Ultrastructure of the Infectious and Reproductive Forms of *Holospora obtusa*, a Bacterium Infecting the Macronucleus of *Paramecium caudatum*

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The ultrastructure of the infectious and reproductive forms of *Holospora obtusa*, a bacterium colonizing the macronucleus of *Paramecium caudatum*, was analysed with the aid of ultrathin sections, freeze-fracture, freeze-etching and cytochemical electron microscopical techniques. The infectious form differed considerably from the reproductive form. The bacterial cytoplasm of the infectious form was confined to less than half of the cell, the rest being occupied by a voluminous periplasmic space. The periplasmic space contained a highly osmiophilic fine granular material. At the end of the cell distal from the cytoplasm, a tip containing less osmiophilic fine granular material was observed. Freeze-fracture and freeze-etching studies revealed differences in the patterns of intramembranous particles between the two forms. It is suggested that some of the structures characterizing the infectious form have a function in the infection process.

INTRODUCTION

The mechanism of invasion of eukaryotic cells by bacteria is not understood in most cases. In particular, little is known about how bacteria may leave a phagosome after being phagocytosed and how they are eventually transported to the nuclei or other compartments of the host cells. Some bacteria develop special infectious forms, e.g. the initial bodies of *Chlamydia* (Moulder, 1984), and it must be assumed that the infectious forms have special signals necessary for recognition by the host cell.

Holospora obtusa infects the macronucleus of *Paramecium caudatum* (Protista, Ciliata). The bacteria of the genus *Holospora* show a developmental cycle with reproductive and infectious forms (for reviews see Preer & Preer, 1984; Görtz, 1986). In the host nucleus the reproductive (vegetative) form multiplies by binary division, whereas the infectious form does not divide. During division of the host nucleus the reproductive forms enter the daughter nuclei. The infectious forms, however, remain in the connecting piece of the dividing nucleus and are released from the host cell after its division. Only the infectious forms are able to infect other paramecia, where they leave the phagosomes and enter the nuclei (Ossipov *et al.*, 1975; Görtz & Wiemann, 1989).

Fine structural features of *H. obtusa* have been reported (Gromov *et al.*, 1976; Görtz, 1983). After a description of the infection process by Görtz & Wiemann (1989) and initial biochemical investigations on these bacteria (Görtz *et al.*, 1988) a more complete analysis became necessary. In the present work, the ultrastructure of the reproductive and infectious forms of *H. obtusa* was studied by means of various electron microscopic methods.

METHODS

Organisms. *Paramecium caudatum* (strain ATCC 50009) infected with *Holospora obtusa* was grown in a Cerophyl medium (Görtz & Dieckmann, 1980) inoculated with *Enterobacter aerogenes* as a food organism.

Abbreviations: IF, infectious form; IMPs, intramembrane particles; RF, reproductive form.

Thin-section electron microscopy. Cells were fixed for 30 min with 2.5% (v/v) glutaraldehyde in 0.01 M-PIPES buffer pH 7.2, postfixed with 1% (w/v) OsO_4 , dehydrated with ethanol and embedded in Epon 812. Thin sections were stained with lead citrate and uranyl acetate.

Freeze-fracture electron microscopy. Either paramecia infected with bacteria, or bacteria isolated from the nuclei of paramecia and concentrated on a Percoll (Pharmacia) gradient (Schmidt *et al.*, 1987), were studied. Probes with concentrated cells of a culture (100 ml) were fixed with 3% glutaraldehyde in 0.01 M-PIPES-buffer pH 7.2. Prefixed cells were washed and treated for at least 2 h with 30% (v/v) glycerol in distilled water. Samples were frozen in Freon 22 at -156°C and immediately transferred into liquid nitrogen (-196°C). Replicas were made according to standard techniques on a Balzers BA 300 freeze-fracture apparatus (Balzers AG, Liechtenstein) equipped with electron gun evaporators and a quartz crystal thickness monitor. The fracture faces were coated with platinum/carbon at an angle of 45° followed by carbon at 90° . The replicas were cleaned overnight in household bleach and washed in distilled water. They were picked up on 200 mesh copper grids and examined in a Philips EM 301 electron microscope at 60 kV.

Freeze-etching electron microscopy. For freeze-etching the fixed samples were transferred into distilled water and then frozen and fractured as described above. The probes were fractured as described above. Subsequently, the temperature of the probe was raised from -100°C to -90°C for 3 min. The faces were then shadowed with platinum/carbon as described above.

Cytochemistry with RNAase-gold. Colloidal gold was prepared following the method of Frens (1973) and coupled to RNAase A (Sigma) as described by Bendayan (1984). Cells were fixed with glutaraldehyde as described above but were not postfixed with OsO_4 . Thin sections were rinsed with phosphate-buffered saline (PBS; 0.01 M, pH 7.2) containing polyethylene glycol (0.02% M_r 20000) and pretreated with 3% (w/v) bovine serum albumin. The sections were then incubated with RNAase-gold, rinsed with buffer (three times) and stained with uranyl acetate and lead citrate.

RESULTS

Thin-section electron microscopy

Infected macronuclei contain up to several hundred cells of *H. obtusa*. The ratio of reproductive (RF) and infectious forms (IF) differs depending on the physiological conditions of the host cell (Ossipov *et al.*, 1975; Görtz, 1983), but both forms (and intermediate forms; not shown) are present simultaneously. The two forms could easily be distinguished by their different cytology. In thin sections of the RF, ribosomes and floccular material appeared evenly distributed throughout the cytoplasm. The cytoplasm was surrounded by two membranes with a thin dark layer between them, resembling various Gram-negative bacteria. However, the inner membrane was hardly visible in conventional thin sections (Fig. 1). Proof for the location of the inner membrane came from the freeze-fracture preparations (see below).

Fig. 1. Electron micrograph of the RF of *H. obtusa*. Bar, 0.5 μm .

Fig. 2. Electron micrographs of the IF of *H. obtusa*. (a-c) Cross-sections of the IF at the levels indicated by arrows in (d), which is a longitudinal section of two IF cells, one with the cytoplasmic part shown, the other with the periplasmic part. P, periplasm with osmiophilic material; T, tip with less osmiophilic material; C, cytoplasm. Bar 0.5 μ m.

The morphology of the IF of *H. obtusa* differed considerably from that of the RF in that the cell was subdivided into several compartments. One compartment, surrounded by a membrane, contained densely packed granules, presumably ribosomes (Fig. 2). This compartment is assumed to be the condensed bacterial cytoplasm (Gromow *et al.*, 1976; Görtz, 1983). The surrounding membrane must be regarded as the inner bacterial membrane. Between this membrane and the outer membrane the IF contained osmiophilic material, most of which was

Fig. 3. Freeze-fractures and freeze-etching of the IF. (a, b) Freeze-fractures; bar 0.5 μ m. (c) Freeze-etching; bar 1 μ m. Ei, external face of the inner bacterial membrane; Eo, external face of the outer membrane; Pi, protoplasmic face of the inner membrane; Po, protoplasmic face of the outer membrane.

located in one half of the cell (Fig. 2). The polar tip of this half of the cell contained less osmiophilic material (Fig. 2*d*, compare also Fig. 6), which was not separated from the osmiophilic material by a membrane. One or more thin tubes extending from the main body of the cytoplasm were frequently found to project far into the periplasm, sometimes up to the polar tip. In cross-sections of the IF these tubes were seen as small circles (Fig. 2*a*). Beneath the outer membrane a dense layer surrounding the osmiophilic material and the less osmiophilic material was visible (Fig. 2). This layer may be regarded as part of the cell wall.

Fig. 4

Fig. 4. Freeze-fracture of the RF. Pi, protoplasmic face of the inner membrane; Po, protoplasmic face of the outer membrane. Bar 0.5 μ m.

Fig. 5

Fig. 5. Freeze-etching of the IF. Note the pore-like structures (arrows) which were only found at the pole of the IF. Bar, 0.5 μ m.

Fig. 6. *H. obtusa* in the macronucleus of *P. caudatum* treated with gold-labelled RNAase. In the IF, only the compartment encircled by the inner membrane is labelled, indicating the presence of RNA. In the RF, the label is distributed throughout the cell. Unstained section. Bar, 1 μ m.

Freeze-fracture and freeze-etching electron microscopy

To characterize the membrane layers of the bacteria more precisely, the freeze-fracture technique was used. Freeze-fracture of the outer membrane of the IF of *H. obtusa* revealed that the external face was almost devoid of intramembranous particles (IMPs), and only a few particles were present on the protoplasmic face (Fig. 3*a, b*). Also, freeze-etching preparations of the IF exhibited a smooth particle-free external surface (Fig. 3*c*). In contrast, the protoplasmic face of the outer membrane of the RF contained high numbers of IMPs (Fig. 4).

Fig. 7. Cytological organization of the IF of *H. obtusa*. cw, cell wall; cy, cytoplasm; im, inner membrane; om, outer membrane; p, osmiophilic periplasm; t, less osmiophilic tip.

The fracture faces of the inner membranes of both the RF and IF were studded with many IMPs (Fig. 3*a,b*), the protoplasmic faces exhibiting a denser population of particles than the external faces. Even with the freeze-fracture method no membrane could be seen between the osmiophilic material and the less osmiophilic material in the polar tip, thus confirming the thin-section observations. At one pole of the IF, numerous pore-like structures with a diameter of about 12 nm could be observed (Fig. 5). Replicas of longitudinally oriented IFs showed these pore-like structures only at one end.

Cytochemistry

Although the observations by conventional electron microscopy and freeze-fracture preparations indicated that the compartment surrounded by the inner membrane is the cytoplasm of the IF, we wanted to prove that the electron-dense material is not part of the cytoplasm. Therefore, the distribution of RNA was investigated using gold-labelled RNAase. Epon-embedded cells clearly showed labelling of the nucleoli of the host nuclei and of host cytoplasmic ribosomes (results not shown). IFs were strongly labelled over the suggested cytoplasm surrounded by the inner membrane. No gold label was found over the osmiophilic material or over the less osmiophilic tip (Fig. 6), indicating that no RNA is present in these compartments. The cytological organization of the IF is summarized in Fig. 7.

DISCUSSION

The IF and RF represent two stages of the life cycle of *H. obtusa* (Ossipov *et al.*, 1975). The extreme structural differences between the two forms, which are visible with the light and electron microscope, correspond to significant differences of protein patterns between the two forms (Görtz *et al.*, 1988) and indicate the existence of a dramatic type of cellular differentiation unique for bacteria. Ultrathin sections, freeze-fracture, freeze-etching and most clearly the cytochemical localization of RNA with gold-labelled RNAase show the unusual structural organization of the IF of *H. obtusa*.

The differences in outer membrane structure of IF and RF are consistent with the concept that the IF is metabolically inactive, whereas the RF is active and multiplies (Ossipov *et al.*, 1975; Görtz & Dieckmann, 1980; Gromov & Ossipov, 1981). The density of IMPs indicates that the outer membrane of the RF is active in the transport of metabolites. The IF, which is also a resting stage (Ossipov *et al.*, 1975; Görtz, 1983), may have little or no transmembrane transport. The IF is resistant to antibiotics, which kill the RF (Görtz & Dieckmann, 1980; Görtz, 1983). Also, some lytic enzymes, which could digest components of the host chromatin (Görtz, 1983), may be excreted by the RF.

The most remarkable feature of the IF is its huge amount of periplasmic material. Although a number of proteins are located in the periplasm (Görtz *et al.*, 1988; and unpublished), the nature and significance of the periplasmic material are largely unknown. During the infection process the IF always leaves the phagosome and enters the nucleus with the polar tip leading (Görtz, 1983; Fujishima & Fujita, 1985; Görtz & Wiemann, 1989). The polar tip is reminiscent of the acrosome of sperm cells or the rhoptries of *Plasmodium* or other apicomplexa (Görtz & Wiemann, 1987). The significance of the pore-like structures observed at one pole of the IF is

unknown, and it remains to be clarified whether these structures are located at the same pole of the IF as the polar tip. Cytological observations suggest that components of periplasmic material are exported at the polar tip during the infection process (Görtz & Wiemann, 1989).

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