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Enteroaggregative *Escherichia coli* induced increase in intracellular calcium concentration modulates cytoskeletal F-actin rearrangement and bacterial entry in INT-407 cells

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ABSTRACT

Background: Enteroaggregative *Escherichia coli* (EAEC) is an emerging enteric pathogen, associated with cases of acute and persistent diarrhoea worldwide. The pathogenesis of EAEC is yet to be understood. In intestinal epithelium, an increase in $[Ca^{2+}]_i$ has been attributed due to the action of different enteric pathogens. EAEC was shown to increase $[Ca^{2+}]_i$ in HEp-2 cells.The present study was undertaken to investigate the effect of EAEC induced increase in $[Ca^{2+}]_i$ oncultured human intestinal epithelial cells.
Methods: INT-407 cells were infected with EAEC (T8 strain) in the absence and presence of dantrolene (inhibitor of release of Ca^{2+} from intracellular stores)/verapamil (L-type Ca^{2+} channel blocker)/BAPTA-AM (Ca^{2+} chelator)/U73122 (PLC inhibitor)/Cytochalasin-D (inhibitor of actin polymerization). $[Ca^{2+}]_i$ was estimated using Fura-2/AM. Cytoskeletal rearrangement was assessed by F-actin staining using TRITC-phalloidin. The invasiveness of EAEC-T8 to INT-407 cells was checked by electron microscopy and invasion assay.
Results: A significant increase in $[Ca^{2+}]_i$ was observed in EAEC-T8 infected INT-407 cells, which was reduced in presence of dantrolene/verapamil/U73122. EAEC-T8 could induce cytoskeletal F-actin polymerization in INT-407 cells and was found to be invasive in nature. The cytoskeletal rearrangement as well as invasion of EAEC-T8 was attenuated in presence of U73122/dantrolene/BAPTA-AM/verapamil/cytochalasin D.
Conclusions: EAEC induced increase in $[Ca^{2+}]_i$ seems to play a major role in host cytoskeletal F-actin rearrangements leading to invasion of the organism.
General significance: Our study undoubtedly will lead to an improved understanding of EAEC-pathogenesis.

1. Introduction

Enteroaggregative *Escherichia coli* (EAEC) is rapidly gaining recognition as an important pathogen causing diarrhoeal disease in multiple epidemiologic and clinical settings [1]. The pathogenicity of EAEC has been attributed to the characteristic aggregative adherence (AA) property followed by colonization and ultimately damage to host mucosa, apparently via elaboration of different toxins. The AA phenotype of EAEC is known to be associated with a 65 MDa plasmid (pAA). Most of the putative virulence factors of the organism were shown to be localized on pAA, thereby indicating the importance of the plasmid in the virulence of the organism [2].

Enteric bacterial pathogens have been shown to interact with the host via intimate biochemical crosstalk leading to stimulation of signaling cascades resulting in rearrangement of the host cytoskeleton and infection. It has been reported that Enterohemorrhagic *E. coli* and enteropathogenic *E. coli* rely on the type III secretion apparatus, which translocates bacterial effector proteins into host cells resulting in the formation of actin pedestals [3,4]. The induction of cytoskeletal rearrangement to facilitate invasion of host cell is a common strategy in bacterial pathogenesis. Pathogens like *Salmonella* and *Shigella* could induce self internalization into intestinal epithelial cells in a process involving cytoskeletal reorganization that could lead to the formation of ruffle at the site of bacterial contact with the host cells [5,6].

However, EAEC induced alteration in host cell cytoskeleton has not been reported so far, although it was shown that invasion of EAEC (strain 162) in HeLa cells could be inhibited in presence of

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cytochalasin D [7]. Further, it has also been reported that Pet (the plasmid encoded toxin, an autotransporter protein) of EAEC (042 strain) could cause cytoskeletal disruption through degradation of spectrin and fodrin (nonerythroid spectrin), connected with the cytoplasmic actin network [8]. EAEC is generally considered as a non-invasive pathogen present only in the lumen of the intestine and attached to the epithelial cells [2]. However, some strains of EAEC have been reported to be protected in the gentamicin invasion assay [9]. It is possible that the attachment, invasion and intracellular persistence may be a strategy of these organisms to evade expulsion from the intestine and contribute to persistent diarrhoeal disease.

Intracellular calcium, the universal second messenger is known to regulate various cellular processes and its cytosolic concentration has been found to be tightly regulated [10]. EPEC was shown to induce an increase in $[Ca^{2+}]_i$ in HEp-2 cells which could result in the formation of the characteristic lesion by calcium-dependent activation of actin- proteins, with eventual loss of absorptive capacity [3]. Increased intracellular Ca^{2+} has been found to be associated with the microfilament-dependent invasion of *Salmonella typhimurium* in cultured eukaryotic cells [11]. Further, it was reported that *Campylobacter jejuni* induced mobilization of Ca^{2+} from host intracellular stores was an essential event in the invasion of intestinal cells by this pathogen [10]. However, no such report exits in case of EAEC. In the present study, an attempt has been made to investigate the effect of EAEC induced increase in $[Ca^{2+}]_i$ oncultured human intestinal epithelial cells.

2. Methods

2.1. Chemicals

All the chemicals used in this study were of analytical grade. Dantrolene (inhibitor of release of calcium from intracellular stores), verapamil (L-type Ca^{2+} channel blocker), BAPTA-AM (calcium chelator), U73122 (phospholipase C inhibitor) and Cytochalasin-D (inhibitor of actin polymerization) were procured from Sigma Aldrich (USA).

2.2. Cell culture

INT-407 cell line (an epithelial cell line derived from human embryo small intestine) was procured from National Centre for Cell Sciences (Pune, India).The cells were propagated in Minimum Essential Medium (MEM) supplemented with 2 mM L-glutamine, Earle's balanced salt solution (Gibco BRL, USA), Sodium bicarbonate (1.5 g/l), penicillin (50 U/m), streptomycin (50 μg/ml) and 10% heat inactivated fetal calf serum (FCS, Biological Industries, Israel) at 37 °C in a humidified atmosphere of 5% CO_2 - 95% air. Cells from exponentially growing cultures were used in all the experiments.

2.3. Bacterial strains

The EAEC-042 (prototype strain) and EAEC-T8 (a clinical isolate harbouring AAF II) were procured from National Institute of Cholera and Enteric Diseases (Kolkata, India). Fim H^+ strain of *S. typhimurium* was a kind gift from Dr. Philip T. Loverde (State University of New York, Buffalo). The strains were grown at 37 °C in Luria–Bertani medium. All the strains were characterized by the characteristic plasmid profile, PCR using EAEC specific primers [12] as well as on the basis of HEp-2 adherence assay [13]. The T8 strain was cured of its plasmid by repeated passage in increasing concentration of acriflavin [14] and designated as pT8. The curing of plasmid was confirmed by the absence of plasmid as well as EAEC specific PCR product in agarose gel electrophoresis.

2.4. Infection

Unless otherwise mentioned, bacteria were added to the INT-407 cells grown to about 80% confluence in 6 well/24 well tissue culture plates at 1:100 multiplicity of infection in MEM medium without serum and antibiotics. The cells were incubated in the CO_2 incubator at 37 °C for different time intervals as required for each experiment.

2.5. Estimation of intracellular free calcium ion concentration $[Ca^{2+}]_i$

INT-407 cells (10^6/ml) were triggered with EAEC-T8 (10^7/ml) for different time intervals (5 min/10 min/20 min/40 min/60 min/120 min/180 min/240 min/270 min/300 min). Cells without bacteria were used as controls. The cells were washed, resuspended in fresh medium and loaded with 2 μM Fura-2/AM (Sigma, Chemicals, prepared in DMSO) at 37 °C for 1 h. Unabsorbed dye was removed by washing the cells thrice with 10 mM HEPES (pH 7.4)/20 mM NaCl/115 mM KCl/0.5 mM $MgCl_2$/5 mM glucose. Finally, the cells were resuspended in 1 ml of the same buffer and fluorescence of the cell suspension was measured in the quartz cuvette at an excitation wavelength of 340 nm. The emission spectrum was recorded at 510 nm. After measuring the basal fluorescence (F), 2 mM EGTA [prepared in 1 M Tris/HCl (pH 8.8)] was added to the suspension to bring pH of the solution to 8.3. A rapid and sustained decrease in fluorescence after addition of EGTA was attributed to extra Fura-2/AM. Digitonin (10 nM/L) was then added to permeablize the cells and release the trapped dye, which resulted in maximum fall in fluorescence (F_{min}). Then, 5 mM $CaCl_2$ was added to saturate Fura-2/AM and EGTA. The resultant fluorescence signal was designated as F_{max}. The intracellular free Ca^{2+} concentration was calculated by the equation [15]

$$\left[Ca^{2+}\right]_i = 224 \times (F - F_{min})/(F_{max} - F),$$

where 224 is the Kd value of Fura-2/AM.

In a separate set of experiments, the cells were pretreated with dantrolene (20 μM, 15 min, 37 °C/verapamil (50 μM, 15 min, 37 °C)/U73122 (2 μM, 15 min, 37 °C) prior to infection with bacteria for 40 min and $[Ca^{2+}]_i$ was measured in each case. The inhibitors/blocker used in this study were at their reported IC50 concentration. Cells treated with EAEC-T8 were used as controls. All sets of the experiments were performed at least thrice (each time in duplicate).

2.6. Detection of host cell-cytoskeletal rearrangement

INT-407 cells (10^5/ml/well) grown on glass coverslips (Bluestar, India) in six well tissue culture plates were triggered with EAEC-T8/pT8 strain (10^7/ml/well) at 37 °C (in a CO_2 incubator) for 5 h. The cells were fixed in PBS containing 4% paraformaldehyde (HiMedia, India) for 15 min at 25 °C, washed and permeabilized in PBS containing 0.1% Triton X-100 (Sigma, USA) for 5 min at 37 °C. After washing, the cells were stained for F-actin using TRITC-phalloidin (0.65 μg/ml, Sigma, USA). Cells were washed, mounted onto slides in 80% glycerol containing DABCO (1, 4-Diazabicyclo [2.2.2] Octane, 10 mg/ml) and examined under an Olympus BX 61 epifluorescence microscope (Japan). Cells without bacteria were used as controls. For the analysis of F-actin stress fiber disassemble and F-actin ruffling, the scoring method was used as described by Kotani et al. [16]. Briefly, cells with well-organized F-actin network were scored as 1 point. Cells showing atypical or equivocal F-actin disorganization were scored as 0.5 point. Cells which showed no well-organized F-actin network were scored as 0. More than 200