

INTRACELLULAR LOCALIZATION OF *RICKETTSIA*
TSUTSUGAMUSHI IN POLYMORPHONUCLEAR LEUKOCYTES*

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Because rickettsiae proliferate exclusively in the cytoplasm of the host cells, rickettsial invasion into the host cell cytoplasm is an essential step in rickettsial infections. Although this process is incompletely understood, two possible mechanisms that have been suggested are: the phagocytosis of the rickettsiae by the host cells followed by their escape from the phagosomes (1) or the direct penetration of the rickettsiae through the plasma membrane of the host cells (2, 3). Light microscopical studies on the uptake of the rickettsiae cannot distinguish the phagosome-confined rickettsiae from those directly in the host cell cytoplasm as a result of resolution limitations. To date, only a few ultrastructural studies in rickettsial entry into host cells have been reported. Andrese and Wisseman (3) found that human peripheral macrophages would take up *Rickettsia mooseri* and found them in phagosomes and in the cytoplasm. However, in their brief report, the process was not described in detail. A recent study by Ewing et al. (4) illustrated the apparent *in vivo* infection cycle of *R. tsutsugamushi* in mouse peritoneal mesothelial cells. Budding rickettsiae from infected cells were phagocytized by other mesothelial cells while still enclosed in the original host cell membrane. These enveloped rickettsiae were then observed to escape from the phagosome into the new host cell cytoplasm.

The present study takes advantage of the active and abundant phagocytosis of *R. tsutsugamushi* by polymorphonuclear leukocytes (PMN) and describes the ultrastructural features of rickettsial release and localization in the host cell cytoplasm.

Materials and Methods

Rickettsial Seed Suspensions. The stock of *R. tsutsugamushi* (Gilliam strain) used in this study was obtained from the Walter Reed Army Institute of Research Washington, D.C. The rickettsiae had been passed 131 times in yolk sacs in a sucrose-potassium glutamate solution (5). This seed material was passed several times in the specific pathogen-free yolk sac of 6- to 7-d embryonated chicken eggs (SPAFAS, Norwich, Conn.) in our laboratory. A 50% suspension of infected yolk sacs was distributed to vials which were quick-frozen in a dry-ice alcohol mixture and stored at -70°C . These seed samples were thawed as needed and used for infecting cultured cells.

Rickettsial Culture. *R. tsutsugamushi* was propagated serially in monolayers of BHK-21 clone 13 (Lister Institute, London) cells in Falcon (Falcon Labware, Div. of Becton, Dickinson, & Co., Oxnard, Calif.) tissue culture flasks (growth area 75 cm^2). They were cultivated in modified Eagle's minimum essential medium with Hank's balanced salt solutions (Grand Island Biological Co., Grand Island, N. Y.) containing 10% heat-inactivated fetal calf serum (Microbiological Associates, Walkersville, Md.) and $50\text{ }\mu\text{g/ml}$ streptomycin. The BHK-21 cells were inoculated with a stock culture of *R. tsutsugamushi* harvested from chick yolk sacs. After the population of

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rickettsiae reached a plateau (6–8 d as determined by Giemsa and Gram stains) the cells were incubated with 0.25% trypsin (Microbiological Associates) at 35°C for 5 min, and harvested by centrifugation at 500 *g* for 3 min. Aliquots of the infected cells were used to inoculate the serial passages in BHK-21 cells. Infected BHK-21 cells were detached from the culture flask with a rubber policeman, and soon thereafter interacted with the leukocytes.

Polymorphonuclear Leukocytes. Polymorphonuclear leukocytes were obtained from 350 to 400 g guinea pigs (Hartley Strain) after intraperitoneal inoculation of 2.4 g sodium caseinate (Tokyo, Kasei, Ltd., Toyoshima, Tokyo) as described by Sbarra and Karnovsky (6). After 14 h, peritoneal fluid which contained 4×10^8 to 10^9 cells in 20 ml of fluid was withdrawn and the PMN were harvested and washed three times in ice-cold 0.85% NaCl by centrifugation at 500 *g* for 3 min at 4°C and filtered through nylon mesh to remove large contaminants. Before incubation with rickettsiae, the PMN were washed once with the incubation medium at 4°C.

Incubation of PMN with Rickettsiae. A suspension of 3×10^6 infected BHK-21 cells in ≈ 200 μ l of the modified rubella medium was incubated with 1×10^7 guinea pig peritoneal PMN at 35°C for 30 min before fixation for electron microscopy. The incubation medium was a modification of the rubella medium (Flow Laboratories, Inc., Rockville, Md.). The basic component used was Eagle's minimum essential medium made up with Earle's balanced salt solution (Microbiological Associates). This solution was enriched twofold with nonessential amino acids and the vitamin mixture and 0.2 mM L-glutamine, all from the same source. In addition, the medium contained 10% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.), 10% inactivated fetal bovine serum (Microbiological Associates), and 0.3% glucose (Fisher Scientific Co., Pittsburgh, Pa.). To complete the medium, 50 μ g/ml of streptomycin (Eli Lilly & Co., Indianapolis, Ind.) was added.

Electron Microscopy. The specimens were fixed in a solution containing 1.2% wt/vol formaldehyde, 2.5% wt/vol glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.), and 0.03% wt/vol trinitrocresol in 0.05 M cacodylate buffer (pH 7.4), and 0.03% wt/vol CaCl_2 (7) at room temperature for 1 h or at 4°C overnight. The cells were washed three times in the 0.2 M cacodylate buffer pH 7.4, fixed in 1% wt/vol OsO_4 in 1.5% wt/vol potassium ferrocyanide (8) for 1 h at room temperature. This treatment preserves the glycogen granules in the PMN which are lost if conventional buffered osmium fixation is used. Samples were treated with propylene oxide and embedded in a low viscosity epon mixture of 1,2,7,8-diepoxyoctane (Aldrich Chemical Co., Milwaukee, Wis.) and nonenyl succinic anhydride (Electron Microscopy Sciences, Fort Washington, Pa.) (9). Ultrathin sections (60–90 nm) were cut on a Porter-Blum MT-1 or MT-2 Ultramicrotome (Ivan Sorvall, Inc. Norwalk, Conn.) with a diamond knife, picked up on uncoated copper grids, and stained with a saturated solution of uranyl acetate diluted with equal parts of acetone and then with lead citrate. The specimens were examined in a JEOL 100B or 100S electron microscope operated at 80 kV.

Results

Uptake of Rickettsiae by Polymorphonuclear Leukocytes. When heavily infected BHK-21 cells are examined by electron microscopy, most of the cultured cells contain a few rickettsiae and some have many. Intracellular rickettsiae are located without specific localization in the cytoplasm of BHK-21 cells but in some cells rickettsiae bulged or protruded from the surface. Truly extracellular rickettsiae enveloped in BHK-21 cell membrane were not apparent. No ultrastructural evidence of direct egress or entry of *R. tsutsugamushi* through the BHK-21 cell plasma membrane was found in this study.

Infected BHK-21 cells centrifuged at low speed formed pellets which contained infected host cells, partially ruptured cells with rickettsiae, and extracellular rickettsiae in various stages of degeneration. When these infected BHK-21 cell preparations were incubated in a small volume of medium with the PMN for 30 min, many of the PMN were found to have taken up few or no organisms whereas some contained several rickettsiae per sectioned cell profile. About one-half of the rickettsiae in the PMN were still within phagosomes and the remainder appear to have escaped from the phagosome into the host cell cytoplasm (Fig. 1). Although rickettsiae in phagosomes

and others free in the cytoplasm could be found within the same host cell, it was more common to find rickettsiae within a particular PMN to be localized either in the phagosome or in the cytoplasm. Another characteristic feature of rickettsial phagocytosis is that intact, apparently viable rickettsiae were taken up singly in phagosomes, whereas partially lysed, degenerating rickettsiae were phagocytized in clusters. The phagocytosis of rickettsiae appears to be selective because nonrickettsial debris was excluded from the rickettsia-containing phagosome and there was an apparent preferential uptake of rickettsiae because more rickettsiae than cell debris were found in the PMN (Fig. 1).

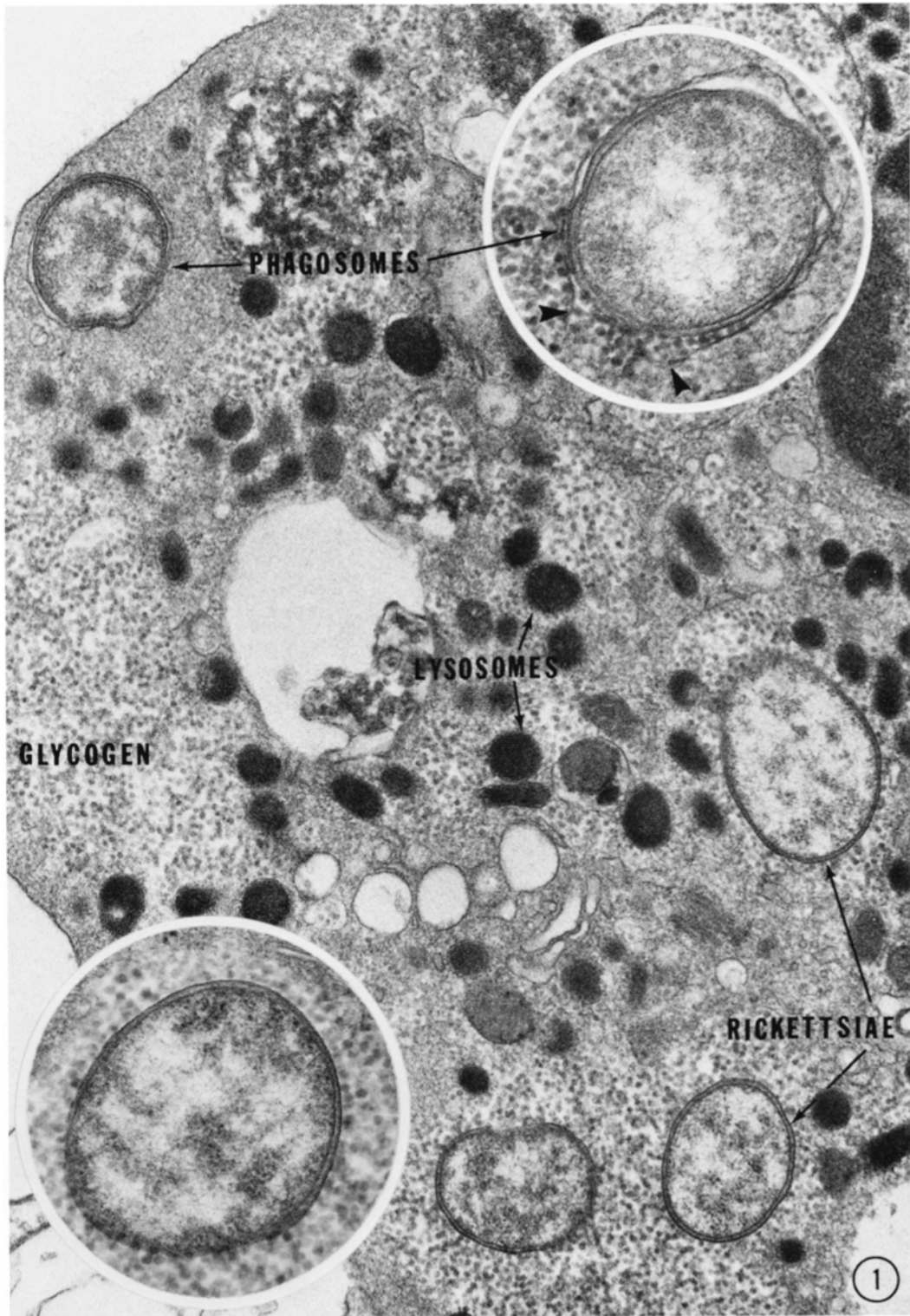
The focal localization of rickettsiae within the PMN is characteristic and fairly consistent. Phagosomes containing rickettsiae were usually found in the ectoplasmic filamentous areas containing vacuoles and lysosomes (Fig. 1). Mitochondria, endoplasmic reticulum, and ribosomes are rare in these cells. Near the cell center with the Golgi complex and centrioles, free rickettsiae in glycogen areas were observed frequently. Guinea pig PMN contain many glycogen granules which are located in pools and it is in these regions where the extraphagosomal rickettsiae are preferentially localized. These glycogen-rich areas are devoid of other cytoplasmic structures and in routine preparations appear as empty spaces as a result of the dissolution of the glycogen during specimen preparation. In such material the rickettsiae are found in clear, vacuole-like spaces which are not segregated by a membrane from the rest of the cytoplasm. With appropriate fixation techniques, the glycogen granules are preserved as characteristic particles $\cong 40$ nm in diameter. The free rickettsiae in the glycogen areas retained their bacillary shape and contained a normal array of fine DNA filaments and ribosomes. The association of glycogen particles to the surface of the rickettsial cell wall was very close and virtually no recognizable space between the outer surface of the rickettsiae and the tightly apposed glycogen particles could be demonstrated (Fig. 1, inset). Rickettsiae in phagosomes were not found in the glycogen lakes.

Phagocytized rickettsiae in a zone adjacent to the glycogen areas were usually enclosed by an intact phagosomal membrane. However, some had phagosomal membranes enclosing rickettsiae which were discontinuous and apparently ruptured. In these examples, glycogen particles were adjacent to the rickettsiae (Fig. 1, inset). Various degrees of disrupted phagosomal membranes were observed.

Discussion

The consistent observation that viable appearing *R. tsutsugamushi* were phagocytized by PMN selectively by tightly surrounding phagosomal membrane suggests that rickettsiae possess some ligands or factors which stimulates their uptake. Our present observation indicated that rickettsiae can escape from phagosomes and become localized in the glycogen-rich areas of PMN. Some rickettsiae bordering the glycogen areas were found in partially disrupted phagosomes into which glycogen granules appear to have entered. These images suggest the rupture of phagosomal membranes as a result of the presence of rickettsiae, entry of glycogen into the broken phagosomes, and the eventual release of the rickettsiae in the glycogen areas. No images suggesting the direct entry or release of rickettsiae through the intact host cell membrane were found in this study. Our studies on the phagocytosis and escape of rickettsiae with electron dense tracers (which are in progress) further support this interpretation.

The possibility that a rickettsia still enveloped in a host cell membrane enters the



cytoplasm of another cell by fusion of the membrane of both host cells cannot be entirely eliminated in our present studies. However, the finding that the host cell-free rickettsiae harvested from the culture medium are phagocytized and escaped into the cytoplasm (data not shown) suggests that the direct fusion of the BHK-21 cell plasma membrane with the PMN is not an obligatory mechanism of rickettsial entry. Once in the host cell cytoplasm, the preferential localization of rickettsiae in the glycogen-rich areas of the PMN is conspicuous, but how they seek these areas is not clear. Some indication of the limited rickettsial mobility in the host cell cytoplasm has been demonstrated by time-lapse cinematography (10) and it is possible that rickettsiae may be able to move to these sites.

The escape of a particular rickettsiae from a phagosome occurs soon after phagocytosis either before or after fusion with lysosomes, if escape is to occur at all. Among PMN which contained numerous rickettsiae, about one-half had already escaped into the cytoplasm within 30 min, which was the earliest time period studied.

Because phagosomes are formed from the plasma membrane, the selective breakage of phagosomal membranes by rickettsiae is puzzling. However, it has recently been shown (11, 12) that the PMN phagosomal membrane has different immunolabeling characteristics from that of the plasma membrane. The phagocytized rickettsiae may be able to recognize these characteristics or be capable of inducing further changes which allow them to escape from phagosomes.

The PMN does not seem to be an effective host cell for *R. tsutsugamushi*, even though they may reside in the cytoplasm, because the average half-life of leukocytes in blood is only 6 h and limited to 1–2 d in human tissues (13). Because the generation time of rickettsiae has been determined to be about 9 h for *R. prowazeki* (1), there seems to be inadequate time for significant proliferation of rickettsiae in these phagocytes, though some undergoing division were observed. Furthermore, there is a consistent loss of viable rickettsiae as they are phagocytized, because only about one-half of these manage to escape into the cytoplasm. Assuming that upon death and disruption of the PMN the rickettsiae are immediately phagocytized by another PMN, the number of viable appearing rickettsiae would be reduced to one-half of the original population at each cycle. This process of phagocytosis and escape of rickettsiae into the cytoplasm appears to shorten the life span of PMN because many of these cells are disrupted when heavily infected. The efficacy of PMN as effective ultimate host cells may be very limited, but they may play a positive role in rickettsial infection. Polymorphonuclear leukocytes harboring rickettsiae may serve as temporary reservoirs and by their mobility transfer the viable microorganisms to other sites and infect host cells with longer life spans and result in a typical rickettsial infection.

FIG. 1. Part of a guinea pig peritoneal polymorphonuclear leukocyte illustrating rickettsial uptake after incubation with BHK-21 cells heavily infected with *R. tsutsugamushi*. The rickettsia in the tightly fitting phagosome at the upper left is localized in a filament-rich area near the plasma membrane. Three rickettsiae near the lower right are located directly in the host cell cytoplasm in glycogen-rich areas. Note that the glycogen is present in pools or areas devoid of other cytoplasmic organelles. Numerous lysosomes are present as well as part of a Golgi complex and a centriole ($\times 26,000$). The circular inset at the upper right shows a rickettsiae within a partially disrupted phagosomal membrane. At the site of the discontinuous membrane (arrowheads) glycogen granules are present between the phagosomal membrane and the rickettsiae ($\times 60,000$). The inset at the lower left is an enlarged view of a rickettsia in a glycogen-rich area. Note the intimate association of the glycogen granules to the outer membrane of the rickettsiae ($\times 60,000$).

Summary

Rickettsia tsutsugamushi (Gilliam strain) was serially propagated in BHK-21 cell cultures and incubated with guinea pig peritoneal polymorphonuclear leukocytes to study the ultrastructural features of rickettsial uptake and entry into the leukocytes. Significant numbers of rickettsiae were phagocytized selectively by these leukocytes within 30 min. About one-half of these rickettsiae remained sequestered in phagosomes but the other one-half were free from the phagosome and localized directly in the polymorphonuclear leukocyte cytoplasm. Various stages of rickettsial release from the phagosomes were observed. Once free within the polymorphonuclear leukocyte cytoplasm, the rickettsiae were preferentially localized in the glycogen-packed areas which are devoid of lysosomes and other cytoplasmic organelles. This study indicates that rickettsiae phagocytized by polymorphonuclear leukocytes can escape from the phagosome into the cytoplasm.

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