INFECTION AND IMMUNITY, Dec. 1978, p. 804-809
0019-9567/78/0022-0804$02.00/0

Vol. 22, No. 3
Printed in U.S.A.

Ultrastructural Studies on the Interaction Between *Salmonella typhimurium* 395 M and HeLa Cells

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Received for publication 11 September 1978

The interaction of *Salmonella typhimurium* 395 MS and its rough Rd-mutant 395 MR10 with HeLa cells was studied by transmission and scanning electron microscopy. The bacteria attached to central as well as more marginal positions of the HeLa cell surface. Bacteria associated preferentially to HeLa cells with a relatively low number of microvilli, in which they often were entangled. Bacteria attached to the cell border were sometimes surrounded by membrane folds, possibly as a response to their attachment. Infected cells had longer and more slender microvilli compared with noninfected cells. Some parts of the attached bacteria were in close contact with the HeLa cell membrane, whereas other parts were separated from the latter by a gap. Bacteria adhered preferentially to microvilli without obvious membrane damage. Most of the intracellular bacteria were surrounded by a membrane, often appearing as a vacuole, which sometimes contained more than one bacterium. Intracellular bacteria seemed to be morphologically intact. We propose that *S. typhimurium* enter HeLa cells by a process of phagocytosis.

The early events in the pathogenesis of salmonellosis have during the last years received increasing attention. The tendency to interaction between host cell surface and bacteria leading to attachment to and internalization into the host cells is profoundly influenced by the bacterial cell surface (12, 22, 25). Nonspecific physicochemical factors therefore seem to influence the interaction between host and microbe (4, 26, 27). The association of *Salmonella typhimurium* with HeLa cells is, however, impeded, without concomitant alterations in physicochemical bacterial surface properties, when the bacteria are exposed to heat or UV irradiation (12). Thus, when essential metabolic processes in the bacteria are interrupted, there is a pronounced reduction in the tendency to adhere. This raises the question of whether the bacteria enter HeLa cells by active penetration or as a consequence of phagocytic activity by the HeLa cells. Phagocytosis in professional phagocytes induces specific metabolic (28) and ultrastructural (9) features. In penetration, where the incentive is with the bacterium rather than with the host cell, the process should be more independent of host cell activity. Studies on the effects of metabolic inhibitors on the internalization of *S. typhimurium* into HeLa cells have given results similar to the phagocytosis of particles by professional phago cytes (13). To further elucidate the mechanisms of attachment, internalization, and the intracellular fate of bacteria, the present communication shows at the ultrastructural level the interaction between *S. typhimurium* and HeLa cells.

MATERIALS AND METHODS

Bacterial strains. The highly mouse-virulent smooth (S) strain *S. typhimurium* 395 MS and the rough (R) mutant 395 MR10 (chemotype Rd) derived from it have been described earlier (5, 8, 14).

Cultivation of bacteria. All strains were kept at 4°C on agar slants before use. The bacteria were inoculated into 15 ml of glucose broth and incubated at 37°C for 18 h. The bacteria were harvested by centrifugation (1,100 × *g* for 15 min), washed twice in phosphate-buffered saline solution (pH 7.3), and suspended to 4×10^7 bacteria per ml (estimated with a Turner spectrophotometer at 650 nm) in Earle balanced salt solution, pH 7.3 (Flow Laboratories, Irvine, Scotland).

Mammalian cell culture and its interaction with bacteria. The HeLa cell line (ATCC strain CCL2, human serum research grade) and the cell culture material were purchased from Flow Laboratories. Specimens of cells were kept in liquid nitrogen and thawed at intervals not longer than 3 months. The cells were grown in Eagle minimal essential medium supplemented with 10% fetal bovine serum, 100 IU of penicillin, and 100 IU of streptomycin per ml. Cells were maintained as monolayers in glass bottles in a humidified incubator with an atmosphere of 5% CO_2–95% air at 37°C (ASSAB, Sundbyberg, Sweden). The monolayers were treated with 0.25% trypsin to detach the cells, and the detached cells were used to

prepare new monolayers. Monolayers to be used for transmission electron microscopy were cultivated in glass petri dishes. HeLa cells for scanning electron microscopy were cultivated on cover slips, 10 mm in diameter, attached with petrolatum to the bottom of a plastic tissue culture petri dish (50 by 13 mm). The petri dishes were seeded with 2 ml of HeLa cell suspension and 1 ml of fresh medium. The dishes were incubated for approximately 72 h in the humidified incubator with changes of medium every 24 h, the last change without penicillin and streptomycin. The cells were not contaminated with mycoplasma, as regularly tested by staining with orcein (7). After incubation for 72 h, the medium was poured off from the petri dishes, the bottoms were rinsed three times with 37°C phosphate-buffered saline (pH 7.3), 3 ml of bacterial suspension was added, and the petri dishes were incubated again in the humidified incubator. After 3 h of incubation with bacteria, the liquid was removed, and the petri dishes were rinsed three times with 37°C phosphate-buffered saline (pH 7.3) and prepared for electron microscopy. As a control HeLa cells not exposed to bacteria were treated identically.

Transmission electron microscopy. The monolayers were fixed in situ with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 30 min at 20°C and for another 30 min at 4°C, rinsed in 0.1 M cacodylate buffer (pH 7.2), and postfixed in 1% osmium tetroxide in Veronal buffer (pH 7.2) for 30 min at 4°C. They were rinsed with Veronal buffer (pH 7.2), stained for 30 min with uranyl acetate, and dehydrated in an acetone gradient. Gelatin capsules filled with Vestopal W were inverted over the monolayer. After polymerization, the embedded cells were separated from the petri dish by breaking off the capsules. Thin sections were cut with a diamond knife in an LKB Ultratome parallel to the cell surface. The sections were stained with uranyl acetate and lead citrate and examined in a Philips 300 electron microscope at 80 kV.

Scanning electron microscopy. The monolayers on cover slips were fixed in situ with 2% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.2) for 1 h at 37°C, after which the fixative was replaced, and fixation continued at 20°C to a total of about 60 h. The specimens were rinsed in 0.15 M cacodylate buffer (pH 7.2), postfixed in 1% osmium tetroxide in 0.15 M cacodylate buffer (pH 7.2) for 30 min at 20°C, and rinsed in 0.15 M cacodylate buffer (pH 7.2). They were then dehydrated in a graded series of acetone and dried in a critical-point drying apparatus (Polaron E 3000) with liquid CO_2 as the transitional fluid. The cover slips were mounted on stubs and coated with carbon and gold and examined in a scanning electron microscope (JEOL JSM-S1) at 10 kV and at a tilting angle of 45°.

RESULTS

Transmission electron microscopy. Bacteria were seen attached to the HeLa cell membrane and in different locations intracellularly (Fig. 1 and 2). At locations on the HeLa cell membrane where bacteria were observed, numerous projections appeared. These projections ran in different directions because they were seen sectioned perpendicularly as well as parallel to their long axis in the same section (Fig. 1). At different distances from the bases of the projections, bacteria were in contact with the HeLa cell membrane. The contact always appeared as close between some parts of the bacterial and HeLa cell surfaces. Other parts of the bacteria were separated from the cell membrane by a narrow gap (Fig. 1).

Bacteria were also observed intracellularly. They were localized from close to the plasma membrane to the perinuclear region (Fig. 2). All bacteria, with few exceptions, were enclosed in a membrane structure, indicating that they were contained in vacuoles (Fig. 2). Most of the vacuoles contained only one bacterium, but several enclosed two, three, or in exceptional cases even four bacteria (Fig. 2). Some vacuoles also contained finely dispersed electron-dense material (Fig. 2). Of the intracellular bacteria, the majority seemed morphologically intact (Fig. 2). Rarely, structures with a partly multilayered and granular appearance were observed in vacuoles (Fig. 3). Microtubules and microfilaments were seen in the vicinity of intracellular bacteria (Fig. 2).

The tendency to interaction with bacteria seemed to vary from cell to cell. Some cells were free from bacteria. HeLa cells that had interacted with MR10 bacteria often did so with more than one bacterium.

Scanning electron microscopy. Noninfected HeLa cells showed similar surface topography, as reported by others (21, 23). The number of cell surface protrusions varied from cell to cell. The cell surface protrusions of noninfected cells in interphase consisted of relatively short microvilli, ruffle-like processes, and exceptional blebs of different sizes (data not shown). Furthermore, the peripheral parts of the cells were often not in contact with the substrate.

No gross morphological changes deviating from normal HeLa cells were observed on cells exposed to bacteria. Minor differences were however observed already after 3 h of bacterial incubation. The most conspicuous difference of HeLa cells exposed to MR10 bacteria was the appearance of longer and more slender microvilli anchoring the bacteria to the cell surface as the number of shorter microvilli, ruffle-like processes, and blebs were reduced (Fig. 4).

No obvious differences could be observed in the pattern of interaction for MS and MR10 bacteria in this study. Very few MS bacteria could be observed associated with HeLa cells, agreeing with earlier observations (11–13). Therefore, most of the data are based on observations on live MR10 bacteria.

The bacteria were found associated preferentially to HeLa cells with a relatively low number

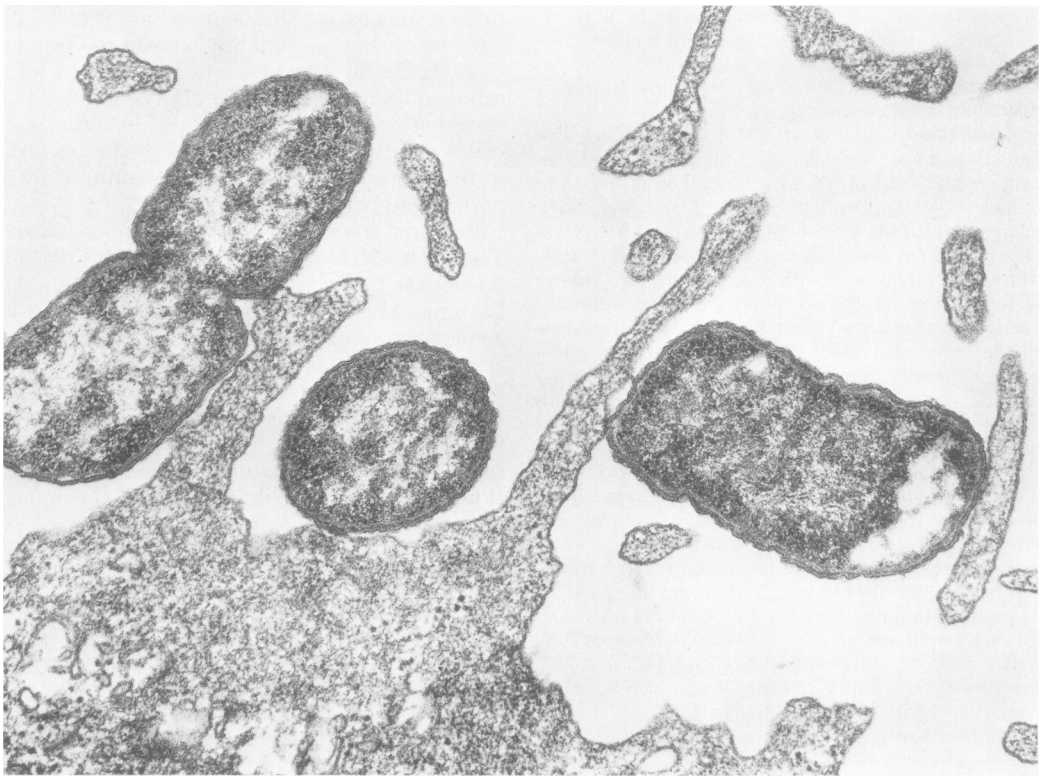

FIG. 1. *Micrograph depicting HeLa cell exposed to viable MR10 bacteria. Close contact is seen between some parts of the bacterial cell surface and the HeLa cell membrane. HeLa cell protrusions are sectioned in different planes.* ×*47,000.*

of microvilli or in some cases even to cells almost devoid of microvilli. Association to cells with numerous microvilli was infrequent. Cells in mitosis never showed associated bacteria. The attached bacteria most often adhered to microvilli (Fig. 4) even when the HeLa cell showed few microvilli. Bacteria attached to the cell border were sometimes partly covered by membrane folds (Fig. 5). Bacteria were also occasionally seen partly embedded in the HeLa cell without distinct membrane folds.

DISCUSSION

Microorganisms and other particles may attain an intracellular localization in different kinds of epithelial cells. Whether this is a consequence of endocytosis has been studied by the ultrastructural appearance of the interaction process. Although not quantitative, electron microscopic data conclusively demonstrate an endocytosis-like process by which microorganisms (3, 6) and biologically inactive material (2, 30, 32) are ingested into nonprofessional phagocytes. However, it cannot be excluded that microbial products (soluble or membrane bound) may enhance the phagocytic process, as has been proposed for *Toxopolasma gondii* (10).

The attachment of a negatively charged *S. typhimurium* 395 MR10 bacterium (16) to a negatively charged cell surface may imply electrostatic repulsion. A low radius of curvature of the contacting surfaces will reduce the repulsive forces and promote cell contact (31). As shown in Fig. 1, the bacteria tended to attach to cell surface projections or by their polar ends to the cell membrane, rather than to stick to flat areas of the cell membrane. This may be a way of overcoming the repulsive forces. When HeLa cells from a suspension culture were spread on a glass surface, the histochemical staining reaction for surface sialic acid was reduced (17). This might indicate a structural rearrangement of sialic acid during cell spreading and retraction, leading to decreased negative surface charge density on cells forming a monolayer, thereby promoting cell contact.

Bacteria attached to different areas of the cell surface (Fig. 4) and showed differences in the mode of interaction. Most of the bacteria were entangled in microvilli (Fig. 4), whereas others,

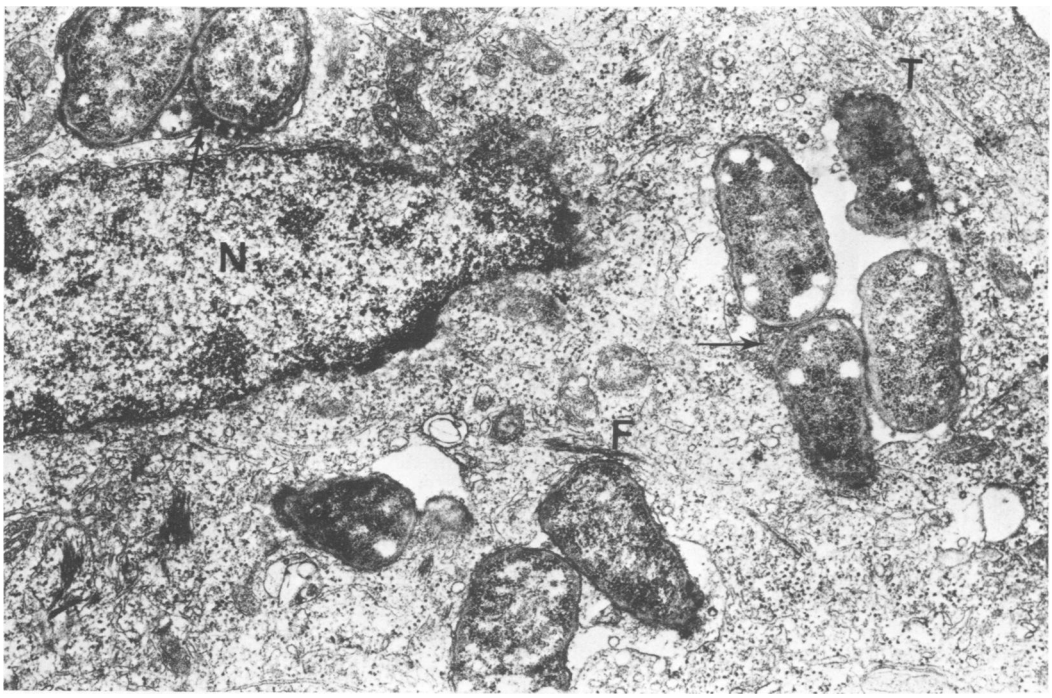

FIG. 2. *HeLa cell exposed to viable MR10 bacteria. Intracellular membrane-enclosed bacteria in the perinuclear region. One vacuole contains four bacteria; the others contain one or two. Most bacteria appear morphologically intact. Electron-dense material is seen in the vacuoles (arrows). Microtubules (T) and microfilaments (F) are found in the vicinity of the bacteria. N, nucleus. ×27,000.*

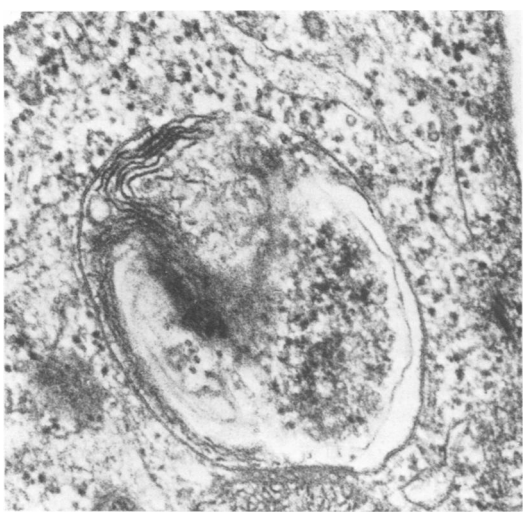

FIG. 3. *Micrograph showing a vacuole containing a myelin-like figure and dense granulated material from a HeLa cell exposed to viable MR10 bacteria. ×62,000.*

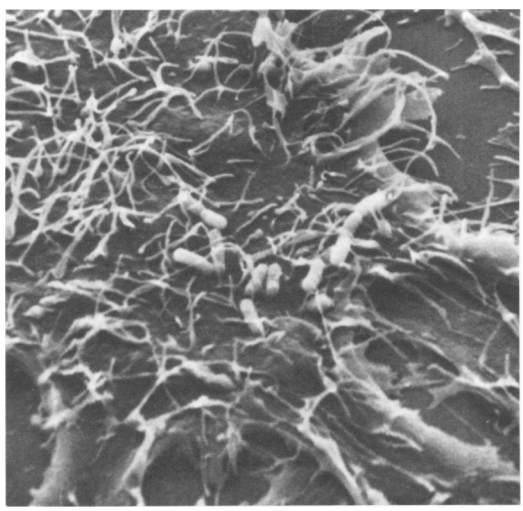

FIG. 4. *HeLa cell exposed to viable MR10. The bacteria are attached to the cell surface in clusters at central as well as marginal positions and seem entangled in long slender microvilli. ×3,400.*

especially those attached to the cell border, were more embedded in membrane folds (Fig. 5). Rather similar sheets folding around opsonized erythrocytes being phagocytosed by macrophages were observed by Orenstein et al. (18). This is considered to be an early step in the

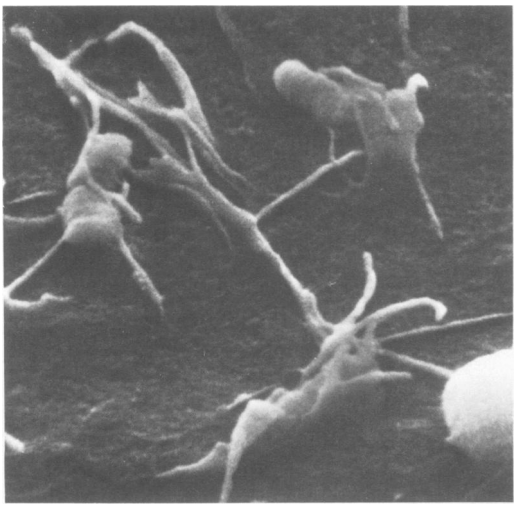

FIG. 5. *HeLa cell exposed to viable MR10. Attached bacteria are found partly covered by membrane folds.* ×7,900.

ingestion phase.

Because MS and inactivated bacteria interacted with HeLa cells to a lesser extent than viable MR10 (11, 12), an active interaction-promoting role for certain bacteria is indicated. This role may imply active bacterial penetration with host cell membrane lysis and attaining an intracellular localization with minor contribution from the host cell. When *S. typhimurium* bacteria come within a critical proximity (35.0 nm) to the guinea pig small intestine, the microvilli degenerate, which may enhance the attachment (22), and the bacteria become enclosed by membrane-bound intracellular vesicles(29). We have not been able to confirm cell surface degeneration, but found intracellular bacteria enclosed in vacuoles. Differences in the bacterial cell surface properties and between the microenvironment of intestinal mucosa in vivo and HeLa cells in vitro may explain the differences between Takeuchi's (29) and our results. We propose that viable MR10 bacteria are superior to MS and inactivated bacteria in triggering the phagocytic capacity of HeLa cells. The nature of this triggering mechanism is still obscure. It is of interest, however, that the release of lipopolysaccharide and of periplasmic enzymes such as alkaline phosphatase is greater from *S. typhimurium* LT2 chemotype Rd_2 than from the isogenic smooth strain of the same organism (15). Alkaline phosphatase is also released to a greater extent from *S. typhimurium* 395 MR10 than from MS (Kihlström, unpublished data). Osada and Ogawa have found an extracellular product from virulent *Shigella flexneri* 2a to stimulate the phagocytic activity of HeLa cells (19). Furthermore, phospholipase activity has been demonstrated in the outer membrane of *S. typhimurium* (20). The presence of viable bacteria is, however, not a sine qua non for attachment and intracellular localization, because inactivated bacteria are also ingested, although to a lesser extent. This indicates a phagocytic process, rather than a penetration. Furthermore, HeLa cells treated with phagocytosis-inhibiting metabolic agents, such as iodoacetic acid and cytochalasin B, ingest fewer bacteria than untreated cells (13).

Most of the intracellular bacteria appeared to reside in cytoplasmic vacuoles (Fig. 2). Structures reminiscent of damaged bacteria, partly containing multilayered myelin-like figures, were occasionally seen in vacuoles (Fig. 3) (1). Similar figures were also found in noninfected cells. Thus, some of these structures might represent degenerated bacteria, whereas others certainly are of different origins. In no case could fusion with cytoplasmic organelles be ascertained, but this may be difficult with the method used. However, some intracellular membrane-limited bacteria were surrounded by electron-dense material which may represent discharge of lysosomal contents into the phagosome (Fig. 2) (24, 32).

We thus conclude that bacteria interacting with HeLa cells in culture will reside within membrane-limited vacuoles. This process has many similarities with the uptake of bacteria by professional phagocytes.

ACKNOWLEDGMENTS

We thank Björn Afzelius, The Wenner-Gren Institute, University of Stockholm, Stockholm, Sweden for valuable discussions. The skilled technical assistance of Kerstin Hagersten, Margareta Lindroth, Marianne Oldbäck and Gunilla Ökvist is gratefully acknowledged.

This work was supported by grants 16X-2183 and 12X-734 from the Swedish Medical Research Council.

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