SCANNING ELECTRON MICROSCOPIC STUDY OF HeLa CELL INVASION BY SALMONELLA TYPHI

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ABSTRACT

Invasion of cultured non-phagocytic cells by salmonella is illustrated by a scanning electron microscopic study of HeLa cell invasion by *S. typhi*. This study shows that after bacterial adherence the HeLa cell cytoplasmic membrane shows ruffling and formation of filopodia which gradually engulf the bacterium and draw it into the cytoplasm. The available literature is reviewed and the probable mechanism underlying phagocytosis is hypothesized.

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KEY WORDS : HeLa cells; *S. typhi*.

Introduction

Salmonella infections result when ingested bacteria pass through the stomach and are engulfed by the epithelial cells of the ileum. The bacteria enter and travel through the epithelial cell within a vacuole and cause cytotoxic damage to the epithelium. The species causing enteric fever proceed from the epithelium into the deeper tissues before entering the reticulo-endothelial system where they multiply and disseminate. The species causing gastroenteritis do not penetrate the basement membrane of the epithelium [1]. Knowledge of interactions between salmonella species and non-phagocytic cells is fragmentary and little is known of microbial determinants, cell receptors involved, and their relation to host specificity.

S. typhi, the causative organism of typhoid fever is a human pathogen with no animal reservoir. Absence of an animal model for *S. typhi* infections have necessitated the use of in vitro techniques for the study of microbial pathogenesis in typhoid fever. In vitro studies on non-phagocytic cells to quantify adherence and intracellular invasion by *S. typhi* have been carried out on various cell lines like HeLa, Vero and MDCK [2,3].

However no studies which have visualized the interaction between the bacterium and the epithelial cell line by scanning electron microscopic (SEM) could be traced in world literature. This

study was carried out to try and understand and illustrate the interaction between *S. typhi* and HeLa cells.

Material and Methods

The study was done at the 7 Air Force Hospital, Kanpur. Scanning electron microscopy was carried out at the Defence Material Stores Research and Development Establishment, Kanpur.

S. typhi used in the study were obtained from blood cultures of patients suffering from typhoid fever. They were identified by routine biochemical and serological methods [4]. Antimicrobial susceptibility testing was done by the comparative Stokes method [5]. Both sensitive and multiple drug resistant *S. typhi* isolates were used.

HeLa cells were maintained as monolayers in minimum essential medium (MEM) with Earles salts, 2 mM glutamine, 5 per cent bovine serum albumin, and 10 µg/ml Gentamicin in 25 cm² flasks. For sub-culturing, MEM was removed and monolayers rinsed in 10 mL of phosphate buffer saline (PBS). One mL of 0.1 per cent trypsin ethylenediamine tetraacetic acid in PBS was added and incubated at 37°C for 1 minute. Five mL of growth medium was added and cells dispersed by pipetting. Cells were counted using a haemocytometer and density adjusted to 6x10⁵ cells/mL. For adhesion and invasion studies 100 µL of the

above suspension was deposited into each well of a 12-well flat bottom tissue culture plate and incubated at 37°C in 5 per cent CO₂ (approximately 60,000 cells per well). Pieces of glass cover-slips were flamed and added to each well. The monolayers formed over the glass slips. Prior to addition of *S. typhi* the cells were washed with Dulbecco's PBS.

Late log phase *S. typhi* used for adhesion and invasion studies were obtained by inoculating the bacterial cells in 2 mL of Luria Bertanni broth which was modified by addition of 1 mM Ca⁺⁺ [6] and incubated for 6 hours. Bacteria were washed in saline and resuspended in MEM to an approximate concentration of 10⁸ cells/mL. Ten µL were inoculated into each well of a 12-well tissue culture plate with HeLa cell monolayer previously prepared as above (cell : bacterial ratio of 1:33).

Monolayers were washed thrice with PBS after 15 minutes to remove non-adherent bacteria and incubated at 37°C in 15 per cent CO₂ with fresh MEM. To study the stages of invasion after 15, 30 and 45 minutes, monolayers in columns 1,2 and 3 respectively of the culture plate were washed thrice with PBS and one mL of buffered glutaraldehyde [7] was added. Fixation was done at 2 hours with cells in column 4 to visualize invasion. The cells on the cover slip were dehydrated in graded alcohols and air dried. The specimens were coated with gold and examined under the scanning electron microscope (Joel 500). The angle of observation was 45 degrees. The gun voltage employed was 10 kV.

Results

Representative scanning electron microscopic photographs are shown as Figs 1-4 and show various stages of adhesion and invasion of HeLa cells by *S. typhi*. Fig 1 shows a normal HeLa cell monolayer at low magnification. Fig 2 taken at 15 minutes of incubation shows adhesion of *S. typhi* to the HeLa cells. The HeLa cell membrane appears smooth and relatively unruffled. Fig 3 shows the response of HeLa cells to the adherent *S. typhi* after 30 minutes. The cytoplasmic membrane shows ruffling and formation of many filamentous filopodia. A hollow is formed below the bacterium and the filopodia begin to encircle the organism. They increase in number and the bacterium is soon

buried within the cytoplasm (Fig 4 taken at 45 minutes). The stage after intracellular invasion is unremarkable on SEM, except that there is a considerable rounding-off and dehiscence from the wells and are probably due to the cytopathic effect of the intracellular bacterium. The isolates of sensitive and resistant *S. typhi* induced similar changes on the HeLa cell membrane.

Discussion

The transepithelial transport process by which invasion of the host occurs appears to be dependent on microbial factors and is designated 'parasite-directed endocytosis' to distinguish it from

Fig. 1 : (Magnification 600 x). The photograph shows normal HeLa cells.

Fig. 2 : (Magnification 20000 x) The photograph taken at 15 minutes of incubation shows *S. typhi* adherent on a HeLa cell. The cell membrane is smooth and does not show ruffling or pseudopodia formation.

Fig. 3 : (Magnification 20000 x) The photograph taken at 30 minutes shows ruffling of the cytoplasmic membrane, formation of filopodia and formation of a hollow around the bacterium adherent on the cytoplasmic membrane prior to ingestion.

Fig. 4 : (Magnification 15000 x) The photograph taken at 45 minutes shows the organism almost buried within the cytoplasm.

'host-directed endocytosis' by cells such as macrophages that eventually degrade the parasites. Initial invasion of the host by a number of human pathogens, e.g. *N. meningitidis*, *H. influenzae*, *L. monocytogenes*, *Shigella*, *Salmonella*, and strains of enteroinvasive *E. coli* is accomplished by parasite directed endocytosis [8].

Takeuchi [9] was the first to study experimental salmonella infection by *S. typhimurium* in guinea pigs using transmission electron microscopy. Salmonellae invading the epithelial brush border induced the degeneration of the microvilli, apical cy-

toplasm and the terminal web. A cavity formed in the apical cytoplasm before the advancing bacterium developed into a bacterium-containing membrane bound vacuole, incorporating degenerated microvilli and pinched-off apical cytoplasm. The vacuoles then were transported to the supranuclear region of the epithelial cells where they decreased in size. They then passed into the subnuclear region where they were enclosed in either a tight membrane-bound vacuole or surrounded by dense osmophilic material. He also noticed organisms within phagocytes in the lamina propria tightly enclosed within membrane bound vacuoles. Bacteria were also seen lying free within the cytoplasm. Yabuchi et al [10] studied the invasion of HeLa cells by *S. typhi* using light microscopy. They used Giemsa stain to differentiate between internalized and external bacteria. They found that formalin-killed strains were still adhesive while those killed with boiling water were neither adhesive nor internalized and concluded that internalization of *S. typhi* by HeLa cells should be regarded as invasion and not phagocytosis and could be used as an indicator to study invasiveness of the *S. typhi* isolates. Many studies on epithelial cell line adhesion and invasion by salmonellae have been reported (2,3,11,12). However only a few have studied invasion and adherence by *S. typhi* [6]. No report on SEM study of *S. typhi* invasion of HeLa cells could be traced in literature and the present study is unique in this respect.

Galan et al [13] isolated a set of three genes from *S. typhimurium*, *invA*, *invB*, *invC* and *invD* responsible for invasion of tissue culture cells. Recently Altmeyer et al [14] have identified a chromosomal gene *invH* responsible for efficient adherence and entry of *S. choleraesuis* and *S. typhimurium* into cultured epithelial cells. Hybridization studies have shown these genes to be present in all the salmonella strains tested including *S. typhi*. It has been shown that the expression of *S. typhimurium* genes required for invasion is regulated by changes in supercoiling [15]. This is shown by de-novo synthesis of *Salmonella* proteins on exposure to epithelial cell surface [16]. Bett et al [17] in a study using tn10 mutants of *S. typhimurium* have shown that invasiveness is multifactorial with at least 6 distinct genetic loci. Intact motility enhances invasiveness of cultured

cells and invasion loci involved in uptake into epithelial cells are also needed for uptake into cultured phagocytic cells. This suggests a similar molecular response mechanism during parasite endocytosis in both phagocytic and non-phagocytic cell types and possibly a role of common mediators and cell receptors.

It has been traditionally believed that liberation of lipopolysaccharide (LPS) occurs only after death and lysis of bacteria. Recent data however have shown that LPS release can occur from viable bacteria as a function of the growth phase. Both smooth and rough forms of LPS were released and bound with serum HDL and acute phase proteins [18]. Alpuche-Aranda et al [19] have shown that bone marrow macrophages exposed to *S. typhimurium* displayed generalized plasma membrane ruffling and macropinocytosis. Ikewaki et al [20] showed that U937, a monocyte-like cell-line, when cultured for 48 hours with LPS resulted in increased adhesion with induction of numerous filamentous filopodia on the cell surface, with enhanced expression of CD16 and CD23. They showed that these changes were mediated via the Cd11b molecule.

Reitmeyer et al have shown that the salmonella cytotoxin is a component of the outer membrane [2] and is involved in the interaction between the host cell and organism. Salmonella cytotoxins have been shown to be proteinaceous, immunologically distinct from shiga toxin and closely related to shiga-like toxins produced by *E. coli* [22]. Further studies have shown the salmonella cytotoxin to inhibit protein synthesis.

The present study demonstrates induction of plasma membrane ruffling and formation of filamentous pseudopodia (filopodia) in epithelial cells by *S. typhi* prior to phagocytosis. It is hypothesized that for induction of phagocytosis of the bacterium by the non-phagocytic mammalian cell both LPS and OMP play a dual role. The LPS released into the microenvironment causes ruffling, macropinocytosis, and filopodia formation on the adjacent cytoplasmic membrane, while the OMP cytotoxin prevents ruffling of the cell membrane underlying the adherent bacterium by inhibiting protein synthesis. The final result is filopodia surrounding the bacterial cell which rests in a hollow on the cyto-

plasmic membrane where protein synthesis has been inhibited thus inducing phagocytosis.

Further studies on identifying the non-phagocytic cell receptors for LPS, molecular mechanisms underlying induction of filopodia formation, and the nature of receptors on the cell surface responsible for protein synthesis inhibition may provide a pointer to alternative strategies for prevention of salmonellosis. All this can be conveniently accomplished by using cultured mammalian epithelial cell lines, a convenient experimental model.

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