Acta virol. **26**: 506—511, 1982

SURFACE MORPHOLOGY OF *RICKETTSIA TSUTSUGAMUSHI*-INFECTED MOUSE FIBROBLASTS[1])

T. TSURUHARA, H. URAKAMI, A. TAMURA

Department of Microbiology, Niigata College of Pharmacy, Kamishinei-cho, Niigata, Niigata 950-21, Japan

Received April 29, 1982

Summary. — Sequential morphological changes of the L-cell surface after infection with *Rickettsia tsutsugamushi* (Gilliam strain) have been examined by scanning electron microscopy and ruthenium red staining technique. Adherence of inoculated rickettsiae to the host-cell surface and their engulfment by the cell were seen at 30 min and have still proceeded at 24 hr post infection (p.i.). Progeny rickettsiae which were lifting up the host cell membrane by budding were observed on the cell surface at 48 hr p.i. The budding of rickettsiae increased gradually in time and, at 96 hr after infection, covered almost the all host-cell surface except of the cell margin. Numerous microvilli observed on the surface of uninfected L-cells decreased gradually p.i.; they had almost disappeared when progeny rickettsiae occurred. Ruthenium red staining specimens clearly showed that the budding rickettsiae were surrounded with the host cell membrane. The following layers were distinguished from outside on: (1) ruthenium red positive fuzzy coat (25 nm thick); (2) a triple-layered cell membrane (5—6 nm); (3) outer and inner leaflets of the rickettsial cell-wall (7—8 nm and 2-2.5 nm, respectively); (4) periplasmic space (15—20 nm); (5) a triple-layered rickettsial cytoplasmic membrane (5—6 nm).

Key words: *Rickettsia tsutsugamushi*; *surface morphology*; *budding rickettsiae*; *ruthenium red staining*

Introduction

The rickettsiae, obligatory intracellular parasites, are morphologically similar to bacteria but smaller in size. On regards their ultrastructure and multiplication in host-cells, Silverman and Wisseman (1979), Silverman *et al.* (1980) have reported observations of the *Rickettsia rickettsii*- and *prowazekii*-chicken embryo fibroblast (CEF) systems describing by electron microscopy the sequential changes from the early stages of infection to the

[1]) A part of this work was presented at the 27th Annual Meeting of the Society of Japanese Virologists, 1979.

host-cell lysis and death. Ewing *et al.* (1978) showed the hypothetical scheme for infection cycle of *Rickettsia tsutsugamushi* in BALB/c mouse peritoneal mesothelial cells, suggesting that budding rickettsiae from the cell surface entered other mesothelial cells by phagocytosis. Rikihisa and Ito (1980) reported detailed investigations of rickettsial entry into guinea pig polymorphonuclear leukocytes by using electron-dense tracers. Thus, it is very interesting to investigate the uptake, penetration phase and budding or late phase in rickettsial infection.

We have reported an easy method for *R. tsutsugamushi* infectivity titration (Tamura and Urakami, 1981), for its purification (Tamura *et al.*, 1982) and demonstrated the intranuclear localization of this rickettsia (Urakami *et al.*, 1982). Present study shows the morphological findings by scanning electron microscopy of the development of *R. tsutsugamushi* in mouse L-cells from the penetration of inoculated rickettsiae to the release of progeny microorganisms and also the appearance of ruthenium red-stained coat on the budding rickettsiae by transmission electron microscopy.

Materials and Methods

Rickettsiae and cell culture. R. tsutsugamushi, Gilliam strain, kindly supplied by Dr. A. Shishido, National Institute of Health, Japan, was passaged for several times in chick embryo yolk sacs and then, in this laboratory, in mouse L-cells. Confluent monolayers of L-cells formed on coverslips in Leighton tubes or on glass surface of 2-oz prescription bottles were inoculated with purified *R. tsutsugamushi* inoculum. The inoculum was prepared according to the method described by Tamura *et al.* (1982). After adsorption for 1 hr at 37 °C, the inoculum was removed and the cell monolayers were further incubated at 37 °C in fresh Eagle's minimum essential medium containing 2% calf serum.

Scanning electron microscopy. Cells cultured on coverslips were washed twice with phosphate buffered saline, fixed with 2% glutaraldehyde in 0.1 mol/l cacodylate buffer (pH 7.2) for 20 min and postfixed with 1% osmium tetroxide in the same buffer for 15 min at 4 °C. They were dehydrated through a graded series of alcohol and up to 100% isoamyl acetate before drying at the critical point in Hitachi HCP-2 apparatus with liquid CO_2. Specimens were coated with carbon, followed by a thin film of gold in a vacuum evaporator (JEOL JEE-4×) and examined with a JEM-100CX ASID electron microscope operated at 10 kV.

Transmission electron microscopy. For staining with ruthenium red, our technique was essentially based on the method described by Luft (1966). Cells in prescription bottles were doubly fixed *in situ* with 2% glutaraldehyde and 1% osmium tetroxide as described above, except that the both fixatives contained ruthenium red dye (Wako Pure Chemical Industries, LTD.) at a concentration of 1 mg/ml. Then the cells were scraped off from glass surface with a rubber policeman, centrifuged, and the resulting pellets were stained *en bloc* with 2% uranyl acetate, dehydrated in a graded series of ethanol solutions and embedded in Epon 812 (Luft, 1961). Thin sections were prepared with an LKB-IV ultratome and examined with a JEM-100 CX electron microscope operated at 80 kV.

Control specimens not stained with ruthenium red were handled as above omitting the dye; the thin sections were contrasted on grids with leas citrate (Sato, 1968).

Results

Scanning electron microscopy

Unifected cells. The monolayers of L-cells examined for up to 96 hr after sham-inoculation displayed similar appearances with little variation in size, shape or surface architecture. The L-cells at 24 hr (Fig. 1) were generally

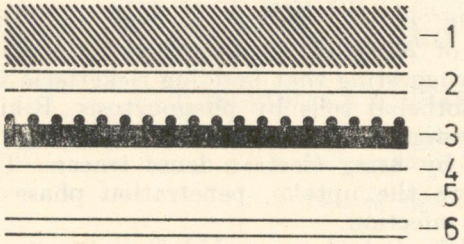

Fig. 11.

Schematic representation of the budding rickettsial envelope
1 — ruthenium red positive fuzzy coat (25 nm); *2* — cell membrane (5—6 nm); *3* — outer leaflet (7—8 nm); *4* — inner leaflet (2—2.5 nm); *5* — periplasmic space (15—20 nm); *6* — cytoplasmic membrane (5—6 nm)

flattened, grew with some overlapping, and showed numerous microvilli on their surface. The microvilli were about 0.1 μm in diameter and varied in length from one to several micrometers, erecting almost vertically from the surface. The cells were attached to the coverslip and to each other by numerous filipodia which were longer and thinner than microvilli. These differ-ferent kinds of projections have been recognized by Porter *et al.* (1972, 1973) in cultured rat sarcoma cells and chinese hamster ovary cells.

Rickettsia-infected cells. At 30 min after inoculation (Fig. 2A), inoculum--rickettsiae 0.5—0.7 μm in diameter, were found on the surface of many cells, especially near microvilli. Some microorganisms were seen to be invaginated by the host cell membrane (Fig. 2B). At 24 hr after inoculation (Fig. 3), clumps of rickettsiae which might had come from the original inoculum, were still seen on the cell surface. The microvilli on cell surfaces diminished gradually after infection, and the cells showed a tendency to round up, while long filipodia still attached to the substrate.

At 48 hr after infection (Fig. 4), progeny rickettsiae of rod-shape in budding form, 0.5—0.7 μm × 1.0—1.5 μm in size, were recognized on the cell surface, distinguishable from the round-shaped inoculum-rickettsia (Fig. 3). At 96 hr after infection (Fig. 5), numerous rod-shaped rickettsiae of budding form covered over almost all the rounding up-cell surfaces except of the cell margin (Fig. 5A). At a higher magnification (Fig. 5B), some microorganisms prior to budding were observed to be oblong below the host cell mebrane, but budding form of numerous riskettsiae seemed to be perpendicularly to the host cell surface.

Transmission electron microscopy

When uninfected L-cells were stained with ruthenium red, a darkly stained layer at the cell surface (about 25 nm thick) was clearly distinguished (Fig. 6). This layer was also seen in the cells at 96 hr p.i. as shown in Fig. 7, when many budding rickettsiae located on the cell surface were surrounded with this darkly stained layer. Staining effect was only observed on the cell surface, suggesting that the dye could not penetrate into intracellular portion. Un-stained specimen of cells at the same hr p.i. (Fig. 8) showed that budding microorganisms were lifting up the host-cell membrane, with which they were closely covered. This outermost membrane was not recognized at the microorganisms in the host-cell cytoplasm, which were enveloped by their

own cell-wall and cytoplasmic membrane. At a higher magnification (Fig. 10), the rickettsial cell envelope was composed distinguishably of the outer and inner leaflets of the cell-wall, of a periplasmic space, and a triple-layered cytoplasmic membrane. Subunits (about 7 nm long) of rod-like periodicity were also recognized on the outside of outer leaflet in Fig. 10 (arrowheads).

Based on these electron micrographs, surrounding layers of budding rickettsia appeared to be composed of layers as illustrated in Fig. 11, viz., proceeding from the outside: (1) ruthenium red positive fuzzy coat (25 nm thick); (2) a triple-layered cell membrane (5—6 nm thick); (3) a outher leaflet (7-8 nm thisk); (4) a inner leaflet (2—2.5 nm thick); (5) periplasmic space (15—20 nm thick); (6) a triple-layered cytoplasmic membrane (5—6 nm thick).

Discussion

In the present experiments, morphology of cells infected with *R. tsutsugamushi* was studied by scanning electron microscopy, concentrating attention on the mode of rickettsia-penetration into and -release from the host cells. Additionally, observations of thin sections stained with or without ruthenium red were performed by transmission electron microscopy, concerning on the late stage of infection, especially on budding rickettsiae.

Decrease in the number of microvilli after rickettsial infection was observed by scanning electron microscopy. However, it is difficult to discuss here the mechanism or meanings of these changes, because the discrete relationship between the intracellular events and the surface changes has not been understandable yet.

On the rickettsial entry into host cell, we observed the engulfment of the microorganisms by the host cell membrane (Fig. 2B), suggesting the entry by phagocytosis, as mentioned by Kokorin (1968) in the case of *R. tsutsugamushi*-cultured guinea pig kidney epithelial and reticular cell systems. On the other hand, a small number of the microorganisms adhering to but not engulfed by the host cell membrane was observed in the specimen at 24 hr p.i. (Fig. 3). These microorganisms might be inactive and inviable, and not able to enter into host cells, resulting in their protracted adherence to the cell surface. The failure of inactivated rickettsia to penetrate into cells was recognized by Cohn *et al.* (1959) in the case of *R. tsutsugamushi*-mouse lymphoblast cell system from light microscopic observation, and by us in the same rickettsia-L-cell system by electron microscopic studies (paper in preparation).

In the late stage of infection, the surfaces of infected cells were almost completely covered with budding rickettsiae (Figs 5A and 5B). This might be due to the accumulation of progreny rickettsiae which were formed in the cell cytoplasm, transfered to the cell margin one after another to form budding state and kept for long time on the cell surface. Detachment of budding rickettsiae might be very slow or a little. Our previous studies (Tamura and Urakami, 1981) demonstrated a very slow increase of the percentage of infected cells in culture at low multiplicity of infection, indicating that

secondary infection by spreading of the budding rickettsiae was rare. On comparison with other rickettsiae, *R. rickettsii* grown in cultured chick embryo cells appear to be actually escaping from living infected cells without resulting cell damage (Schaechter *et al.*, 1957; Wisseman *et al.*, 1976), contrasting with *R. prowazekii* in the same cells in which case the progeny rickettsiae accumulate in the cytoplasm and spread after the host cells break down and burst (Wisseman and Waddell, 1975). Hence *R. tsutsugamushi*, in this respect, might be intermediate between *R. rickettsii* and *R. prowazekii*.

As to rickettsial cell envelopes, Anacker *et al.* (1967) described a five-layered architecture in the cell-wall of *R. prowazekii*, and Povov and Ignatovich (1976) clearly observed a microcapsular layer on the surface of *R. prowazekii* by means of ruthenium red staining. In our studies, budding rickettsiae were covered with the host-cell membrane, so that microcapsular-like layer, even if it existed, was not stained with ruthenium red dye (Fig. 9). The outer leaflet of cell wall of *R. tsutsugamushi* was thicker than the inner leaflet as shown in the present study. This was coincident with the result of Silverman and Wisseman (1978), who showed a similar dimension of the outer envelope.

References

Anacker, R. L., Pickens, E. G., and Lackman, D. B. (1967): Details of the ultrastructure of *Rickettsia prowazekii* grown in the chick yolk sac. *J. Bact.* **94**, 260—262.

Cohn, Z. A., Bozeman, F. M., Campbell, J. M., Humphries, J. W., and Sawyer, T. K. (1959): Study on growth of rickettsiae. V. Penetration of *Rickettsia tsutsugamushi* into mammalian cells in vitro. *J. exp. Med.* **109**, 271—292.

Ewing, E. P., Takeuchi, A., Shirai, A., and Osterman, J. V. (1978): Experimental infection of mouse peritoneal mesothelium with scrub typhus rickettsiae: an ultrastructural study. *Infect. Immun.* **19**, 1068—1075.

Kokorin, I. N. (1968): Biological peculiarities of the development of rickettsiae. *Acta virol.* **12**, 31—35.

Luft, J. H. (1961): Improvements in epoxy resin embedding methods. *J. biophys. biochem. Cytol.* **9**, 409—414.

Luft, J. H. (1966): Ruthenium red staining of the striated muscle cell membrane and the myotendial junction, pp. 65—66. In R. Uyeda (Ed.): Proc. 6th Int. Congr. for Electron Microscopy, vol. 2, Tokyo, Maruzen.

Popov, V. L., and Ignatovich, V. F. (1976): Electron microscopy of surface structures of *Rickettsia prowazeki* stained with ruthenium red. *Acta virol.* **20**, 424—428.

Porter, K. R., Kelley, D., and Andrews, P. M. (1972): The preparation of cultured cells and soft tissues for scanning electron microscopy. In *Proc. 5th Annual Stereoscan Colloquium*, Kent Cambridge Scientific, Inc., Morton Grove, Illinois.

Porter, K. R., Prescott, D., and Frye, J. (1973): Changes in surface morphology of chinese hamster ovary cells during the cell cycle. *J. Cell Biol.* **57**, 815—836.

Rikihisa, Y., and Ito, S. (1980): Localization of electrondense tracers during entry of *Rickettsia tsutsugamushi* into polymorphonuclear leukocytes. *Infect. Immun.* **30**, 231—243.

Sato, T. (1968): A modified method for lead staining (in Japanese). *J. Electr. Microsc.* **17**, 158—159.

Schaechter, M., Bozeman, F. M., and Smadel, J. E. (1957): Study on the growth of rickettsiae. II. Morphologic observations of living rickettsiae in tissue culture cells. *Virology* **3**, 160—172.

Silverman, D. J., and Wisseman, C. L., Jr. (1978): Comparative ultrastructural study on the cell envelopes of *Rickettsia prowazekii*, *Rickettsia rickettsii*, and *Rickettsia tsutsugamushi*. *Infect. Immun.* **21**, 1020—1023.

Silverman, D. J., and Wisseman, C. L., Jr. (1979): In vitro studies of rickettsia-host cell interactions: Ultrastructural changes induced by *Rickettsia rickettsii* infection of chicken embryo fibroblasts. *Infect. Immun.* **26**, 714—727.

Silverman, D. J., Wisseman, C. L., Jr., and Waddell, A. (1980): In vitro studies of rickettsia-host cell interactions: Ultrastructural study of *Rickettsia prowazekii*-infected chicken embryo fibroblasts. *Infect. Immun.* **29**, 778—790.

Tamura, A., and Urakami, H. (1981): Easy method for infectivity titration of *Rickettsia tsutsugamushi* by infected cell countig (in Japanese). *Jap. J. Bacteriol.* **30**, 783—785.

Tamura, A., Urakami, H., and Tsuruhara, T. (1982): Purification of *Rickettsia tsutsugamushi* by percoll density gradient centrifugation. *Microbiol. Immunol.* **26**, 321—328.

Urakami, H., Tsuruhara, T., and Tamura, A. (1982): Intranuclear *Rickettsia tsutsugamushi* in cultured mouse fibroblasts (L cells). *Microbiol. Immunol.* **26**, 445—447.

Wisseman, C. L., Jr., and Waddell, A. D. (1975): In vitro studies on rickettsia-host cell interactions: Intracellular growth cycle of virulent and attenuated *Rickettsia prowazeki* in chicken embryo cells in slide chamber cultures. *Infect. Immun.* **11**, 1391—1401.

Wisseman, C. L., Jr., Edlinger, E. A., Waddell, A. D., and Jones, M. R. (1976): Infection cycle of *Rickettsia rickettsii* in chicken embryo cells and L-929 cells in culture. *Infect. Immun.* **14**, 1052—1064.

Explanation of Electron Micrographs (Plates LIV—LVIII):

Figs. 1—5 show scanning electron micrographs.

Fig. 1. Uninfected L-cell monolayer 24 hr after shaminoculation. Numerous microvilli extend almost vertically from the cell surface and many filipodia stretching from the cell margin are seen. × 1,800.

Fig. 2. Portion of a cell at 30 min after inoculation of *R. tsutsugamushi*. 2A — the inoculated rickettsiae are adhering to the cell surface (× 5,400); 2B — the microorganism partially surrounded by the host cell membrane are indicated by arrow (× 18,000).

Fig. 3. Cells 24 hr p.i. Clumps of inoculated rickettsiae (arrows) still can be observed on the cell surface. Decrease in the number of microvilli is significant in comparison with Fig. 1. × 1,800.

Fig. 4. Portion of a cell at 48 hr p.i. Progeny of rod shaped budding rickettsiae* on the cell surface is distinguishable from the round shaped inoculum rickettsiae (compare Fig. 3). × 9,000.

Fig. 5. Cell surfaces at 96 hr p.i. Numerous budding rickettsiae are seen on the cell surface in Fig. 5A (× 1,800). At a higher magnification (Fig. 5B), microorganisms just budding and elevating the host-cell membrane (arrows) can be recognized near the clumps of rickettsiae finishing the budding process. × 9,000.

Figs. 6—10. Electron micrographs of thin sections.

Fig. 6. Uninfected L-cell stained with ruthenium red. Darkly stained surface layer is clear. × 30,000.

Fig. 7. Infected cell at 96 hr p.i. stained with ruthenium red. Budding rickettsiae seen on the cell surface (arrowheads) are covered with the dye staining the host cell membrane. × 15,000.

Fig. 8. Rickettsiae budding from the cell surface at 96 hr p.i. not stained with ruthenium red. All budding rickettsiae are surrounded with the host cell membrane (arrowheads) which is not seen around the intracellularly localized rickettsiae. × 19,800.

Fig. 9. Higher magnification of the lower left portion from Fig. 7, showing ruthenium red positive fuzzy coat (arrowheads). × 150,000.

Fig. 10. A budding rickettsia at high magnification, no ruthenium red staining. Host-cell membrane, rickettsial cell-wall and its plasma membrane are clearly distinguishable; subunit structure of rod shape is seen on the surface of outer leaflet of the cell-wall (arrow-heads).

Abbreviations used in Figures: Ri = rickettsia; M = mitochondria; MV = microvilli; fp = = filipodia; r = ruffle. Bars in μm as indicated.