JOURNAL OF BACTERIOLOGY, May 1983, p. 879–892
0021-9193/83/050879-14$02.00/0

Vol. 154, No. 2

Growth Pattern of *Rickettsia tsutsugamushi* in Irradiated L Cells

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Received 29 June 1982/Accepted 10 February 1983

Irradiated L cells infected with *Rickettsia tsutsugamushi* were studied under the electron microscope to define the morphological growth pattern of the organism. For 2 days after inoculation, no rickettsiae were found either extra- or intracellularly; after 2 days multiple rickettsiae appeared within the host cells without morphological evidence of their entry. These observations showed that the rickettsiae within the cell were assembled in situ by segregation of portions of the granular cytoplasm and subsequent internal differentiation and surface membrane assembly of the segregated bodies. The protoplasmic (P) bodies, which seemed to be formed by shedding infected-cell granular cytoplasm, consistently appeared on the surface and within the phagosomes of the host cells. Rickettsiae were occasionally seen entering host cells in the later phase of infection; these were apparently the ones assembled within the P bodies. This suggested that the P bodies, and not the rickettsiae, were the major infectious particles that transmitted the rickettsial genetic substance among the host cells. On the basis of the present morphological observations, viral-type multiplication for *R. tsutsugamushi* is proposed.

Like viruses, rickettsiae are generally conceived as being obligatory intracellular parasites; however, although the biological process of intracellular parasitism is well known for viruses, for rickettsiae it remains obscure. Ultrastructurally, rickettsiae resemble certain gram-negative bacteria (3); however, their mode of entry into host cells and their subsequent growth and multiplication there is still poorly understood (2).

Rickettsia tsutsugamushi grows luxuriantly in irradiated L cells in tissue culture, and the system can be used as an experimental model for rickettsial infection (4). The growth pattern of the organism in the system at an ultrastructural level has not been reported. Therefore, this study examined the infectious process of *R. tsutsugamushi* in irradiated L cells with the electron microscope to define the morphological growth pattern of the organism in the tissue culture system.

MATERIALS AND METHODS

Rickettsiae. The stock inoculum was an infected yolk sac suspension (passage no. 164, plaque titer 2.2 $\times$ 10^8/ml) stored in ampoules at −75°C at the Walter Reed Army Institute of Research.

L cell infection. L929 cells free of mycoplasm were obtained from the American Type Culture Collection, Rockville, Md., and propagated in the Department of Rickettsial Diseases, WRAIR. The L cells were cultured in an antibiotic-free growth medium, consisting of medium 199 with modified Earles salts and 10% fetal bovine serum. The cell suspension in the same medium was subjected to 3,000-rad irradiation in a ^{60}Co gamma irradiator, and approximately 1.5 $\times$ 10^7 cells were dispensed into each 150-cm^2 plastic flask. The cells were infected 24 h after irradiation by adding 2 ml (plaque titer, 4.4 $\times$ 10^7) of diluted inoculum, which was prepared by diluting the stock inoculum 10 times with brain heart infusion broth. Control irradiated L cells were treated in the same manner with uninfected yolk sac suspension. The cells were incubated at 37°C with gentle rocking for 1 h; the supernatant was discarded, and the monolayer cultures were washed twice with Hanks solution. Fresh culture medium was added, and the cells were again incubated at 37°C. The cells were harvested daily after inoculation by the addition of medium containing 0.125% trypsin for 5 min. The harvested cells were washed with medium and processed for electron microscopy.

Electron microscopy. The harvested cells were pelleted by centrifugation at 600 $\times$ *g* for 10 min, fixed in half-strength Karnovsky fixative for 2 h at room temperature or overnight in a refrigerator, washed with cold 0.1 M cacodylate buffer (pH 7.4), postfixed in 1.3% collidine-buffered osmium tetroxide, dehydrated, and embedded in araldite 502. Thin sections were cut on a Porter-Blum MT-2 ultramicrotome, placed on uncoated grids, stained with uranyl acetate and lead citrate, and examined with a Hitachi H-500 electron microscope at 50 and 75 kV.

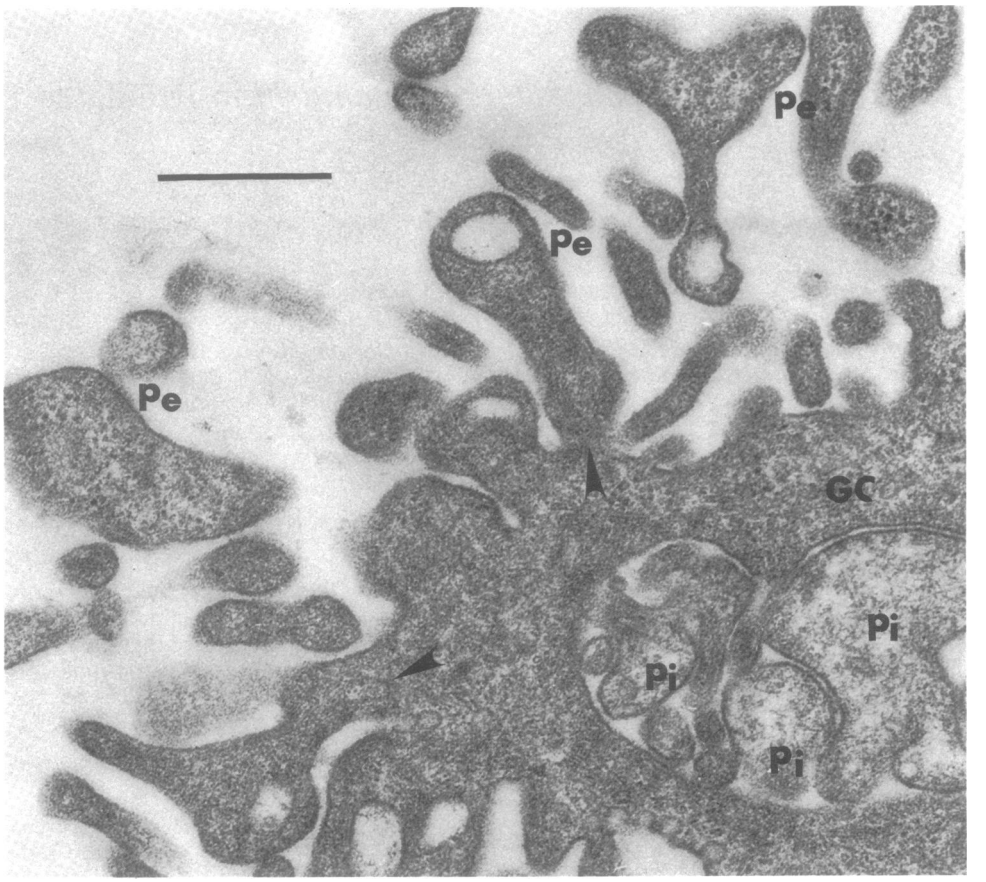

FIG. 1. Pe bodies on the surface and Pi bodies within what appears to be a phagocytic vacuole in the GC of an infected cell, 3 days p.i. Arrows indicate sites where cytoplasmic processes appear to be breaking away to form Pe bodies. The Pi bodies within the phagosome show rarefaction. Bar, 500 nm.

RESULTS

The irradiated L cells at the initiation of the experiment had large multiform nuclei and abundant, moderately electron-dense cytoplasm in which tubular and saccular segments of rough endoplasmic reticulum and small oval or rod-shaped mitochondria were dispersed. The cell surfaces had various numbers of slender microvilli. In addition, the cells expressed small numbers of C-type viruses on the surface and within phagosomes.

Both the infected and control L cells up to 48 h postinoculation (p.i.) had various pieces of cell debris on their surfaces and within phagosomes; these probably originated from the yolk sac inoculum and degenerated tissue culture cells. Compared with the control, the infected L cells generally showed distinct cytopathological changes, such as cytoplasmic edema, fragmentation and cystic dilatation of rough endoplasmic reticulum, and swelling and dissolution of the mitochondria. An amorphous granular material accumulated in the cytoplasm, replacing disrupted and dissolving subcellular structures. Eventually, many infected L cells contained these amorphous granular areas, in which multiple rickettsiae would appear as described below. In the initial phase of infection, no extracellular particles characteristic of the infected tissue cultures were recognizable, and, despite a diligent search, no intact rickettsiae were found.

After day 3 p.i., characteristic differences appeared between the infected and control L cells. Whereas the surface of the control L cells became increasingly clean, with regularly arranged microvilli and club-shaped cytoplasmic processes, those of many of the infected cells became increasingly crowded, with disorderly, scattered cytoplasmic pieces, here called protoplasmic (P) bodies (Fig. 1 and 2). The P bodies were seen both extracellularly (Pe) over the cell

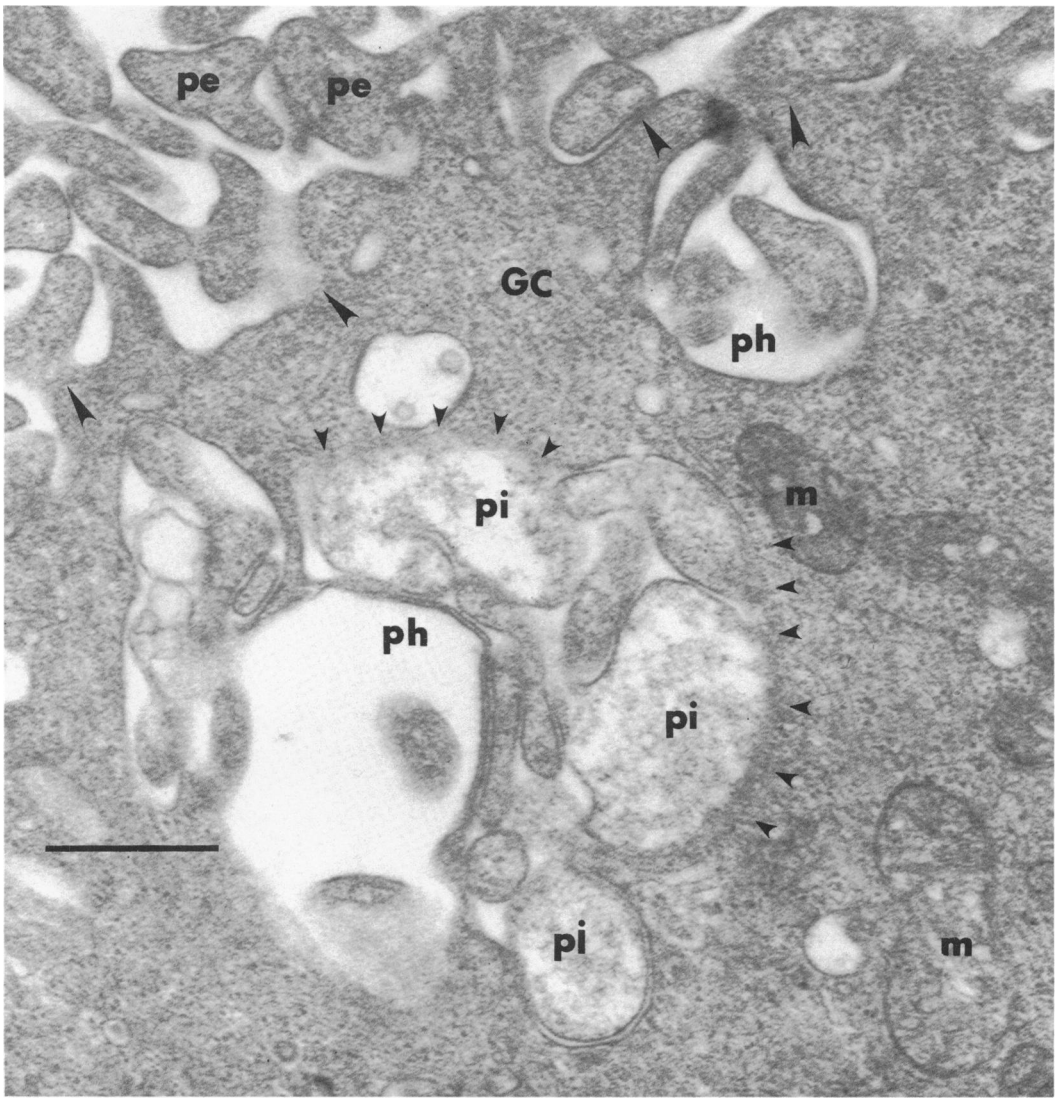

FIG. 2. Pe and Pi bodies on and in the GC of an infected cell, 4 days p.i. Large arrows indicate sites where the limiting membranes of the Pe bodies appear to be fusing to the GC; small arrows indicate sites where the limiting membranes of the Pi bodies and the phagosomal membranes appear to be fusing and dissolving. m, Mitochondrion; ph, phagosome. Bar, 500 nm.

surface and intracellularly (Pi) within phagosomes (Fig. 1 and 2). The Pe bodies were usually round or oval structures bounded by unit membranes; however, they showed marked variation in size and shape, particularly on the cell surface. They were characterized by a high concentration of ribosomes, which frequently formed polysomes. In the early phase of infection, the Pe bodies tended to be small, many of them the size of obliquely cut or cross-cut microvilli; however, the Pe bodies were differentiated from the latter by their conspicuous ribosomes and polysomes. Some Pe bodies also contained vesicles. The Pe bodies were particularly numerous over the granular cytoplasm (GC), which became increasingly prominent in infected cells at this stage; some were connected with the GC by narrow stalks and appeared to be breaking away from the cytoplasm (Fig. 1), and some seemed to be fusing to the cells by lying flat and fuzzing or obliterating their membranes at the contact site (Fig. 2). P bodies in the process of being phagocytized were also found (Fig. 2). The Pi bodies within phagosomes frequently appeared rarefied

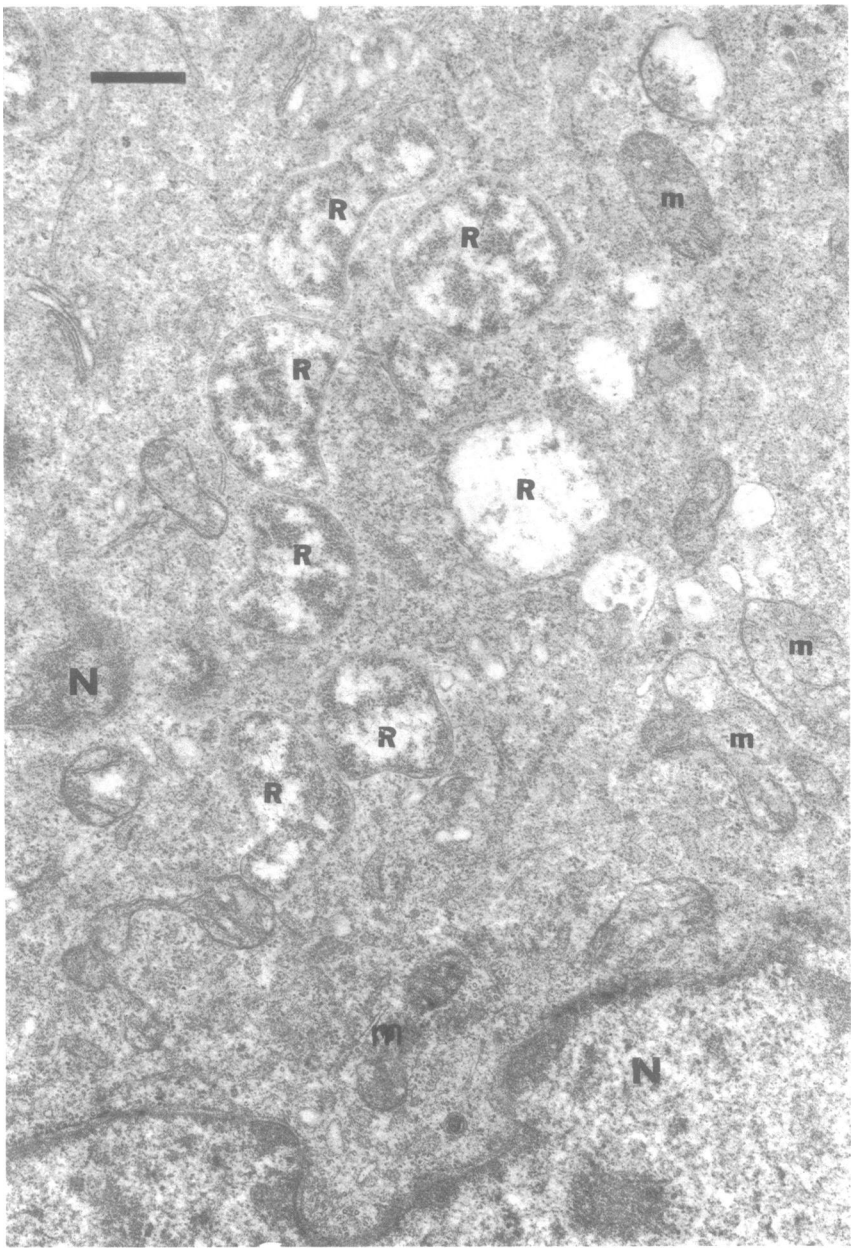

FIG. 3. Appearance of rickettsiae (R) in the cytoplasm of an infected cell, 3 days p.i. Rickettsiae are bordered by fuzzy zones or faint double membranes; none are within phagosomes. Note the replacement of subcellular structures with amorphous granular material. m, Mitochondrion; N, nucleus. Bar, 500 nm.

and swollen (Fig. 1 and 2). The membranes at the contact sites between the Pi bodies and the phagosomal walls were often obliterated by fusion (Fig. 2).

Concurrently, multiple rickettsiae appeared within the GC of infected cells from day 3 on. In the early phase of infection, multiple rickettsiae were seen tightly embedded in the cytoplasm, usually near the nucleus (Fig. 3 and 4). No rickettsiae were found on the cell surface or within phagosomes at this stage, and therefore there was no morphological evidence that intracellular rickettsiae originated extracellularly. The rickettsiae appeared subtly within the GC;

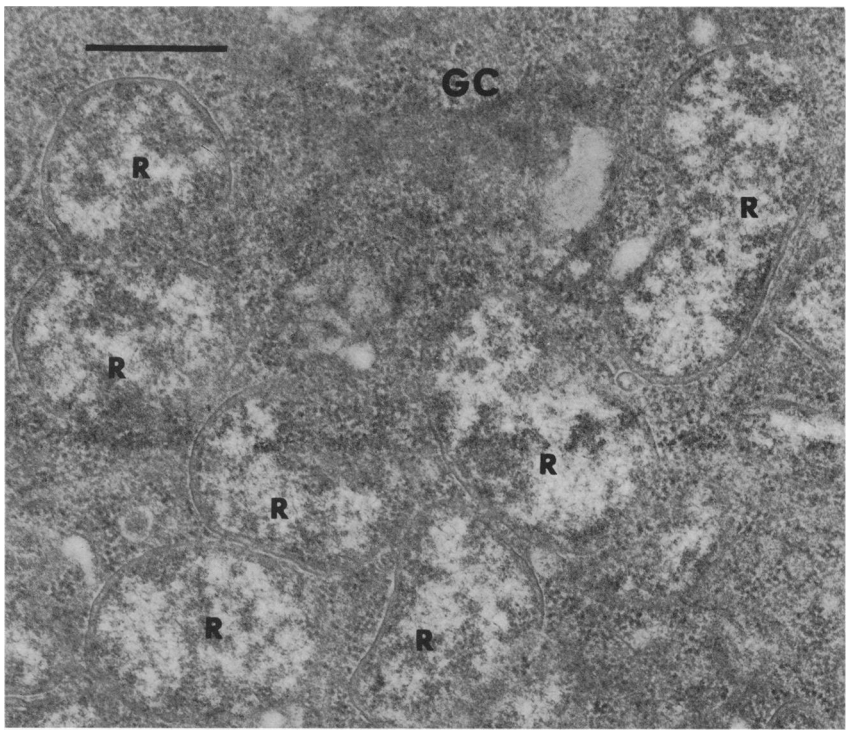

FIG. 4. Multiple rickettsiae (R) embedded in the dense GC, 4 days p.i. Rickettsiae are partially delineated from the surrounding cytoplasm by fuzzy zones or faint, delicate double membranes. The rest of the rickettsial bodies frequently blended with the surrounding cytoplasm. Bar, 500 nm.

the earliest morphological change in their appearance was seen there as small portions of GC segregated to form foci of differentiation into electron-lucent filamentous and electron-dense granular areas. These segregated bodies were only partially bordered by zones of mildly electron-dense fuzzy material or faint, delicate double membranes. The segregated bodies then seemed to differentiate further to assume the characteristic appearance of rickettsiae; at the same time, double membranes were assembled on the peripheries of the nascent rickettsiae. Rickettsiae within the GC of infected cells showed marked variations in internal differentiation and external membrane coverage.

Progeny rickettsiae appearing within the GC of infected cells were poorly delineated by faint, incomplete membranes and appeared more or less blended into the GC (Fig. 4). The extent of membrane coverage for individual rickettsiae was difficult to assess, mainly because of out-of-phase phenomena. Nevertheless, many of the rickettsiae that showed complete profiles, suggesting that they were cut near the center, still had no limiting membranes over parts of the periphery, and these membraneless parts frequently blended with the surrounding GC (Fig. 4). Therefore the partial absence of limiting membranes was too extensive and too pervasive to be simply an out-of-phase phenomenon, and many rickettsiae lacked limiting membranes over parts of the periphery. Electron micrographs of the progeny rickettsiae taken at various degrees of tilt also showed the nature of the double membranes (Fig. 5). In the existing double membrane, the outer membrane representing the cell wall was usually very thin and was frequently interrupted by membraneless areas where membrane could not be brought into phase at any angle (Fig. 5, R1). The inner membrane representing the cytoplasmic membrane was either highly discontinuous or simply unrecognizable. Some rickettsiae were covered almost entirely by fuzzy zones, with little membrane structure appearing on the periphery (Fig. 5, R2). As the infection progressed, intracellular rickettsiae tended to show clearer internal differentiation and more definitive membrane coverage, although they still displayed what appeared to be segmental and focal membrane defects (Fig. 6). The findings indicated that the rickettsiae matured within the GC through progressive

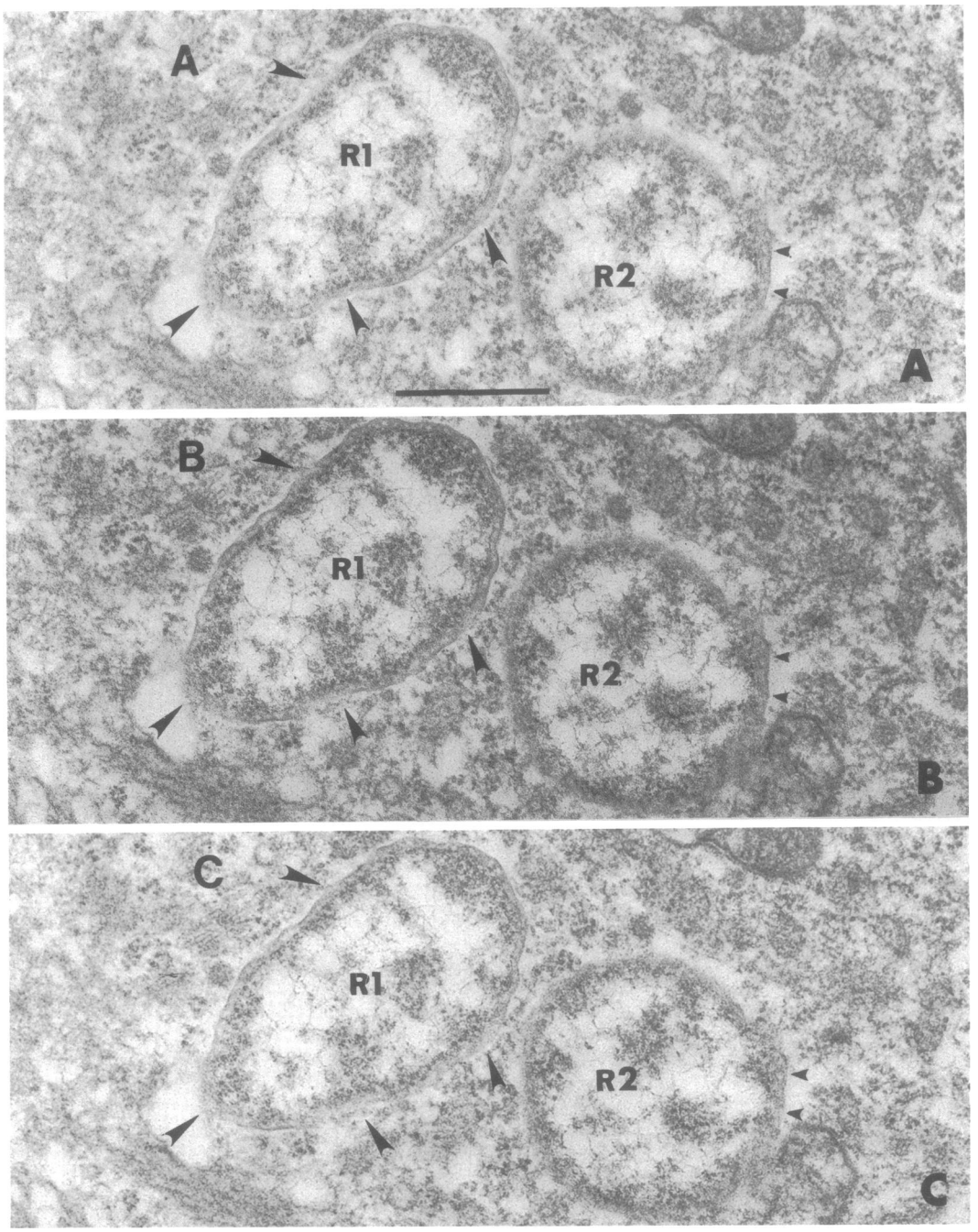

FIG. 5. Two rickettsiae (R1 and R2) in the GC, 4 days p.i., at tilt angles of +20 (A), 0 (B), and −20° (C), with the tilt axis running from the lower right to the upper left at 45°. R1 is covered by a thin, delicate double membrane; the large arrows indicate areas where the membrane was faint or absent at different angles, suggesting inherent membrane defects. R2 is covered with a fuzzy zone, in which membrane is recognizable only in a small segment (small arrows). Bar, 500 nm.

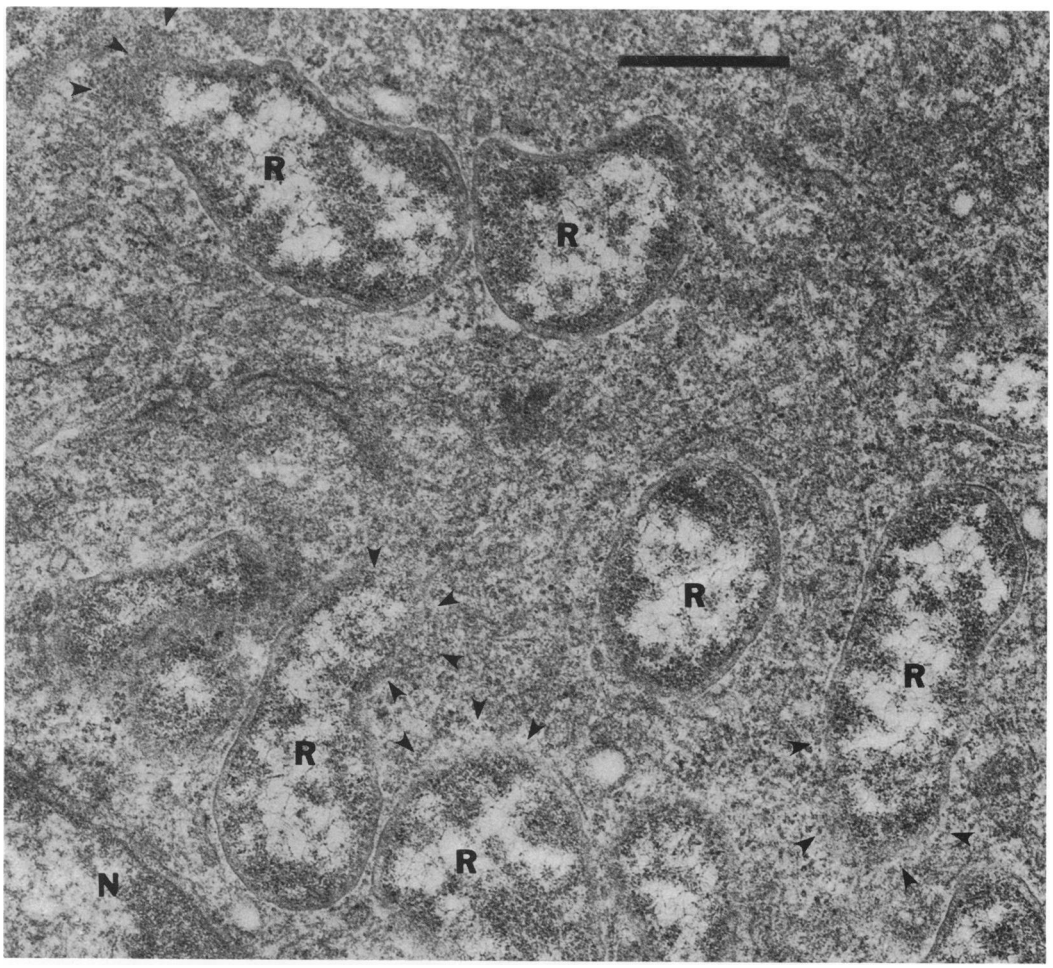

FIG. 6. Rickettsiae (R) within the GC, 5 days p.i. The rickettsiae show clearer internal differentiation and more distinctive membrane coverage than in the preceding periods, although membrane coverage appears to be still incomplete (arrows). Bar, 500 nm.

internal differentiation and external membrane coverage.

In the later phase of infection, numerous P bodies persisted on the surface of infected cells (Fig. 7). Generally, they were larger than those seen in the early phase of infection and showed internal differentiation, with ribosome aggregation and the appearance of electron-lucent areas in which a faint filamentous network was recognizable (Fig. 7A). Some P bodies had relatively electron-lucent or even vacuolar interiors in which single or multiple vesicles were seen (Fig. 7B). The same kinds of vesicles also appeared free in the extracellular space (Fig. 7B). Some P bodies were quite large, and they occasionally contained rickettsiae (Fig. 8 and 9). These had features typical of nascent rickettsiae, with poorly differentiated interiors and rudimentary or incomplete double membranes. Examination with tilting also indicated that the membrane coverage of these rickettsiae was incomplete (Fig. 9). In addition to the extracellular rickettsiae contained in P bodies, extracellular rickettsiae covered with plasma membranes were occasionally found on the cell surface and within phagosomes (Fig. 9 and 10A); they had faint, thin double membranes of their own beneath the plasma membrane. These rickettsiae appeared to be still immature; they were probably derived from those seen in the P bodies, since rickettsiae leaving host cells with host plasma membrane covers were not found on the cell surface. Occasionally these rickettsiae were phagocytized by host cells with the plasma membrane

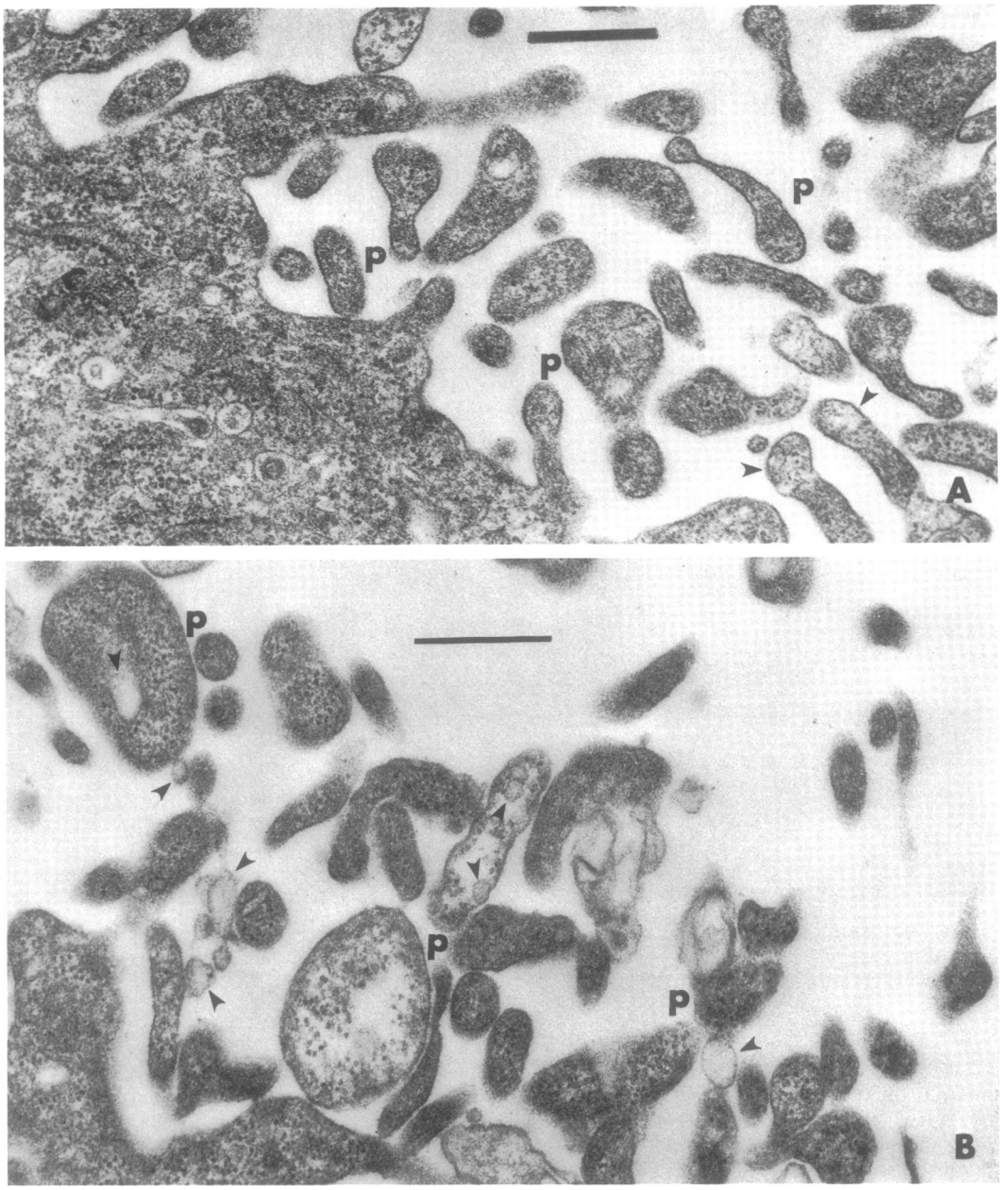

FIG. 7. P bodies (P) on the surface of infected cells, 5 days p.i. (A) P bodies showing variable internal differentiation. Arrows indicate electron-lucent areas where a faint filamentous network is recognizable. (B) Vacuolar P bodies containing vesicles (arrows). Note the same kinds of vesicles in the extracellular space (arrows). Bar, 500 nm.

covers intact (Fig. 9 and 10A). Phagocytized rickettsiae seemed to disintegrate within phagosomes and eventually to dissolve into the cytoplasm in the same manner as the Pi bodies (Fig. 10B).

Concurrently with these changes on the surface of infected cells, a majority of the cells developed dense, amorphous GC in which multiple rickettsiae of different assembling stages were seen (Fig. 11). On day 7, approximately 10% of the infected L cells had clear cytoplasm depleted of granular material, in which multiple

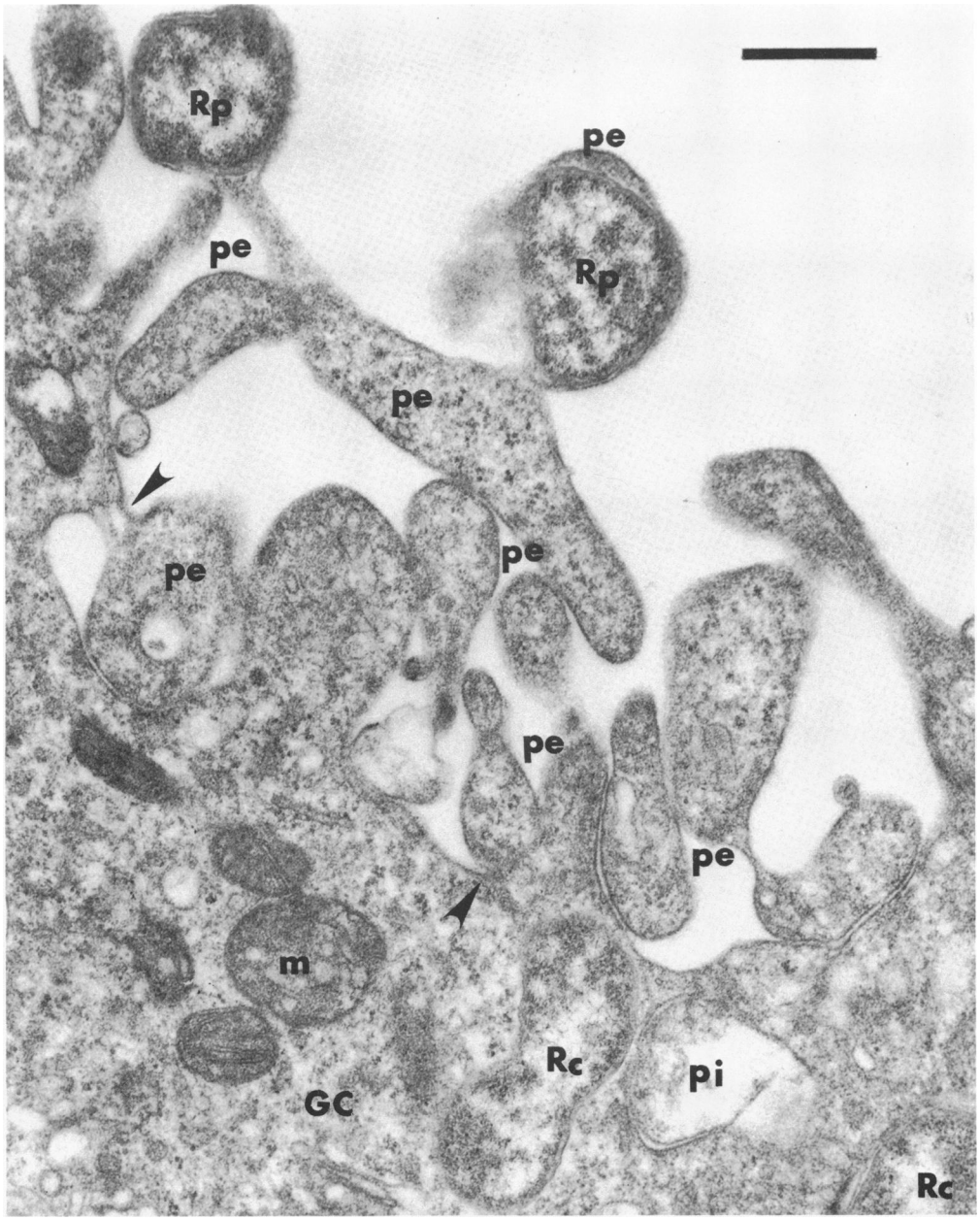

FIG. 8. Surface of an infected cell, showing multiple P bodies, 6 days p.i. Some Pe bodies show adhesion of their limiting membranes to the plasma membrane (arrows). Two Pe bodies contain nascent rickettsiae (Rp), which have poorly differentiated interiors and show indistinct membrane coverage. m, Mitochondrion; Pi, dissolving phagocytized P body; Rc, intracellular rickettsia. Bar, 500 nm.

individual rickettsiae were seen (Fig. 12). These cells were apparently degenerating as the result of rickettsial infection. In early-degenerating cells, rickettsiae were crowded in the loosely filamentous cytoplasm, in which islands of remaining granular material were scattered (Fig. 12A). In late-degenerating cells, individual rickettsiae were seen free in the vacuous cytoplasm (Fig. 12B).

DISCUSSION

In irradiated L cells infected with *R. tsutsugamushi*, rickettsiae were not detectable in the

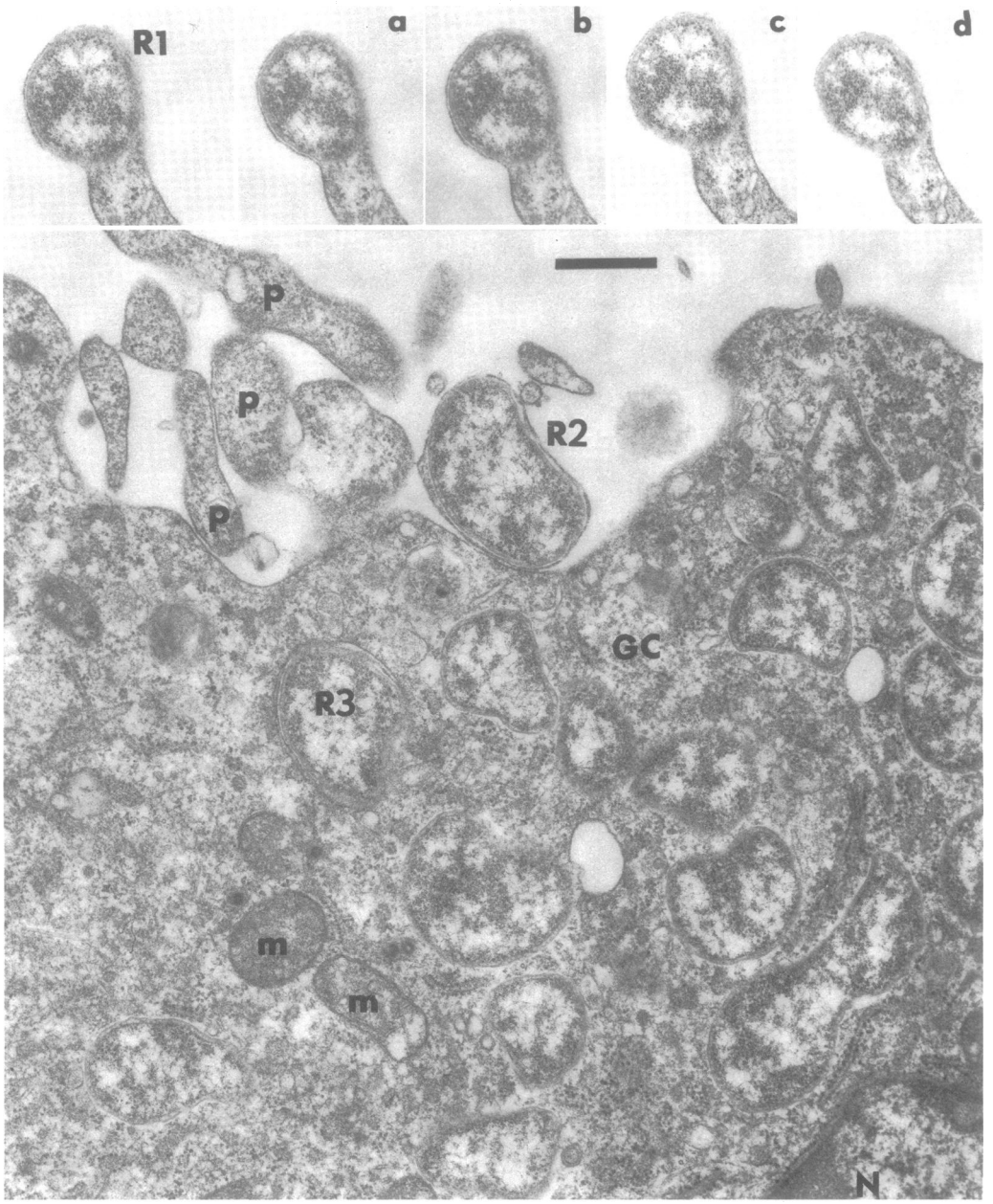

FIG. 9. Rickettsia-infected cell, 6 days p.i. R1, Nascent rickettsia within a P body (P) at 0° tilt and at +40, +20, −20, and −40° tilt (insets a through d, respectively), with the tilt axis running from the upper right to the lower left at 45°. Note the incomplete membrane about R1. R2, Extracellular rickettsia covered by plasma membrane; R3, phagocytized rickettsia similar to R2. The other rickettsiae are embedded in the GC and are probably assembled in situ. m, Mitochondrion; N, nucleus. Bar, 500 nm.

tissue culture system for the first 2 days p.i.; multiple rickettsiae appeared in the cytoplasm of host cells from day 3 on, without morphological evidence of rickettsial entry into the cells. The absence of rickettsiae in the first 2 days could be attributed to a numerical factor, although it contrasted sharply with the later abundance of intracellular rickettsiae. It is difficult to account

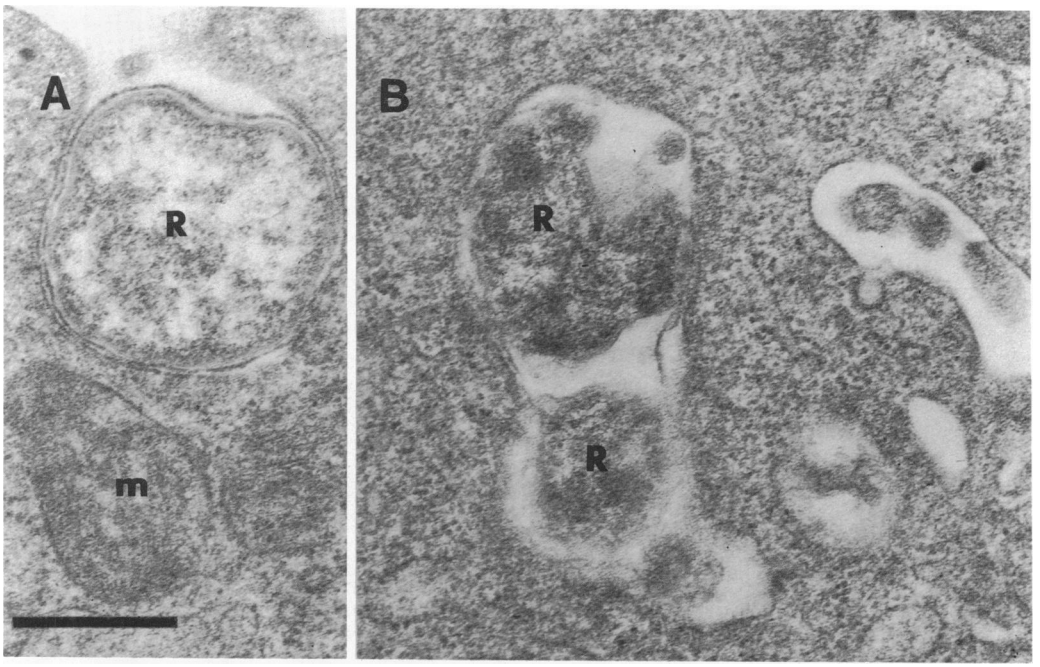

FIG. 10. Phagocytosis of rickettsiae, 6 days p.i. (A) Rickettsia (R) with the plasma membrane cover in the process of being phagocytized. m, Mitochondrion. (B) Phagocytized rickettsiae which appear to be disintegrating (?). Bar, 500 nm.

for the sudden appearance of multiple rickettsiae within host cells on day 3 on the basis of rickettsial entry and multiplication in the absence of any indication of rickettsial entry into the cells. Moreover, the morphological observations clearly showed that the rickettsiae within the GC were in various stages of assembly. Obviously, the intracellular rickettsiae originated from small portions of GC that had segregated from the rest; the subsequent transition of the segregated bodies into nascent and then mature rickettsiae apparently occurred through internal differentiation and surface membrane assembly.

The finding that progeny rickettsiae are formed within the cytoplasm of host cells is completely new, and a working hypothesis seems to be needed for further investigations. The most feasible hypothesis seems to be that, like viruses, rickettsiae somehow release their genetic substance into the host cell cytoplasm. The amorphous GC that develops in host cells after rickettsial inoculation is then most likely rickettsial material, analogous to viroplasm in viral infection, since nascent rickettsiae appear within it. It is likely, therefore, that the rickettsial genetic substance replicates and eventually assembles progeny rickettsiae within the GC of host cells.

In the proposed viral-type mode of multiplication for *R. tsutsugamushi*, extracellular infectious particles, which were presumably involved in transmission of the rickettsial genetic substance among host cells, were not definitively identifiable. This study seemed to show rather convincingly that rickettsiae were not the particles involved in this process; at the same time, it revealed that the only extracellular particles consistently appearing on the cell surface and within the phagosomes of infected cells were the P bodies, indicating that the P bodies were the principal particles that vigorously entered the cells. The morphological observations suggested that the P bodies were formed through the shedding of small portions of the infected-cell GC and that they re-entered host cells through fusion of their limiting membranes with the plasma or phagosomal membrane of the host cells. It is conceivable, therefore, that the P bodies carry the rickettsial genetic substance among the host cells. This idea was supported by the fact that some P bodies contained nascent rickettsiae, indicating that the P bodies carried the rickettsial genetic substance and that rickettsial assembly occurred within some of them. The present observations therefore strongly suggest that P bodies were the extracellular infectious particles of *R. tsutsugamushi*, which carried and transmitted the rickettsial genetic substance among host cells in the tissue culture system.

It is known that large numbers of rickettsiae occur in degenerated tissue culture cells in the

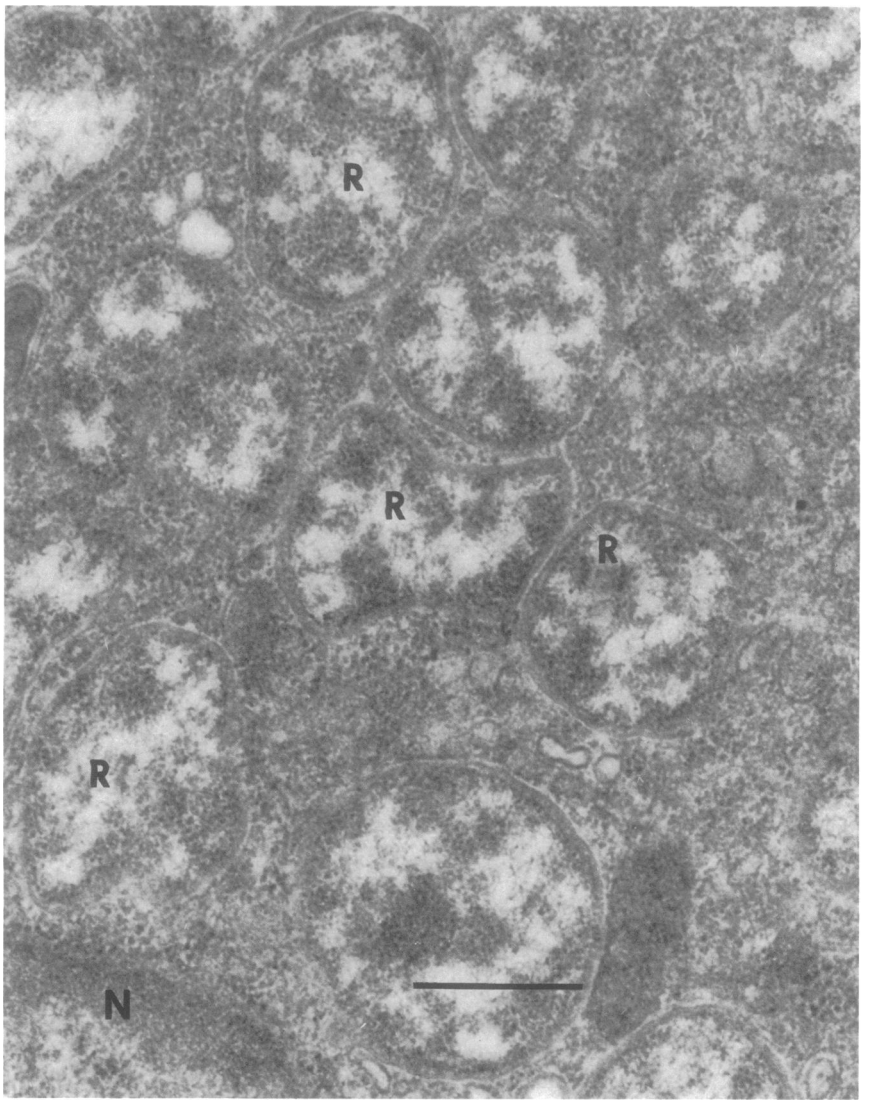

FIG. 11. Multiple rickettsiae (R) in various stages of assembly in the dense GC, 6 days p.i. N, Nucleus. Bar, 500 nm.

later period of infection (2); yet strangely the entry of these rickettsiae into new host cells has not been successfully demonstrated, and therefore the mode of entry remains unknown. In a high-dose intraperitoneal infection of mice, Ewing et al. (1) reported that rickettsiae enclosed by host plasma membrane were phagocytized by mesothelial cells. However, this type of rickettsial entry was found too infrequently in relation to the overall infection to be considered the mode of rickettsial entry (2). In a mixed culture of rickettsia-infected BHK-21 cells and guinea pig polymorphonuclear leukocytes, Rikihisa and Ito (2) showed that the leukocytes phagocytized the rickettsiae released from BHK-21 cells; however, as they admitted, it is doubtful that any appreciable rickettsial proliferation occurred in the leukocytes. Conceivably, free rickettsiae are phagocytized by phagocytes; on the other hand, if phagocytosis is the mode of entry, rickettsiae should be easily found in tissue culture cells in the initial phase of infection. The absence of rickettsiae in this phase and the subsequent appearance of multiple rickettsiae

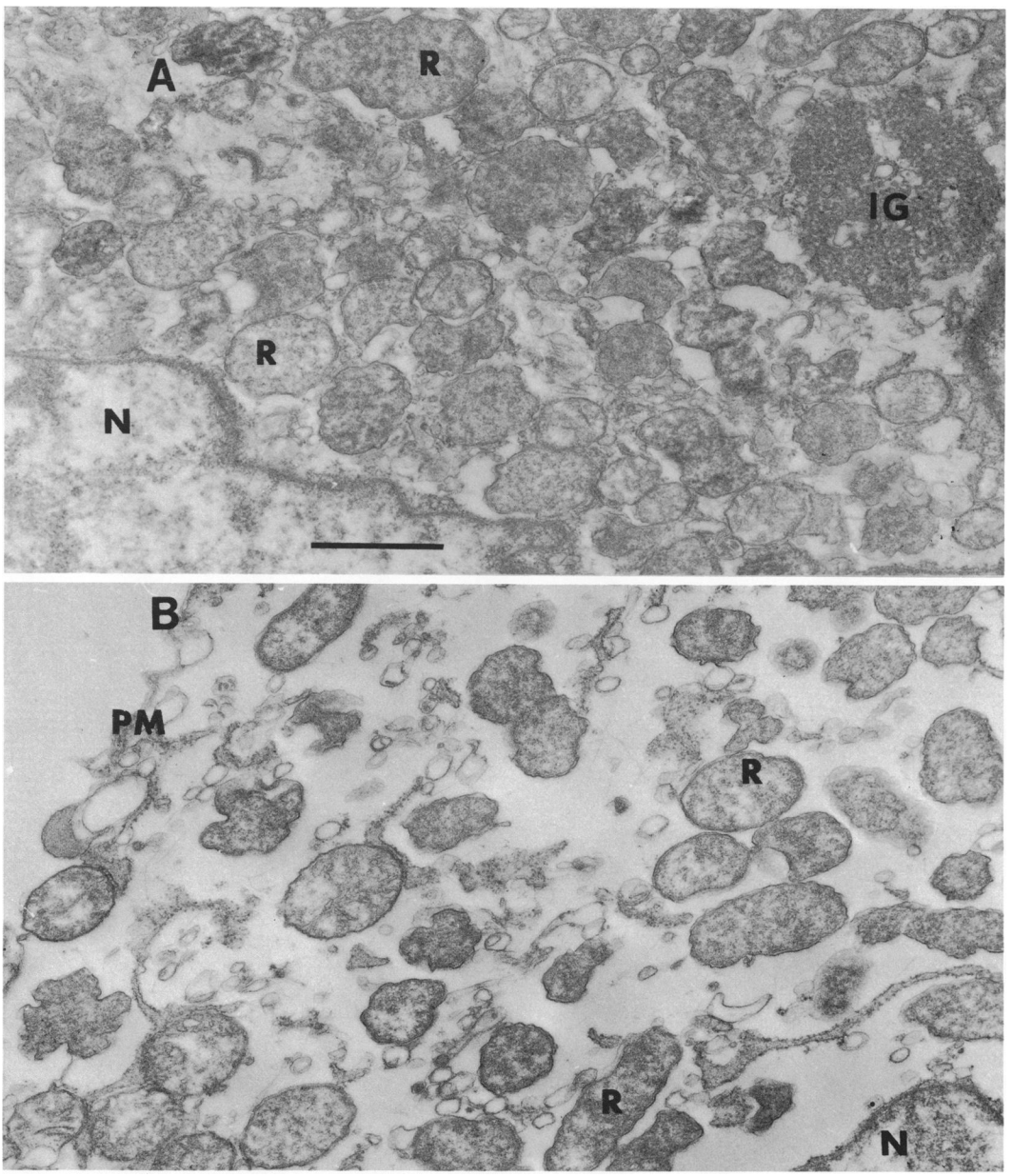

FIG. 12. Rickettsiae (R) in the cytoplasm of degenerating cells, 7 days p.i. (A) Early-degenerating cell containing multiple rickettsiae in the cytoplasm depleted of granular material. IG, Island of remaining granular material; N, nucleus. (B) Degenerated cell containing multiple rickettsiae in the vacuous cytoplasm. N, Nucleus; PM, plasma membrane. Bar, 1 μm.

within host cells therefore suggested that the rickettsial genetic substance was introduced into host cells by some other vehicle. If the P bodies were the vehicles in this process, it seems impossible to morphologically differentiate these highly pleomorphic structures from the cell debris that appeared abundantly in both the infected and control cultures in the initial phase of infection. This might very well explain the difficulties encountered in elucidating the infectious process in the initial phase. The characteristic infectious process in this tissue culture system

seemed to emerge only after the cell debris introduced at the initiation of infection had been cleared.

ACKNOWLEDGMENTS

I thank Jeffrey A. Havenner, Department of Rickettsial Diseases, WRAIR, for supplying the tissue culture materials and JoEtta Hubbard for her technical assistance in electron microscopy.

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