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ELECTRON MICROSCOPIC ANALYSIS OF IN VITRO INTERACTION OF *RICKETTSIA PROWAZEKII* WITH GUINEA PIG MACROPHAGES. I. MACROPHAGES FROM NONIMMUNE ANIMALS

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Summary. — Monolayer cultures of peritoneal macrophages of intact guinea pigs were infected with *Rickettsia prowazekii* (strain Breinl) and examined by electron microscopy after 30 min, 4 and 24 hr post-infection (p.i.). Three parallel processes developed in infected macrophages: reproduction of rickettsiae in macrophage cytoplasm, destruction in phagolysosomes and production of spheroplast-like forms. Reproduction of rickettsiae yielded 2 cell types: those with dense and with light cytoplasm; they were located side by side in the microcolony and seemed to have a common capsule-like coat. Relatively small spheroplast-like forms of about 1 μm in size were regularly detected. Addition of immune serum to macrophages increased the number of rickettsiae, both of rod-shaped as well as of spheroplast-like ones located within phagosomes, but elicited no increase in the number of digested pathogen cells.

Key words: Rickettsia prowazekii; macrophages; reproduction; destruction; spheroplast; nonimmune guinea pigs

Introduction

Macrophages are known to contribute immensely to the resistance against infection, such as epidemic typhus. Thus, for instance, as early as in 1959, Kokorin showed in scrapings of guinea pig peritoneum that macrophages from immune animals digested rickettsiae without any damage to their own integrity. Meanwhile, rickettsiae reproduced well in macrophages from intact guinea pigs, thereby causing death of macrophages. Later, on studies on interaction of rickettsiae and macrophages in culture (Vovk *et al.*, 1980; Kekcheeva *et al.*, 1981) confirmed the ability of *Rickettsia prowazekii* to reproduce in macrophages from intact animals and demonstrated active digestion of the pathogen by the cells of immune animals. Correlation between digestive activity of macrophages and extent of immunity in the animal as

well as the significance of immune serum for the digestion process were established.

For understanding of processes developing in macrophages of intact and immune animals we examined the cells infected with *Rickettsia prowazekii* by electron microscopy. The present paper deals with interaction of rickettsia with the macrophages from nonimmune (intact) animals.

Materials and Methods

Peritoneal macrophages from guinea pigs. Macrophages were obtained from intact animals as described previously (Kekcheeva *et al.*, 1981). To stimulate the formation for macrophage-containing peritoneal exudate the animals were given 7 ml of thioglycol broth intraperitoneally 3 days prior to autopsy.

Macrophage cultivation. Macrophages were cultivated in flat-bottom test tubes 11 mm in diameter. For electron microscopic examination 1 ml of medium was introduced into each test tube. For the analysis of macrophages in light microscope, 1.5 ml of cell suspension in Medium 199 (500,000–600,000 cells per 1 ml) was placed into each test tube with cover slip. Macrophages were cultivated in Medium 199 containing 10 % bovine serum without antibiotics for 48 hr at 37 °C.

Preparation of rickettsial culture. Rickettsia prowazekii Breinl strain-infected yolk sacs were broken by shaking with glass beads. The rickettsial egg culture was suspended in saline, centrifuged for 10 min at 1,500 rev/min; the supernatant was used for infection of macrophages. Macrophages were infected at doses of 10^7-10^8 ELD_{50}.

Infection of macrophages. Two variants of experimental design were used, namely in the presence of 10 % normal or immune guinea pig serum; the latter was obtained 2 1/2 or 3 months post-infection (p.i.). After centrifugation, the rickettsial culture was treated with appropriate serum, the mixture was kept for 30 min at room temperature and inoculated onto the macrophage culture. Two hr p.i., the medium was removed and Medium 199 with normal or immune guinea pig serum, respectively, was added. Uninfected macrophages were treated in the same manner as controls.

Light microscopic examination. For assessment of the degree of macrophage damage, cover slips were examined 30 min, 4 hr and 48 hr p.i. The preparations were stained according to Giemsa.

Electron microscopy. At intervals 30 min, 2, 4 and 24 hr p.i. monolayer cultures of infected macrophages were fixed directly in the flat-bottom test tubes with a mixture of formaldehyde, glutaraldehyde and picric acid (Ito and Rikihisa, 1981) in 0.2 mol/l cacodylate buffer (pH 7.4), post-fixed with 1 % OsO_4 solution in the same buffer, treated with 1 % uranylacetate solution in 0.2 mol/l maleate buffer (pH 6.0), dehydrated in ethanol and absolute acetone and directly in the test tube embedded into Araldite M (resin layer was about 3 mm thick). The embedded material was separated from the glass in liquid nitrogen, the appropriate site of the monolayer was marked under light microscopy control. If necessary, the selected area was then sawed out and fixed on standard acrylic or Araldite blocks 8 mm in diameter. Ultrathin sections were prepared with the use of Ultratom-III LBK 8800 (LBK, Sweden), contrasted with aqueous solution of uranylacetate and lead citrate and then examined in electron microscope JEM-100B (JEOL, Japan) with objective aperture of 50 μm in diameter at 80 kV and magnification from 5 000 to 50 000×.

Results

In macrophage culture prepared from intact animals thirty min p.i. with normal serum treated rickettsiae, as a rule, single rickettsiae could be seen ouside cells when examined in the light microscope. A few damaged cells could be seen after 4 hr. After 2 days dead macrophages prevailed; they appeared to contain many rickettsiae. The latter often occurred outside cells. When rickettsiae were treated with immune serum the outcome was different.

Some macrophages contained rickettsiae already after 30 min p.i.. By hr 4 the number of damaged macrophages increased. On day 2 death of macrophages was accompanied with acummulation of rickettsiae; the rickettsiae were located within as well as outside of dead cells.

Electron microscopy showed that already after 30 min p.i. dividing ricketts ae were present in the macrophage cytoplasm (Fig. 1). At that interval andilater on, cytoplasmic rickettsiae were enclosed by a large electrontranslucent area, the so-called halo (Fig. 2). Microcapsule was quite distinct on the surface of the majority of rickettsial cells.

At 4 hr p.i., in addition to dividing rickettsiae, the cytoplasm of macrophages regularly contained phagosomes with rickettsial cells showing signs of destuction indicating that these phagosomes were phagolysosomes. In addition to rickettsiae, these phagolysosomes contained vesicles of different size (Figs. 3, 4). Spheroplast-like rickettsial forms 0.6 to 1 μm in diameter were regularly found also in such macrophages (Fig. 4). Their cell wall membrane usually formed small projections (detaching from the cytoplasmic membrane). The periplasmic space of these forms was somewhat dilated. The cytoplasm of infected macrophages contained a considerable amount of vesicles of different size. The Golgi complex and granular endoplasmic reticulum showed normal appearance.

Twenty-four hr after infection of macrophages from nonimmune animals, in addition to spheroplast-like rickettsial forms and rickettsiae in the state of destruction, another 2 types of rickettsial cells loosely arranged in the cytoplasm were observed: 1) rickettsiae with dense cytoplasm and a cell wall closely adjacent to the cytoplasmic membrane, and 2) rickettsiae with a lighter cytoplasm and a wider periplasmic space (Fig. 5). In type 1 rickettsiae the outer membrane (cell wall) revealed occasional small invaginations. In type 2 rickettsiae the membrane could produce invaginations not followed by the cytoplasmic membrane, whereas in type 1 rickettsiae the cytoplasmic membrane always followed the invaginations (Fig. 5). Rickettsiae of both types were located in the same microcolony within cytoplasm and seemed to be surrounded by a common capsule-like (slime) coat.

Spheroplast-like forms in these cells could be located near the reproducing rickettsiae. Their periplasmic space was enlarged and could form rather significant expansions in the sites of cell wall projections. Some spheroplast-like forms reached substantial sizes (1.5 — 2 μm; Fig. 6). They had a loosened cytoplasm in which nucleoid fibrils could be seen. They were not surrounded by halo, which seems to indicate that these forms do not have a capsule-like coat.

The above-described phenomena were observed during cultivation of macrophages in the medium with normal guinea pig serum. Upon addition of immune serum the above-described processes of reproduction of rickettsiae, destruction in phagolysosomes and spheroplast formation were observed in the macrophage cytoplasm. However, in latter case a significantly higher number of both, bacillus-shaped rickettsiae and spheroplasts long remained morphologically intact within the vacuoles (Fig. 7). Nevertheless,

several spheroplasts within such vacuoles showed signs of degeneration of cytoplasm and nucleoid even 4 hr p.i. (Fig. 7). Cytoplasm of macrophages cultivated with immune serum, as well as those with normal serum, appeared intact.

Discussion

Electron microscopic examination of macrophages infected with the virulent *Rickettsia prowazekii* strain has shown development of 3 parallel processes: reproduction of the agent, its destruction in phagolysosomes and formation of spheroplast-like forms.

Electron microscopic findings indicated that reproduction of rickettsiae proceeded as a rule. Division started as early as after their penetration into macrophage cytoplasm. However, unlike to "nonprofessional" phagocytes from continuous cell cultures — in our systems the endocyto plasmic colonies of *Rickettsia prowazekii* did not reach a considerable size. A similar finding has been previously noted with chlamydiae (Popov *et al.*, 1973).

A peculiar "dimorphism" of rickettsial cells was noteworthy: the presence of dense and translucent cells within a single microcolony at 24 hr p.i. Ultrastructure of translucent cells indicates that their cell wall has a less rigid structure, but this do not justify to group them along with unbalanced growth forms of gram-negative bacteria (Kats *et al.*, 1984). Similar translucent forms of *Rickettsia prowazekii* have been recently described in L-cells and are probably related to the phenomenon of population heterogeneity, which is a characteristic also of nonparasitic bacteria (Popov, 1985).

The fact that some rickettsiae began to divide in the macrophage even within 30 min p.i., while others remained in the cytoplasm for rather a long time without reproduction, indicated that they penetrated the host cell (and consequently, were released from it) at various phases of cell cycle. It can be recalled that the duration of cell cycle in *Rickettsia prowazekii* was about 9 hr (Wisseman and Waddel, 1975).

In the course of morphological destruction rickettsiae have been observed within vacuoles limited by a membrane and generally containing vesicles and concentric membranes. Therefore, these vacuoles were considered for phagolysosomes. Phagolysosomes containing rickettsiae in the course of destruction were detected at all intervals tested. It is evident from electron microscopic findings that addition of immune serum to macrophages from non-immune animals led to an increase in the number of rickettsiae located within phagosomes, but failed to increase significantly the number of digested rickettsiae. The increased number of phagosomes containing rickettsiae can be explained by 2 reasons: 1) immune serum promotes the enhancement of production (or recirculation) of membranes within the macrophage or 2) immune serum inhibits penetration of engulfed rickettsiae from the phagosomes to the cytoplasm.

Similar data, though not supported by morphological evidence, have been obtained in other rickettsial species: *Rickettsia typhi* and *Rickettsia tsutsu-*

gamushi. Pretreatment of rickettsiae with immune serum caused, on one hand, a significant enhancement of phagocytosis, and on the other hand, a subsequent digestion of most of rickettsiae (Gambrill and Wisseman, 1973*a*, *b*; Nacy and Osterman, 1979). Nonspecific treatment of rickettsiae with methylated bovine serum albumin resulted in enhancement of their ingestion by macrophages; however, no destruction occurred, and *Rickettsia prowazekii* cells reproduced in these cells and eventually destroyed them (Gambrill and Wisseman, 1973*b*). On the other hand, antibody treatment was without effect on the growth and reproduction of rickettsiae in chick fibroblast culture, in the yolk sacs of chick embryos, or in the louse intestine cells (Wisseman *et al.*, 1974). In contrast, the ingestion of antiserum-treated rickettsiae to L-929 cells was much less evident (Turco and Winkler, 1982). The absence of ultrastructural findings does not allow conclusions as to the subtle mechanisms underlying these processes.

Spheroplast-like forms were regularly detected in nonimmune macrophages. The relatively small size of these forms was noteworthy: their general diameter was 1 μm, i.e. it was essentially equal to the length of bacillus-shaped rickettsiae. Meanwhile, the diameter of eubacterial spheroplasts was 3 to 10 times as large as that of vegetative cell (Prozorovsky *et al.*, 1981). Morphologically these forms were indistinguishable from rickettsial spheroplasts produced by penicillin treatment, although the latter are larger in size: about 1.4 — 1.8 μm in *Rickettsia akari* (Barkhatova *et al.*, 1984) or even 10 times as large as the regular pathogen size (Gudima *et al.*, 1977), i.e. in latter case corresponding to the size of bacterial spheroplasts.

Thus, electron microscopic examination has shown that virulent *Rickettsia prowazekii* not only reproduced, but were also inactivated in macrophages from intact guinea pigs. Cytologic mechanisms of this inactivation are the same as in non-professional phagocytes (Popov and Barkhatova, 1981, 1984), namely, destruction and spheroplast-formation; however, in macrophages these processes occurred more often and were more intensive, especially in the presence of immune serum.

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Legends to Figures (Plates VII—XI):

Fig. 1. Division of *Rickettsia prowazekii* in the cytoplasm of macrophage of intact animals; 30 min p.i. Magn. ×70,000.

Fig. 2. Rickettsial cells enclosed by halo in the macrophage cytoplasm 4 hr p.i. Magn. ×45,000.

Fig. 3. Phagolysosome containing a rickettsial cell with signs of morphologic destruction and vesicles of different size at 4 hr p.i. Magn. ×70,000.

Fig. 4. A fragment of macrophage cytoplasm containing rickettsiae, spheroplast and phagolysosome with a rickettsial cell in the course of destruction at 4 hr p.i. Magn. ×50,000.

Fig. 5. Two types of rickettsial cells free in the macrophage cytoplasm 24 hr p.i. Magn. ×60,000.

Fig. 6. Spheroplast-like rickettsial form free in the macrophage cytoplasm; beside — a normal-sized rickettsial cell. 24 hr p.i.

Fig. 7. Macrophage after immune serum treatment containing vacuoles with intact rickettsia and spheroplast in the course of destruction, 4 hr p.i. Magn. ×80,000.