely. At 20 h postinfection, [5-^{3}H]uridine (1 mCi/liter) was added, and the infected cultures were reincubated for 24 to 28 h. The remainder of the harvest and purification procedure was the same as above.

Harvest and infection of mouse macrophages. These studies used both nonstimulated and non-specifically stimulated peritoneal macrophages obtained from 8- to 10-week-old, random-bred Swiss-Webster female mice. For stimulated macrophages, 5 days before harvest mice were injected intraperitoneally with 3.0 ml of sterile thioglycolate medium.

The procedure for harvest and infection of mouse macrophages has been previously described (18). Briefly, suspensions of purified chlamydial EB of known particle count were adjusted to the appropriate MOI, i.e., EB-to-macrophage ratio. One milliliter of the ^{3}H-labeled EB suspension in TC199 medium, containing a solution of 15% heat-inactivated fetal calf serum, streptomycin (0.02%), and kanamycin (0.01%), was added to 24-h-old phosphate-buffered saline (PBS)-rinsed macrophage monolayers (5×10^6 macrophages per petri dish [10 by 35 mm]). The petri dishes were centrifuged for 15 min at $280 \times g$ and then incubated at 37°C in an atmosphere of 5% CO_2 for 30 min, this period being designated as the uptake period (18). The infected monolayers were then washed three times in warm PBS, replenished with fresh warm TC199 medium, and returned to the incubator. At timed intervals, fractions of the supernatant and cell pellet were assayed for lactic dehydrogenase (LDH) and radioisotope counts. Each assay consisted of duplicate or triplicate samples.

Determination of ingestion of ^{3}H-labeled EB in macrophages. In previous studies employing [^{3}H]-uridine-labeled *C. psittaci*, EB samples were hydrolyzed with alkali to destroy the labeled ribonucleic acid, thus monitoring only the [^{3}H]uridine-to-thymidine incorporated into deoxyribonucleic acid (18). This treatment resulted in a reproducible loss of 40% of the total isotope counts. With an average specific activity of 1×10^{-4} cpm/EB, radiolabel studies using EB at an MOI of 1:1 were difficult to interpret due to the low counts. Thus, these studies employing radiolabeled EB at MOIs of both 100:1 and 1:1 were performed without alkali hydrolysis.

At the end of the 45-min uptake period, the 1.0-ml supernatant and two 0.5-ml saline washes were combined, placed on ice, and precipitated with an equal volume of cold 10% trichloroacetic acid. A 0.5-ml amount of PBS was added to the macrophage monolayer, and the cells were harvested with a rubber policeman. The petri dish was rinsed with an additional 0.5 ml of PBS. The cells and wash fluid were combined and precipitated with an equal volume of cold 10% trichloroacetic acid. Each sample consisted of duplicate or triplicate petri dishes. For the 2-, 6-, and 10-h samples, the supernatant was removed at 45 min, and the monolayers were washed twice with warm PBS and replenished with 1.0 ml of fresh, warm TC199 medium. These petri dishes were then reincubated for the appropriate timed intervals.

The trichloroacetic acid precipitates from the 45-min supernatants, all macrophage monolayers, and the 1.0-ml inoculum were collected on glass-fiber filters (Reeve Angel), dried, digested with NCS tissue culture solubilizer (Amersham/Searle), and counted in 5.0 ml of a neutralizing toluene-based scintillation mixture. The 2-, 6-, and 10-h nonprecipitated supernatant samples and all trichloroacetic acid-soluble samples were absorbed (1.0 ml) onto 2.5-cm^2 glass-fiber chromatography paper (Whatman, GF82), dried, digested, and counted as above in a Packard Tri-Carb scintillation spectrometer.

The percent ingestion of ^{3}H-labeled chlamydiae by macrophages was designated by the ratio of the trichloroacetic acid-precipitable counts in the macrophage pellet at 45 min to trichloroacetic acid-precipitable counts in the zero-time inoculum. The percent survival of ^{3}H-labeled chlamydiae in macrophages was designated as the ratio of trichloroacetic acid-precipitable counts in the macrophage pellet at 2, 6, and 10 h to the trichloroacetic acid-precipitable counts in the macrophage pellet at 45 min, multiplied by 100.

Viability of macrophages. Loss of viability of host macrophages can be tested by a variety of methods, including trypan blue dye uptake and detachment of cells from a petri dish. Both of these methods were used initially. However, we have found that leakage of LDH, a soluble cytoplasmic enzyme in these cells, is a more sensitive and reliable method for monitoring host cell damage and, thus, will be reported in these studies. The procedure and assay for LDH was performed according to Bergmeyer (3). Controls included: (i) a medium sample for the presence of serum LDH; (ii) 0.003% Triton X-100-treated uninfected macrophages, as a positive control; and (iii) duplicate samples assayed in the absence of pyruvate to screen for released reduced nicotinamide adenine dinucleotide (NADH) oxidase. TC199 medium for these macrophage experiments was devoid of a phenol red indicator, which interferes with the spectrophotometric reading. The results are expressed in terms of enzyme units per milliliter of supernatant; 1 enzyme unit = 1 μmol of β-NADH oxidized/min at 25°C.

Measurement of ingestion of polystyrene-latex spheres. Polystyrene-latex spheres, ca. 0.25 μm in diameter (Dow Diagnostics), were suspended in either TC199 medium or in PBS, placed in a 37°C water bath for 1 h, and then allowed to stand at 4°C overnight. The latex spheres were then diluted to the appropriate MOI (150:1, 300:1, 500:1, and 1,000:1) and inoculated onto thioglycolate-stimulated macrophage monolayers. To account for the difference in density between the 0.25-μm latex sphere and an EB cell, centrifugation was carried out for 30 min at $280 \times g$. The petri dishes were then incubated at 37°C in an atmosphere of 5% CO_2. At timed intervals, fractions of the supernatant medium were assayed for LDH release. The monolayers were thoroughly rinsed with PBS, collected with a rubber policeman, harvested by centrifugation, suspended in deionized distilled water, and sonically

treated for 3 min to disrupt cells and release latex spheres. The sonic extracts were layered onto a discontinuous hypaque gradient (7, 10, and 12% [wt/vol]) and centrifuged at 49,360 × *g* (18,500 rpm) for 4 h. The latex sphere bands were collected, washed in distilled water, resuspended in water, and dried in a 70°C oven. After drying the supernatants, pellets, test samples, and standards were extracted with 1.25 ml of dioxane, and the optical density was recorded at 259 nm. From a standard curve, the concentration of latex spheres per test sample was determined.

RESULTS

Immediate cytotoxicity in macrophages infected with high MOI of fresh, purified *C. psittaci* EB. Addition of fresh, purified EB of *C. psittaci*, at an MOI of 100:1, to mouse peritoneal macrophages in monolayer culture resulted in release of LDH from host cells, beginning 2 h postinfection (Fig. 1). In contrast, in macrophages infected with EB at an MOI of 1:1, release of LDH did not occur until 60 to 72 h, at which time, following intracellular growth and development, some mature infectious EB were emerging from the host cells (Fig. 1). The early damage to host cells resulting from infection with large doses of chlamydiae has been termed "immediate cytotoxicity" (10).

To determine if the host cell damage was parasite mediated, the same experiment was performed substituting polystyrene-latex spheres (ca. 0.25 μm in diameter) for EB. At MOIs of 150:1, 300:1, 500:1, and 1,000:1, there was no release of LDH from thioglycolate-stimulated macrophages up to 6 h postinoculation. With an MOI of 1,000:1, however, there was some release of the enzyme from macrophages by 27 h (Fig. 2). Internalization of the latex spheres in macrophages was demonstrated by dioxane extraction of washed macrophage pellets at 6 h. With MOIs of 500:1 and 1,000:1, 97.4 and 84% of the latex spheres were internalized, respectively. The dioxane-extraction method was not reproducible nor sufficiently sensitive for detection of solubilized polystyrene-latex at lower multiplicities.

Effects of pretreatment of EB on immediate cytoxocitiy and subsequent parasite survival in macrophages. The effects of heat, ultraviolet light, and homologous antibody pretreatment of EB on immediate cytotoxicity and subsequent parasite survival in macrophages are represented in Fig. 3. Pretreatment of EB (MOI, 100:1) with heat (56°C) for 5 and 30 min abrogated immediate cytotoxicity in macrophages, whereas ultraviolet light pretreatment of the EB (MOI, 100:1) did not prevent cytotoxicity. The amount of LDH released from macrophages infected with opsonized EB was the same at 6 h when compared with macrophages infected with untreated EB, but by 24 h there was a 50% reduction in the amount of LDH released.

Survival of EB (MOI, 1:1) in macrophages appeared to be prevented or significantly reduced by pretreatment of the EB with heat or homologous antibody, as monitored by late release of LDH (Fig. 3).

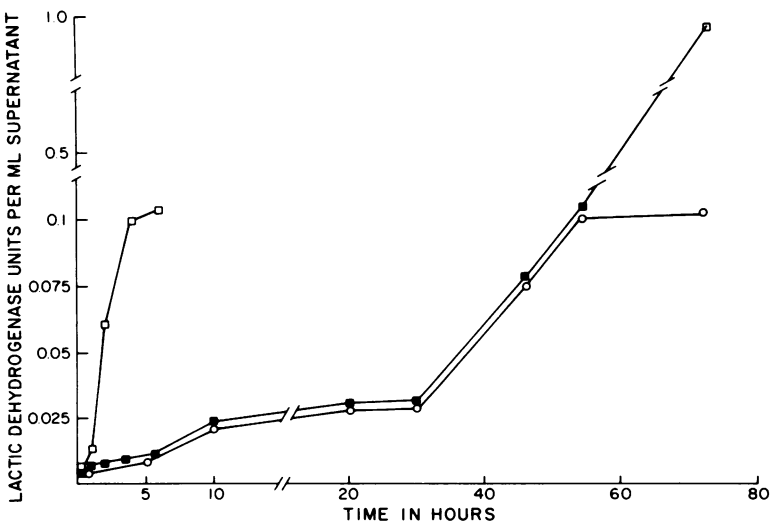

FIG. 1. *Release of LDH from resident mouse peritoneal macrophages infected in vitro with EB of C. psittaci at MOIs of 100:1 and 1:1. Each curve represents the mean values of four separate experiments. The results were almost identical when thioglycolate-stimulated macrophages were used. Symbols:* (□) *MOI, 100:1;* (■) *MOI, 1:1;* (○) *uninfected, control macrophages.*

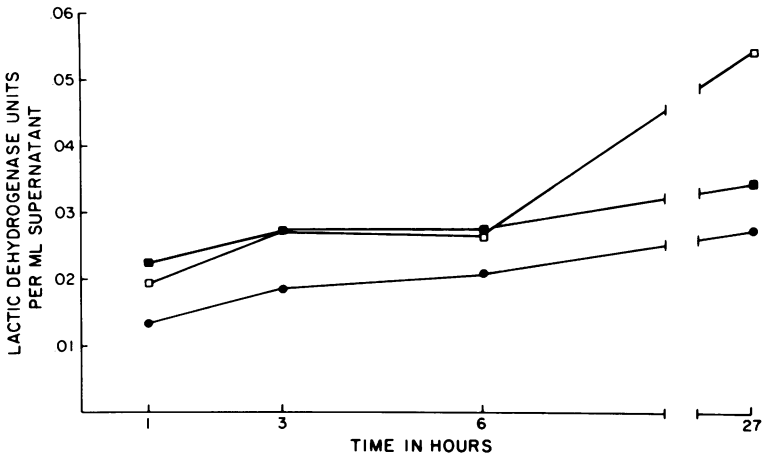

FIG. 2. *Release of LDH from thioglycolate-stimulated mouse peritoneal macrophages inoculated in vitro with 0.25-μm polystyrene-latex spheres at MOIs of 500:1 and 1,000:1. Each curve represents the mean values of two separate experiments. Symbols:* (●) *uninoculated control macrophages. Latex sphere-to-macrophage ratio,* (■) *500:1 and* (□) *1,000:1.*

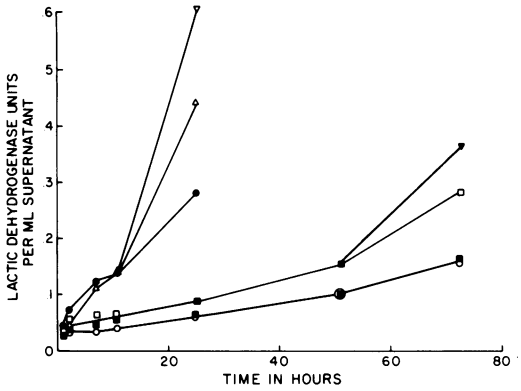

FIG. 3. *Release of LDH from thioglycolate-stimulated mouse peritoneal macrophages infected in vitro with EB of C. psittaci pretreated with heat (56°C for 5 and 30 min), ultraviolet light, and homologous hyperimmune serum. Each curve represents the mean values of a minimum of two separate experiments. The results were similar when resident, unstimulated macrophages were used. Symbols:* (▽) *live, untreated EB, MOI = 100:1;* (△), *ultraviolet light-treated EB, MOI = 100:1;* (●) *antibody-coated EB, MOI = 100:1;* (▼) *antibody-coated EB, MOI = 1:1,* (□) *5-min heat-treated EB, MOI = 100:1,* (■) *EB pretreated with heat for 5 or 30 min, MOI = 1:1, and for 30 min, MOI = 100:1; and* (○) *uninfected, control macrophages.*

Moulder et al. (10) reported that chloramphenicol succinate partially prevented cytoxicity in L-cells infected with various MOIs of *C. psittaci* EB, and the authors referred to the EB-induced lethality in the presence of the drug as "multiplication-independent death." A similar investigation in this laboratory was undertaken with macrophages, but due to irreproducible results in early experiments, pure chloramphenicol (100 μg/ml of culture medium) was substituted for chloramphenicol succinate. The LDH assay system was used. With chloramphenicol in the culture medium, stimulated macrophages, infected with EB at the high MOI, exhibited a 40 to 50% reduction in the amount of soluble cytoplasmic enzyme released at 6 h (Fig. 4). In contrast, with resident unstimulated macrophages, the presence of chloramphenicol did not affect the release of enzyme.

Uptake and intracellular fate of ^{3}H-labeled *C. psittaci* in macrophages. The uptake and intracellular fate of EB in both thioglycolate-stimulated and unstimulated mouse peritoneal macrophages were also monitored radioisotopically. A summary of the percent uptake of EB by macrophages is recorded in Table 1 and that of the intracellular fate, in Fig. 5. In general, the calculated uptake by macrophages of live, untreated EB was almost always greater than the number of calculated IFU in the inoculum. Pretreatment of EB with heat (56°C for 5, 10, and 20 min) increased the uptake, probably nonspecifically, as a result of increased stickiness of the outer envelope. Not surprisingly, opsonization of EB afforded maximum uptake of the infectious agents by the professional phagocytic cells.

Whereas heat treatment and opsonization provided increased uptake of *C. psittaci* in macrophages, these treatments appeared to contribute to increased destruction of the parasite in the host cell (Fig. 5). Trichloroacetic acid-precipitable counts in the macrophage pellet de-

creased with time with a concomitant increase in trichloroacetic acid-soluble counts in the macrophage pellet, followed by an increase in trichloroacetic acid-soluble counts in the supernatant fluid. The increased release of radioisotope from untreated EB at an MOI of 100:1 is probably the result of immediate cytotoxicity in the macrophage.

Another interesting difference between the two macrophage populations was in the apparent handling of untreated chlamydiae at an MOI of 1:1. This difference was consistent in each experiment, and the inoculum for both populations of macrophages came from the same purified preparation of EB. The retention of radioisotope from ^{3}H-labeled EB in the resident macrophages averaged 50% (Fig. 5B) or was, in effect, equivalent to the IFU titer. Therefore, the 50% release of radioisotope into the resident macrophage supernatant at 10 h might correspond to the 50% noninfectious particles present in the inoculum that were readily digested in phagolysosomes. Conversely, the retention of radioisotope from ^{3}H-labeled EB in thioglycolate-stimulated macrophages over a 10-h period averaged 95% (Fig. 5A). This latter population of macrophages was seemingly less efficient in processing the noninfectious EB in the inoculum. It should be pointed out that the presence of trichloroacetic acid-precipitable radioisotope in the pellet reflects only the integrity of the EB particle and cannot be equated with viability.

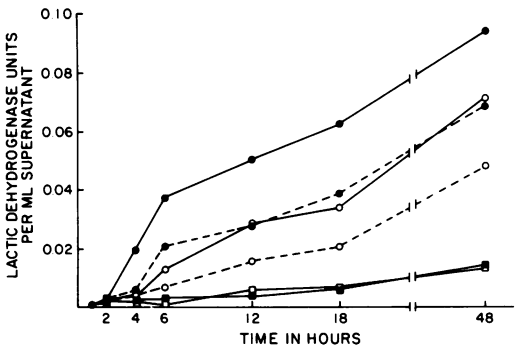

FIG. 4. *Release of LDH from thioglycolate-stimulated mouse peritoneal macrophages infected in vitro with pure chloramphenicol (100 μg/ml)-pretreated EB of C. psittaci. Chloramphenicol (CAP) was also incorporated in the monolayer medium for the duration of the experiment. Each curve represents the mean values of two separate experiments. Symbols:* (●——●) *live EB, MOI = 500:1;* (●- -●) *EB + CAP, MOI = 500:1;* (○——○) *live EB, MOI 200:1;* (○- - -○) *EB + CAP, MOI = 200:1;* (□) *uninfected, control macrophages;* (■) *uninfected, control macrophages + CAP.*

TABLE 1. *Uptake of C. psittaci by thioglycolate-stimulated and unstimulated mouse peritoneal macrophages*

% Uptake[a] (by macrophages)	Condition of EB (MOI)					
	Live		Heated		Opsonized	
	100:1	1:1	100:1	1:1	100:1	1:1
Stimulated	64.4	51.2	83.8	75.1	98.5	93.0
Avg	57.8		79.5		95.8	
Unstimulated	72.0	68.0	93.0	92.0	98.0	93.0
Avg	70.0		93.0		96.0	

[a] Percent ingestion of ^{3}H-labeled chlamydiae by macrophages is designated by the ratio of trichloroacetic acid-precipitable counts in the macrophage pellet at 45 min to trichloroacetic acid-precipitable counts in the zero-time inoculum, multiplied by 100.

DISCUSSION

It is well known that antibodies, both circulating and secretory, appear in response to infections with chlamydiae, but there seems to be no correlation between titer and resistance to infection. In fact, there is substantial evidence that such antibodies provide relatively little or no protection, since, in experimental animal models, relative protection can be overcome by increasing the inoculum concentration (11, 12). Both humoral and cell-mediated immunity operate (17); however, cell-mediated immunity is probably of primary importance. Many previous studies have demonstrated that cellular components, especially macrophage and lymphoid cells, play a decisive role in acquired resistance to facultative and obligate intracellular bacterial infections. We chose, therefore, to begin our investigation by examining in depth the interaction of chlamydiae with macrophages. The present studies provide data on the uptake and intracellular fate of *C. psittaci* in peritoneal macrophages obtained from nonimmune mice.

EB of *C. psittaci* are rapidly phagocytized by mouse macrophages. Maximum uptake by the professional phagocytic cells was obtained, however, with opsonized EB, since Fc receptors on the macrophage surface bind immunoglobulin G and trigger the internalization phase (13). Similar findings have been reported for other intracellular parasites, such as salmonellae (7), rickettsiae, (1, 2), and *Toxoplasma gondii* (8). In the case of EB receptor-epithelial receptor site interaction, however, specific antibody appears to block attachment (4). Intracellular presence of opsonized EB (MOI, 1:1) in thioglycolate-stimulated macrophages was reduced 23% in 10 h when compared with the presence of live, untreated EB at the same MOI. Previous studies

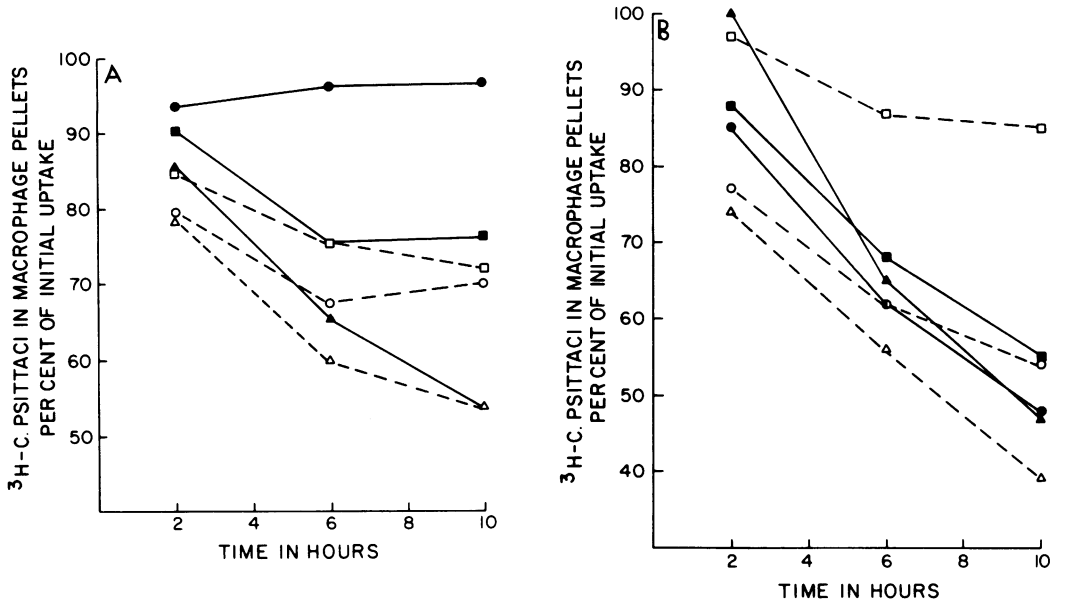

FIG. 5. *^{3}H-labeled C. psittaci EB remaining associated with the (A) thioglycolate-stimulated and (B) resident macrophage pellets after ingestion. The percent survival of ^{3}H-labeled chlamydiae in macrophages is designated as the ratio of trichloroacetic acid-precipitable counts in the macrophage pellet at 2, 6, and 10 h to trichloroacetic acid-precipitable counts in the macrophage pellet at 45 min, multiplied by 100. Each curve represents the mean values of three separate experiments. Symbols: (●) live EB, MOI = 1:1; (○) live EB, MOI = 100:1; (▲) heat-treated EB, MOI = 1:1; (△) heat-treated EB, MOI = 100:1; (■) antibody-coated EB, MOI = 1:1; (□) antibody-coated EB, MOI = 100:1.*

(18) demonstrated that some opsonized EB were clearly destroyed in phagolysosomes. This observation might account, in part, for the limited protection afforded vaccinated individuals by humoral antibody, although some presumably opsonized chlamydiae still manage to escape intracellular destruction.

We have now demonstrated, both by electron microscopic and biochemical studies, that optimal survival of the Cal 10 meningopneumonitis strain in macrophages requires live, untreated EB at an MOI of 1:1 or less. In contrast, if the macrophages are infected with EB at high MOIs (100:1), parasite intracellular survival is reduced as a result of cytotoxic damage occurring in the macrophage as early as 2 h postinoculation. This parasite-mediated damage could be completely abolished by heat pretreatment of EB and significantly reduced by indirect sensitization with anti-EB antibody. Similar results have been obtained by Gardner, using the 6BC strain of *C. psittaci* (M. Gardner, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, D6, p. 70). A similar phenomenon has also been reported in cultivated mouse peritoneal macrophages infected with *Shigella flexneri* (A. W. Pasculle and R. B.Yee, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, B41, p. 22); however, there are some differences between the two systems. Shigella-mediated cytotoxicity was also destroyed by prolonged ultraviolet irradiation, as well as polymyxin B pretreatment, of the bacteria, but was not affected by hyperimmune antiserum. Interestingly, outer envelope fragments isolated from shigella and added to the macrophage monolayers reportedly did not induce a cytotoxic response.

With two exceptions, the interaction of low and high EB MOIs with both resident and thioglycolate-stimulated macrophages was essentially the same. The two exceptions were: (i) the difference in macrophage cytotoxic response in the presence of chloramphenicol, and (ii) the apparent handling of live, untreated EB at an MOI of 1:1. The reason for these differences is not clear at this time. The results of the experiments using chloramphenicol with stimulated macrophages suggested that protein synthesis in chlamydiae is possibly linked to cytotoxicity, perhaps in the replacement of necessary constitutive surface components, and that the kinetics of cytotoxicity are dose and time dependent. The injection of intraperitoneal irritants, such as thioglycolate, results in the appearance of cells with functional and biochemical character-

istics of activated macrophages. These inflammatory macrophages are much more active than resident macrophages in adhering and spreading, pinocytic activity, and ingestion of opsonized substances. The enzyme content of the two cell populations is also different (5). Whereas both cells have been shown to be derived from circulating blood monocytes (16), there may even be a variation in the sequence of differentiation events that culminates either with the activated macrophage or with the resident macrophages. One approach that will be used to clear up these differences will be to isolate the chlamydiae-laden phagosomes or phagolysosomes and examine the resident chlamydiae as well as the vacuole contents and vacuole membranes.

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