INFECTION AND IMMUNITY, Oct. 1978, p. 200-208
0019-9567/78/0022-0200$02.00/0

Vol. 22, No. 1
Printed in U.S.A.

Penetration of Cultured Mouse Fibroblasts (L Cells) by *Rickettsia prowazeki*

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Received for publication 30 May 1978

The association of *Rickettsia prowazeki* with L cells was examined by using a novel radioactive assay in which [α-^{32}P]ATP-labeled rickettsiae were incubated with L-cell monolayers. Rickettsial association with the monolayer involved adherence and internalization steps that could be experimentally distinguished. Since *R. prowazeki* but not L cells possess an ATP-ADP obligate exchange transport system, addition of excess unlabeled ATP resulted in exchange of the labeled ATP from external, adherent rickettsiae but not from internalized rickettsiae. Rickettsial association was temperature dependent and was a linear function of both time and concentration. More than 90% of the biologically active rickettsiae associated with L cells was internalized. Rickettsial internalization required active participation of both rickettsiae and L cells; inactivation of either greatly reduced internalization. Rickettsial adherence to poisoned L cells was a saturable function of time and concentration. Adherence showed less temperature dependence than did internalization, but like rickettsial internalization, the extent of adherence was extremely low at 0°C. The rate and extent of adherence by inactivated and native rickettsiae to inactivated L cells were similar. Although inactive rickettsiae adhered to active and inactive L cells to a similar extent, inactive rickettsiae were internalized poorly by active L cells. These data form the basis for the hypothesis that *R. prowazeki* are internalized by the host cell through a process of "induced phagocytosis" and that inactivated rickettsiae adhere to the host cell differently from native rickettsiae, failing to trigger the endocytosis mechanism.

Rickettsia prowazeki is an obligate intracellular bacterium which parasitizes a variety of eucaryotic cells (5, 9, 19, 20, 24, 26-28). The mechanism by which *R. prowazeki* enters these cells is, however, not known. Cohn et al. (5) showed that penetration of *R. tsutsugamushi* into cultured cells required active participation of both the rickettsiae and the host; heat inactivation of either *R. tsutsugamushi* or cultured mouse lymphoblasts dramatically reduced the number of rickettsiae visualized within the lymphoblasts. Penetration was temperature dependent and was greatly enhanced by the addition of L-glutamic acid, the primary rickettsial energy source, to the incubation medium. Wisseman et al. (27) suggested that rickettsiae attach specifically to host cells before penetration since the presence of host cell membrane fragments inhibited the penetration of *R. prowazeki* into cultured chicken embryo fibroblasts.

Ramm and Winkler (14-16, 21, 23) characterized lysis of sheep erythrocytes by *R. prowazeki*. Rickettsial hemolysis, in which no productive infection occurs, may serve as a model system for host-parasite interactions. The lysis of an erythrocyte required experimentally distinguishable adsorptive and lytic steps. Adsorption was not passive, but was dependent upon the energy of the rickettsiae, most likely a proton motive force (21). Cholesterol was identified as being an integral part of the erythrocyte receptor to which *R. prowazeki* attached (16). The lytic step probably required participation of the erythrocyte since fluoride-treated erythrocytes were not lysed (15). It is not clear, however, that rickettsial hemolysis is an accurate model of invasion of a permissive host cell. Furthermore, the hemolytic system does not permit study of the internalization phase of rickettsial invasion, and the application of the lytic step of hemolysis to the parasitism of the host cell remains to be elucidated.

Previous studies concerning the penetration of permissive cells measured intracellular rickettsiae by light microscopy and did not distinguish between the attachment and the internal-

ization phases of invasion. In fact, there is no direct evidence that rickettsiae attach specifically to the host cell before they are internalized. The inability of light microscopic examination to adequately distinguish between intracellular and adherent rickettsiae necessitates development of a more exact assay system to accurately characterize the invasion process.

The present study examines the invasion of mouse fibroblasts (L cells) by *R. prowazeki* Madrid E strain, using a new radioactive assay system. Rickettsiae were labeled with [α-^{32}P]-ATP and incubated with L-cell monolayers. Because *R. prowazeki* possesses an ADP-ATP obligate exchange transport system (22), labeling was very efficient and there was no efflux of nucleotide from the labeled rickettsiae into the medium. External adherent rickettsiae were distinguished from intracellular rickettsiae by addition of excess unlabeled ATP to the incubation mixture. Because the L-cell membrane is impermeable to ATP, unlabeled ATP exchanged only with the labeled ATP of adherent rickettsiae. Various treatments were used to modify either the host cell or rickettsiae, and the effects of these agents on adherence and internalization of the rickettsiae were assessed.

MATERIALS AND METHODS

Rickettsial preparation and growth. *R. prowazeki* Madrid E strain was propagated in 6-day embryonated, antibiotic-free hen eggs by inoculation from seed pool (yolk sac passage no. 273). Rickettsial suspensions were prepared from heavily infected yolk sacs by a modification of the methods of Bovarnick and Snyder (3) and Wisseman et al. (25) as previously described (21). Only fresh unfrozen rickettsiae were used.

The diluent for the rickettsial inoculum and rickettsial suspension in the purification procedure was a sucrose-phosphate-glutamate (SPG) solution originally described by Bovarnick et al. (2). The diluent for sheep erythrocytes and for L-cell association experiments was SPGMgGlu (SPG containing 0.01 M $MgCl_2$ and 0.1% D-glucose).

Radioactively labeled rickettsiae were prepared by incubation of rickettsiae (8.0 g of original yolk sac equivalents per ml) with [α-^{32}P]ATP (50 μCi/ml, 5.7 Ci/mmol) in the presence of 25 M atractyloside. Under these conditions the rickettsiae contained approximately 4.3×10^{-4} cpm/rickettsia. Rickettsial suspensions were washed to remove extracellular [^{32}P]ATP and resuspended at the indicated concentration.

Cell culture. The L strain of mouse fibroblasts was used in these studies. L cells were cultured in an antibiotic-free growth medium consisting of medium 199 with modified Earle salts and 10% calf serum.

Hemolytic assay. The hemolysis tests were modifications of the method of Snyder et al. (18) as previously described (14–16).

Assessment of rickettsial adherence and internalization. To assess association of rickettsiae with L cells, [α-^{32}P]ATP-labeled rickettsiae in SPGMgGlu (1.5 ml) were incubated with confluent L-cell monolayers in 60-mm culture dishes from which the growth medium had been removed. At the indicated times, the inoculum was aspirated and the monolayer was washed five times with phosphate-buffered saline at the incubation temperature to remove nonassociated rickettsiae. To distinguish intracellular, internalized from external, adherent rickettsiae, 1 mM unlabeled ATP was added to samples 2 min (5 min in those experiments with inactivated rickettsiae and a low temperature) before removal of the inoculum from the monolayer. Previous studies (22) showed that the addition of excess unlabeled ATP results in a complete loss of label from rickettsiae within 1 min. Of the total ATP-labeled rickettsiae associated with the cultured cells, that fraction in which the label was chased represents adherent rickettsiae which were accessible to the unlabeled ATP, whereas that fraction in which the label remained associated after the chase reflects internalized rickettsiae which were inaccessible to the unlabeled ATP due to impermeability of the host cell membrane. Washed L-cell monolayers were extracted with 2 ml of 1% Triton X-100 for 30 min at 37°C, 1.5 ml of each sample was mixed with 5 ml of Aquasol, and radioactivity was determined in a Packard Tri-Carb scintillation counter. Loss of [α-^{32}P]ATP from rickettsiae during incubation was assessed by determination of total and intracellular radioactivity in the inoculum before and after incubation with L cells. To estimate background ^{32}P taken up by L cells, the monolayer was incubated with [α-^{32}P]ATP as described above, but without rickettsiae. The background ^{32}P uptake in each sample was negligible.

L cells were enumerated by direct counting in a Levy Ultraplane counting chamber. Rickettsiae were counted by a modification of the method of Silberman and Fiset (17). Protein was determined by the method of Lowry et al. (11).

Rickettsial treatments. Rickettsiae were inactivated by exposure to UV radiation (8-W germicidal lamp) for 20 min at 0°C and a distance of 12 cm or by treatment for 30 min at 0°C with 1 mM KCN or 3% formaldehyde. Rickettsiae treated with KCN or formaldehyde were washed twice before being used in any experiment. The ability of treated rickettsiae to lyse sheep erythrocytes (14–16) was used as an indicator of rickettsial activity.

L-cell treatments. L cells were inactivated by incubation with 1 mM *N*-ethylmaleimide (NEM) at 37°C; unreacted NEM was neutralized by addition of 10 mM 2-mercaptoethanol. Other treatments included: 40 mM NaF, 1 and 10 μg of cytochalasin B per ml, 1 mM *p*-chloromercuriphenylsulfonic acid (PCMBS), 5 mM colchicine, and 10 and 20 mM NaN_3. All treatments were for 30 min at 37°C. The L-cell monolayers were washed before use except as indicated. The uptake and incorporation of [^{3}H]leucine (0.5 mCi/ml, 58 Ci/mmol) by L cells were measured to assess the effect of treatments on L-cell metabolic activity.

Materials. [α-^{32}P]ATP was obtained from Amersham/Searle, and [^{3}H]leucine was obtained from Schwarz/Mann. Other chemicals and their sources

were: NEM, Schwarz/Mann; PCMBS, colchicine, atractyloside, and ATP, Sigma Chemical Co.; 2-mercaptoethanol, cytochalasin B, and *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid, Calbiochem; and Triton X-100, New England Nuclear Corp.

RESULTS

Association of rickettsiae with L cells. Studies were performed to determine the optimal medium for association of *R. prowazeki* with L cells. Because the incubation medium must be compatible with both rickettsial and L-cell activity, a standard rickettsial suspension medium (SPGMg) was combined with various constituents of L-cell growth medium (Fig. 1). Rickettsiae were not stable in medium 199 alone. Addition of calf serum to SPGMg inhibited the association of rickettsiae with L cells approximately 75%; the inhibitory activity was not removed by dialyzing the calf serum against phosphate-buffered saline. In contrast, bovine serum albumin and dialyzed fetal calf serum were not significantly inhibitory. Neither the addition of vitamins and amino acids or various combinations of $CaCl_2$, NaCl, or KCl to SPGMg nor the substitution of brain heart infusion enhanced rickettsial association above the levels shown in Fig. 1 (data not shown). SPGMg, with glucose added to provide the L cells with an energy source, was the incubation medium in all subsequent experiments.

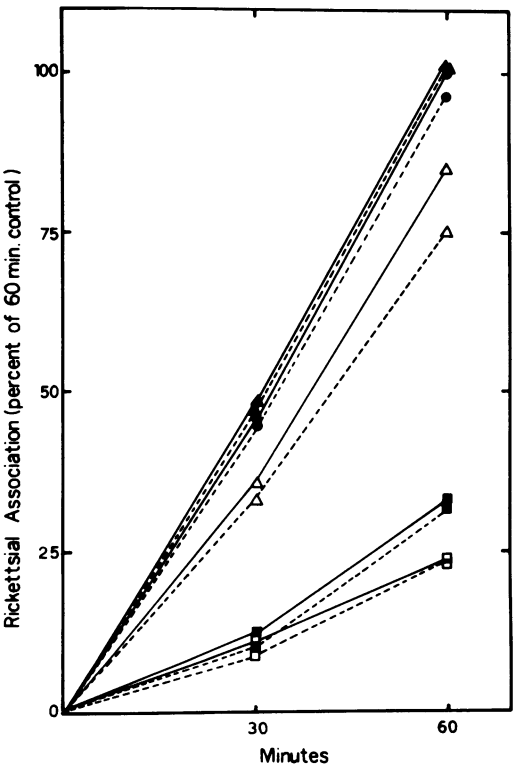

FIG. 1. *Effect of medium composition on rickettsial association and internalization. [α-^{32}P]ATP-labeled rickettsiae at an MOI of 200 were suspended in SPGMg containing the following additions: none (●), 10% calf serum (□), 10% dialyzed calf serum (■), 10% dialyzed fetal calf serum (△), and 7% bovine serum albumin (▲). Solid lines indicate total L-cell-associated rickettsiae, and dashed lines indicate internalized rickettsiae. Results are expressed as the percentage of total L-cell-associated rickettsiae in SPGMg alone at 60 min.*

Rickettsial association with L cells varied directly with multiplicities of infection (MOI), with little evidence of saturation up to a ratio of rickettsiae to L cells of 750:1 (Fig. 2). At this ratio approximately 14 rickettsiae were associated with each L cell after 30 min. This was similar to previous reports that the association of *R. tsutsugamushi* (5), *R. rickettsii* (24), and *R. prowazeki* (27) varied directly with MOI.

Association of *R. prowazeki* with L cells increased linearly for 120 min at 37°C (Fig. 3). More than 90% of the *R. prowazeki* associated with L cells at each time point were inaccessible to the chase with unlabeled ATP and hence were internalized. This indicated that if penetration of L cells, like rickettsial hemolysis (14–16, 21, 23), was a multistep process, internalization occurred more rapidly than did adherence.

Effect of rickettsial viability. Inactivation of rickettsiae, which reduced their hemolytic activity to near zero, inhibited rickettsial association with L cells by 80 to 90% (Table 1). Of the few inactivated rickettsiae associated with L

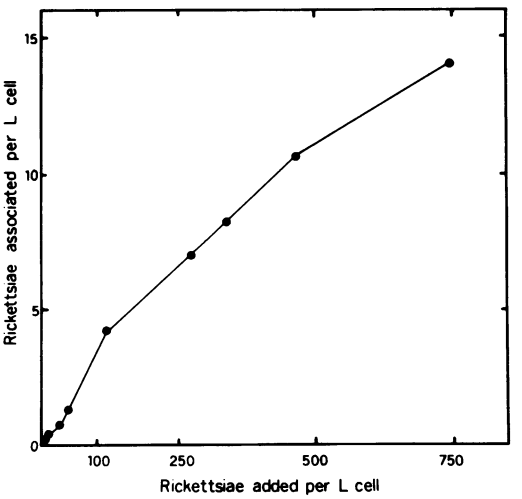

FIG. 2. *Effect of MOI on rickettsial association with L cells. [α-^{32}P]ATP-labeled rickettsiae were incubated with L-cell monolayers at the indicated MOI for 30 min at 37°C as described in the text.*

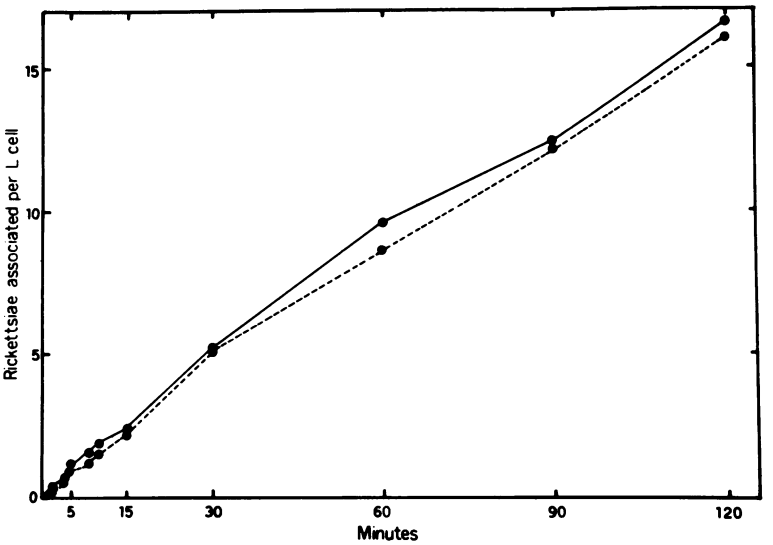

FIG. 3. *Effect of length of incubation time on rickettsial association and internalization. [α-^{32}P]ATP-labeled rickettsiae at an MOI of 180 were incubated with L-cell monolayers at 37°C for the indicated times. The solid line indicates total L-cell-associated rickettsiae, and the dashed line indicates internalized rickettsiae.*

TABLE 1. *Effect of inactivation on rickettsial association and internalization*

Treatment	HE[a]	Rass[b]	Rin[c]	% Adh[d]
None	(100)	(100)	92	8
UV	3	11	9	18
Formaldehyde	1	18	11	39
KCN	0	9	7	22

[a] Rickettsial hemolysis. Results are presented as a percentage of the control rickettsial hemolysis.

[b] L-cell-associated rickettsiae. Results are expressed as a percentage of total L-cell-associated native rickettsiae at 60 min and 37°C.

[c] Internalized rickettsiae. Results are expressed as a percentage of total L-cell-associated native rickettsiae at 60 min and 37°C.

[d] Percent adherence. Results are expressed as the percentage of associated rickettsiae in each sample present as adherent rickettsiae.

cells, a significant fraction was now found as adherent rickettsiae. This indicates that internalization, like rickettsial hemolysis, requires active rickettsiae and is consistent with the report of Cohn et al. (5) that inactivated *R. tsutsugamushi* adhered to cultured lymphoblasts but failed to penetrate.

Effects of L-cell treatment. To determine the role of the host cell in rickettsial adherence and internalization, rickettsiae were incubated with poisoned L cells (Table 2). Rickettsial internalization was inhibited to a similar extent by treatment of L cells with a metabolic inhibitor (NaF), thiol reagents (NEM, PCMBS), or a microfilament and glucose transport inhibitor (cytochalasin B). Colchicine (microtubule inhibitor) and NaN_3 inhibited internalization (and leucine uptake) poorly or not at all. These data indicate that the host cell plays an active role in rickettsial internalization. Because most of the poisoned L-cell-associated rickettsiae were located on the outside of the cell, a convenient method was provided for studying rickettsial adherence in the absence of significant internalization.

TABLE 2. *Effect of L-cell treatments on association and internalization of R. prowazeki*

Treatment	Associated rickettsiae[a,b]	Internalized rickettsiae[a,b]	[^{3}H]Leu uptake
None (30)[c]	100	92 ± 1	100
NEM (19)	22 ± 2	5 ± 1	5
PCMBS (6)	19 ± 3	8 ± 1	6
NaF (5)	42 ± 4	19 ± 5	27
Cytochalasin B			
1 μg/ml (1)	34	19	61
10 μg/ml (3)	25 ± 7	10 ± 2	39
Colchicine	99	99	80
NaN_3			
10 mM (1)	97	74	139
20 mM (1)	80	63	103

[a] Expressed as a percentage of total associated control rickettsiae at 30 min and 37°C.

[b] Mean ± standard error of mean.

[c] Number of determinations is indicated within parentheses.

Studies on rickettsial adherence. Adher-

ence of rickettsiae to NEM-treated L cells was concentration dependent, with maximum adherence at 60 min occurring between an MOI of 400 and 600 (Fig. 4). At this concentration there were approximately three adherent native rickettsiae per NEM-treated L cell. Inactivated and native rickettsiae adhered to NEM-treated L cells similarly as a function of time (Fig. 5). This similarity between active and inactive rickettsiae in their capacity to adhere was in sharp contrast to their relative abilities to penetrate L cells (Table 1). It appears, then, that whereas adherence was independent of rickettsial and host cell viability, maximum internalization required active participation of both the rickettsia and the L cell. However, this requirement for rickettsial viability suggested that inactive rickettsiae may adhere to a different L-cell site or may bind to the same site in a different fashion than do their viable counterparts. If either hypothesis is correct, inactive rickettsiae would not be expected to competitively inhibit the adherence of viable rickettsiae to the same degree as their viable counterparts. To test this hypothesis, excess unlabeled active or inactive rickettsiae (3.8×10^9) were incubated with [^{32}P]ATP-labeled native rickettsiae (9.0×10^8) and L cells (4.0×10^6) for 15 and 30 min at 37°C. Whereas excess unlabeled active rickettsiae did, indeed, significantly inhibit the association of [^{32}P]ATP-labeled rickettsiae (Fig. 6), the same number of unlabeled inactivated rickettsiae was a poor competitive inhibitor. This suggests that the manner of adherence of inactive rickettsiae to L cells differs from that of native rickettsiae.

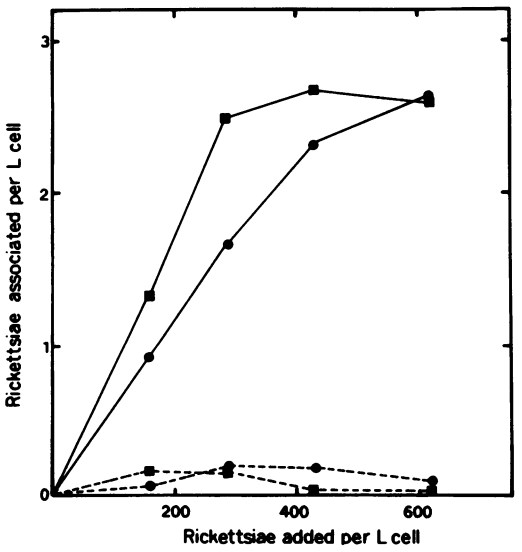

FIG. 4. *Effect of MOI on rickettsial adherence to NEM-treated L cells. [α-^{32}P]ATP-labeled rickettsiae were incubated with NEM-treated L cells for 30 (●) or 60 (■) min as described in the text. Solid lines indicate total L-cell-associated rickettsiae, and dashed lines indicate internalized rickettsiae.*

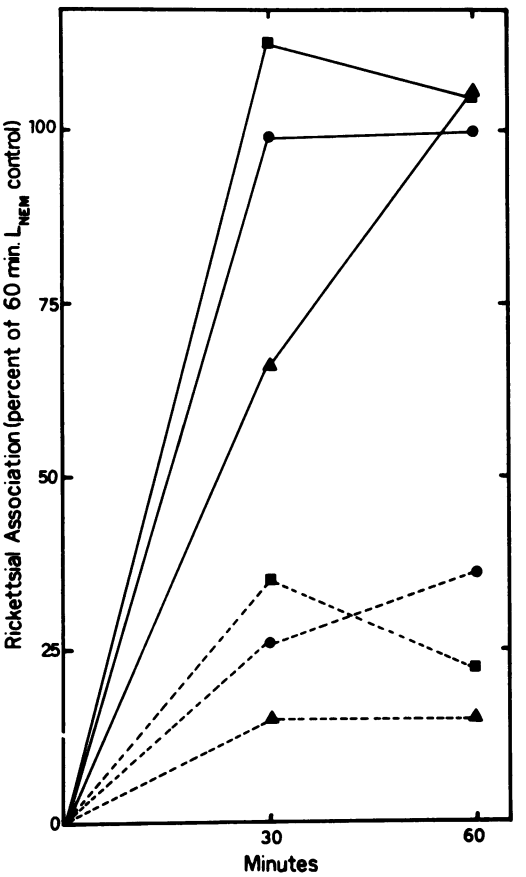

FIG. 5. *Effect of rickettsial inactivation on rickettsial association with NEM-treated L cells. Native (●) and UV (■)- or Formalin (▲)-inactivated rickettsiae were incubated with NEM-treated L cells at 37°C. Solid lines indicate total associated rickettsiae, and dashed lines indicate internalized rickettsiae. Results are expressed as the percentage of total inactivated L-cell-associated native rickettsiae at 60 min.*

Effects of temperature on rickettsial adherence and internalization. Rickettsial suspensions were incubated with L-cell monolayers at the temperatures indicated for 60 min (Table 3), and adherent and internalized rickettsiae were determined. Internalization of native rickettsiae by active L cells was temperature dependent and increased linearly for 60 min when incubated at or above 12°C. At each decreased incubation temperature, the ratio of adherent to internalized rickettsiae increased dramatically; whereas the ratio was 0.1 at 36°C, the ratio was

1.4 at 12°C. This supports the hypothesis that rickettsiae adhere to host cells as part of the invasion process and indicates that internalization is more sensitive to changes in temperature than is adherence. Indeed, adherence of native rickettsiae to inactivated L cells was much less sensitive to temperature effects than was rickettsial internalization. Whereas internalization at 12°C was only 5% of that seen at 36°C, adherence to inactivated L cells at 12°C was 60% of the value obtained at 36°C.

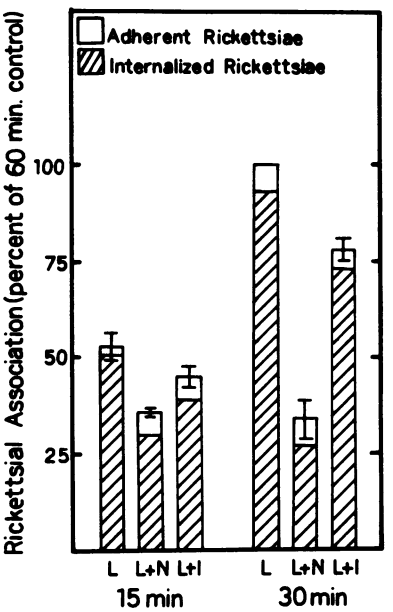

FIG. 6. *Inhibition of rickettsial association and internalization by addition of excess unlabeled rickettsiae. [α-^{32}P]ATP-labeled rickettsiae (9 × 10^6, MOI of 200) were incubated with L cells alone (L) or with L cells plus excess (3.8 × 10^9) unlabeled native (L+N) or inactivated (L+I) rickettsiae. Results are expressed as the percentage of total L-cell-associated control rickettsiae (L) at 30 min ± the standard error of the mean.*

Inactivated rickettsiae were internalized poorly relative to their viable counterparts at all temperatures. As with native rickettsiae, the internalization of inactive rickettsiae was temperature dependent. At each temperature, however, a greater proportion of inactivated rickettsiae was located on the outside of the cell (ratio of adherent to internalized rickettsiae) than was seen with active rickettsiae.

At 0°C, rickettsial adherence and internalization occurred at very low rates. A similar reduction in adherence capacity at low temperature was reported for the intracellular pathogen *Chlamydia psittaci* (8), as well as for the adsorption of *R. prowazeki* to erythrocytes (14).

DISCUSSION

We have utilized a new, radioactive assay to characterize the invasion of L cells by *R. prowazeki*. This assay offers several advantages over the microscopic assays previously used (5, 24, 26, 28) in studies on rickettsial invasiveness. Microscopic assessment of rickettsial invasion into cultured cells is tedious, requiring subjective examination of hundreds of individual host cells to insure reliability. In contrast, our assay was rapid and sensitive; fewer than one intracellular or adherent rickettsia per 20 L cells could be accurately and objectively enumerated. Furthermore, because essentially all of the label from extracellular rickettsiae could be chased out within 60 s, the number of intracellular rickettsiae at any given moment could be accurately determined.

TABLE 3. *Effect of incubation temperature on rickettsial adherence and internalization*

Temp (°C)	Incubation mixture[a]											
	Active R, active L				Inactive R, active L				Active R, inactive L			
	Assoc[b]	Adh[c]	In[d]	Rad/Rin[e]	Assoc[b]	Adh[c]	In[d]	Rad/Rin[e]	Assoc[b]	Adh[c]	In[d]	Rad/Rin[e]
36	(100)	8	92	0.1	10	3	7	0.4	12	10	2	5.0
23	58	10	48	0.2	5	2	3	0.7	9	7	2	3.5
12	12	7	5	1.4	3	2	1	2.0	7	6	1	6.0
0	3[f]				1[f]				2[f]			

[a] Active or inactive rickettsiae (R) were incubated with active or inactive L cells (L) as indicated for 60 min at 37°C.

[b] Total associated rickettsiae. All results are expressed as a percentage of total active L-cell-associated native rickettsiae at 37°C and 60 min.

[c] Adherent rickettsiae.

[d] Internalized rickettsiae.

[e] Ratio of adherent rickettsiae to internalized rickettsiae.

[f] Adherent rickettsiae could not be distinguished from internalized rickettsiae at 0°C.

We have shown that the internalization of *R. prowazeki* into L cells required active participation of both the rickettsia and the host cell. This is consistent with the report of Cohn et al. (5) that inactivation of either rickettsiae or host cells blocked the penetration of *R. tsutsugamushi* into cultured cells. This requirement for host cell participation strongly suggests that the mode of internalization involved phagocytosis. It is apparent, however, that more than normal phagocytosis is involved. Although native and inactivated rickettsiae adhered equally to inactivated L cells, only native rickettsiae were rapidly internalized by active L cells. This suggests that rickettsiae were internalized through a process of "induced phagocytosis." That is, the adherence of a native rickettsia to a specific host cell site signals the host cell to specifically and rapidly phagocytize that rickettsia. Because the presence of excess inactivated rickettsiae inhibited the invasion of L cells by native *R. prowazeki* only slightly, it appears that the adherence of inactivated rickettsiae differed sufficiently from that of their viable counterparts as to fail to trigger the phagocytic mechanism; the exact nature of this difference is not known.

Induced phagocytosis was previously proposed as the mechanism by which *C. psittaci* (4) and *Toxoplasma gondii* (10) enter host cells. The fact that rickettsiae, unlike chlamydiae and *T. gondii,* are not found in phagosomes does not establish that they enter the cells by a different mechanism. It may only indicate that rickettsiae go a step further and rupture the phagosome during or shortly after internalization. We suggest that they may escape via the same lytic mechanism used to lyse erythrocytes.

Cohn et al. (5), in their classic study on invasion of cultured mouse lymphoblasts by *R. tsutsugamushi,* reported that rickettsial "penetration" was linear for only 15 min and was stimulated in the concurrent presence of NaCl, KCl, and $CaCl_2$. In contrast, we found that the rate of *R. prowazeki* internalization by L cells was linear for 120 min at 37°C and that the addition of any combination of KCl, NaCl, and $CaCl_2$ to the standard incubation medium was not stimulatory. Our data compare favorably with the report of Wisseman et al. (24) that the invasion of chicken embryo cells by *R. rickettsii* increased linearly for 180 min at 37°C. Some caution must be exercised in drawing comparisons between the results of Cohn et al. (5) and those reported herein since their results varied greatly with the incubation medium employed. For instance, *R. tsutsugamushi* incubated with cultured lymphoblasts at 4°C were internalized 53% (balanced salt solution), 35% ("complete medium"), or 17% (balanced salt solution plus L-glutamate) as well as those incubated at 37°C. In comparison, *R. prowazeki* suspended in SPGMgGlu were internalized 5% as well at 0°C as those incubated at 37°C. Direct comparison of our results and those of Cohn et al. may also be precluded by the numerous technical differences between the two studies. Cohn et al. (5) examined the internalization of low multiplicities (MOI of 5 and 7) of thawed, semipurified *R. tsutsugamushi* by mouse lymphoblasts in tumbled suspensions; our study examined the adherence and internalization of high multiplicities (MOI of >150) of fresh, purified *R. prowazeki* by L-cell monolayers.

Ramm and Winkler (14–16, 21, 23) characterized the hemolysis of sheep erythrocytes by *R. prowazeki,* which was similar in several respects to rickettsial association with L cells. Both processes involved adherence to the target cell surface (14). We were unable to demonstrate directly that rickettsial adherence to the L cell must lead to internalization since treatments which inhibited the internalization of rickettsiae could not be rapidly reversed, and extracellular rickettsiae were not sufficiently stable at 37 or 23°C to conduct experiments for extended durations. Circumstantial evidence, however, indicates that adherence is part of the invasion process. When rickettsiae were incubated with native L cells at temperatures below 37°C, a significant percentage of the L-cell-associated rickettsiae was adherent to the L-cell surface; at each successive lower incubation temperature, the ratio of adherent to internalized rickettsiae increased dramatically. This was presumably because the L-cell component of internalization (phagocytosis) was more temperature dependent than was the rickettsial component (adherence). Accordingly, the temperature dependence of adherence of *R. prowazeki* to inactivated L cells, which was similar to that reported for adsorption of rickettsiae to erythrocytes (14), was much less than that for rickettsial internalization. Furthermore, Wisseman et al. (27) reported that the presence of membrane fragments inhibited the invasion of chicken embryo cells by *R. prowazeki.* These data strongly suggest that, as with rickettsial hemolysis, rickettsiae adhere specifically to the L-cell membrane as part of the invasion process. Although the relationship of the lytic step to invasion remains to be determined, it is interesting that NaF was a potent inhibitor both of lysis (15) and of L-cell internalization, whereas rickettsial adherence to erythrocytes and L cells occurred in the presence of NaF. Inactivation of rickettsiae, however, reduced adsorption of rickettsiae to erythrocytes

to near zero. This contrasts with the relative abilities of native and inactivated rickettsiae to adhere to L cells and indicates that rickettsial hemolysis and L-cell invasion differ in important respects.

It is interesting that, at saturation, there were only three adherent rickettsiae per inactivated L cell. In contrast, similar studies with mucosal parasites such as *Neisseria gonorrhoeae* (12), group A streptococci (1, 7), and *Escherichia coli* (6) have reported between 75 and 200 bacteria per host cell. This tremendous contrast in adsorptive capacity may reflect fundamental differences between intracellular and extracellular parasitism. Rickettsiae need only to adhere at a rate which approximates the rate of internalization. Extracellular mucosal parasites, on the other hand, must adhere rapidly to avoid elimination via desquamation and fluid flow across the mucosal surface.

These data, along with those of Cohn et al. (5) and Wisseman et al. (24, 26-28), allow the beginnings of a model of rickettsial invasion of host cells. Our results suggest that each native rickettsia adheres specifically to the target cell surface, where it triggers the host endocytic mechanism, and the adherent rickettsia is rapidly phagocytized. Inactive rickettsiae adhere to the host cell membrane but fail to induce rapid phagocytosis and are internalized slowly as are other inanimate particles (13). Once internalized, the native rickettsia rapidly escapes from the phagocytic vacuole; rickettsiae are not seen within target cell phagosomes. Although an L cell parallel to erythrocyte lysis has not been described, this process may be the mechanism by which the rickettsia escapes from the phagocytic vacuole and avoids the lysosomal response.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grant AI-10535 from the National Institute of Allergy and Infectious Diseases. T.S.W. was supported by Public Health Service National Research Award CA09109 from the National Cancer Institute.

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