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Hypermutator *Salmonella* Heidelberg induces an early cell death in epithelial cells

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ABSTRACT

We have previously described that a strain of *Salmonella* Heidelberg with a hypermutator phenotype, B182, adhered strongly to HeLa cells. In this work, we showed that this hypermutator *Salmonella* strain invaded HeLa epithelial cells and induced cytoskeleton alteration. Those changes lead to HeLa cell death which was characteristic of apoptosis. For the first time, we showed that this hypermutator strain induced apoptosis associated with the activation of caspases 2, 9 and 3. Complementation of B182 strain showed a decrease in cells death induction. In the presence of other *Salmonella* Heidelberg with a normomutator phenotype, such as WT and SL486, cell death and caspase 3 were undetectable. These results suggested that early apoptosis and caspase 3 activation were specific to B182. Besides, B182 induced LDH release and caspase 3 activation in CaCo-2 and HCT116 cells. Heat-treated B182 and diffusible products failed to induce this phenotype. Epithelial cells treatment with cytochalasin D caused the inhibition of B182 internalisation and caspase 3 activation. These results showed that this cell death required active *S.* Heidelberg B182 protein synthesis and bacterial internalisation. However *sipB* and *sopB*, usually involved in apoptosis induced by *Salmonella* were not overexpressed in B182, contrary to *fimA* and *fliC*. Comparative genome analysis showed numerous mutations as in *rpoS* which would be more investigated. The role of the hypermutator phenotype might be suspected to be implicated in these specific features. This result expands our knowledge about strong mutators frequently found in bacterial organisms isolated from clinical specimens.

1. Introduction

Salmonella Heidelberg is the third most common serovar causing invasive extra-intestinal infections associated with severe disease symptoms (Wilmshurst and Sutcliffe, 1995). Among natural population of *Salmonella* Heidelberg, some strains display a hypermutator phenotype by harbouring mutations in methyl mismatch repair system (MMR) (Le Gall et al., 2009). Hypermutation allows some bacteria to adapt to adverse environmental conditions, including antibiotic exposure (Blázquez, 2003). The prevalence of strong mutators, which are characterised by an increased frequency of spontaneous mutations, ranges from about 1% among pathogenic strains of *Escherichia coli* to more than 30% among *Pseudomonas aeruginosa* strains isolated from cystic fibrosis patients (Oliver et al., 2000). Identification of hypermutator strains among clinical bacteria has generated many questions of whether this evolution contributes to bacterial virulence (Mérino et al., 2002).

Intestinal epithelial cells are the primary host targets during the initial phase of enteroinvasive *Salmonella* infection (Ohl and Miller, 2001). *Salmonella* are able to replicate inside the intestinal cells and induce cell death (Kim et al., 1998; Paesold et al., 2002). Cell death commonly occurs through pyroptosis or apoptosis. Each of these cell death pathways is regulated by distinct molecular mechanisms. For instance, pyroptosis is an inflammatory-mediated form of cell death characterized by activation of caspase 1, whereas apoptosis is a form of programmed cell death initiated by two major pathways, named the extrinsic and intrinsic pathways. In the case of apoptosis, coordinated activation of two groups of caspases is responsible for apoptotic cellular degradation. Based on

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their position in the apoptotic cascade, caspase 8 or caspase 9 initiates a downstream cascade of effector protease such as caspase 3, which cleaves various substrates and leads to the execution of cell death (Man et al., 2013). *Salmonella* trigger death of cultured epithelial cell lines *in vitro* by apoptosis, whereas in macrophages, *Salmonella* rapidly trigger pyroptosis which is dependent on caspase 1 (Kim et al., 1998; Fink and Cookson, 2005).

Initial studies suggested that Type Three Secretion System (T3SS) was involved in cell death mechanism (Fink and Cookson, 2007). T3SS1 translocates SipA, SipB, SipC and SopB proteins, which are implicated in invasion through the plasma membrane of the host cell. SipB has been shown to induce macrophage apoptosis by activating caspase 1, whereas SopB induces anti-apoptotic activity in infected epithelial cells (Hersh et al., 1999; Knodler et al., 2005).

The role of the strong mutator phenotype in pathogenic bacteria has already been discussed (Jolivet-Gougeon et al., 2011; Le Bars et al., 2014), but the link between this phenotype and virulence is not yet well understood. In the present work, the interaction between epithelial cells and *Salmonella* Heidelberg with hypermutator phenotype was investigated. Indeed, *Salmonella* Heidelberg B182 strain, used as a model in this study, was characterized by a mutation in *mutS* (Le Gall et al., 2009; Le Bars et al., 2012b). Internalised B182 bacteria induced rapid epithelial cell death by activating caspases 2, 9 and 3. This killing of epithelial cells required bacterial protein synthesis and direct interaction with living bacteria.

2. Materials and methods

2.1. Bacterial strains, cell culture and infection protocol

Strains of *S.* Heidelberg previously described (Le Gall et al., 2009) were used in this study: one was a B182, with a hypermutator phenotype (deletion of 12 bp in *mutS*), and the two others were normomutator wild type strains, WT and SL486 (Le Bars et al., 2014). B182 strain trans-complemented with the *mutS* wild-type gene from WT Salmonella Heidelberg (Le Gall et al., 2009) was also used. Heat-killed bacteria were obtained by incubating B182 suspension at 65 °C for 15 min. Fresh bacteria were grown overnight at 37 °C as previously described (Le Bars et al., 2012b).

The human epithelial cell line, HeLa, was grown in DMEM (Lonza, Verviers, Belgium). The HCT116 cell line, derived from human colon carcinoma, was grown in McCoy's 5A modified media (Life technologies, Invitrogen, Saint Aubin, France). CaCo-2 cells, human epithelial colorectal adenocarcinoma cells, were maintained in culture in DMEM (Life technologies) containing 4.5 g/L glucose. HeLa and HTC116 cells were supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin (Lonza) and 1% L-glutamine (Lonza) (Lonza). For CaCo-2 cells, cell culture media were supplemented with 20% FCS, 1% penicillin/streptomycin (Lonza) and 1% L-glutamine (Lonza). Cells were maintained in culture at 37 °C in a humidified atmosphere containing 5% CO_2.

One day before invasion assay, cells were trypsinized and seeded in 6-well or 24-well plates. *Salmonella* infections were conducted at a multiplicity of infection (MOI) of 100 bacteria per cell. Cells monolayers were incubated with *Salmonella* in DMEM at 37 °C in 5% CO_2 for 30 min. After incubation, non-adherent bacteria were removed by washing with phosphate buffer saline (PBS, Lonza) and cells were incubated in DMEM containing gentamicin (Lonza) (100 μg/ml) in order to kill extracellular bacteria.

To inhibit bacterial internalisation, HeLa cells were treated with cytochalasin D (5 μM, Sigma) 1 h prior to infection.

2.2. Confocal imaging and transmission electron microscopy (TEM)

For constitutive expression of GFP, B182 and WT strains were transformed with the modified plasmid pNF8 (pNF8 *gfp-mut1)*. Immunofluorescence studies were performed on HeLa cells fixed in 1% paraformaldehyde (Sigma–Aldrich, L'Isle D'Abeau Chesnes, St. Quentin Fallavier, France). The actin cytoskeleton was labeled using TRITC-conjugated phalloidin (Sigma). CellLight Tubulin-RFP (Life Technologies) was used to visualize tubulin according to manufacturer's instructions. Signals were analysed with a confocal microscope (TCS-SP5; Leica). Images were processed using ImageJ analysis software.

Following different times of infection (1, 2, 3, 6 and 24 h), HeLa cells were also treated for transmission electron microscopy analysis as previously described (Le Bars et al., 2012b). Examination was performed with JEOL 1400 electron microscope operated at 120 kV and pictures were obtained by Orius 1000 Gatan Camera.

2.3. LDH release assay

Lactate dehydrogenase (LDH) was released when cell lost its cell membrane integrity. After cells infection, the supernatant medium from infected cells was collected and the amount of LDH released was measured with a cytotoxicity detection kit (CytoTox 96 Non-Radioactive Cytotoxicity Assay) from Promega (Charbonnières, France). LDH release was calculated as suggested by manufacturer's instructions.

2.4. Measurement of apoptosis by flow cytometry analysis

Discrimination between apoptotic and necrotic cells was performed by staining with annexinV-phycoerythrin (PE) and 7-aminoactinomycin (7′AAD) (BD Biosciences, Le Pont de Claix, France). Briefly, infected HeLa cells were harvested using trypsin and subsequent low-speed centrifugation (3000 × *g*, 10 min). Cell pellets were resuspended at 1×10^6 cells/ml in annexinV binding buffer and 100 μl fractions were then incubated for 15 min with 5 μl of AnnexinV-PE or/with 5 μl with 7′AAD at room temperature in the dark. After the addition of 300 μl from the binding buffer, the samples were analysed by flow cytometry using FCS 500 cytometer (Beckman Coulter, Gagny, France).

2.5. Western blot analysis

Equal amounts of infected cells were centrifuged and the pellet was resuspended in standard Laemmli buffer and boiled for 10 min to denature proteins. Proteins were separated by 12% SDS-polyacrylamide (Sigma) gel electrophoresis under denaturing conditions and transferred onto nitrocellulose membrane (Sigma). The membranes were blocked for 1 h in PBT (PBS with 0.05% Tween-20 (Sigma) containing 5% milk). Immunoblots were probed with rabbit anti-caspases 2 or 3 polyclonal antibodies or with mouse anti-caspases 1, 8 or 9 polyclonal antibodies (Tebu-bio, Le Perray en Yvelines, France) for 1 h. Detection of histone 3 with anti-histone 3 rabbit antibodies was also used as loading control. To detect antibodies reactions, the membranes were incubated for 1 h with alkaline phosphatase-conjugated anti-mouse IgG or anti-rabbit IgG antibodies (Sigma). Caspase proteins (pro- and active-forms) were visualised using 5-bromo-4-chloro-3- indolyl-phosphate/NBT (Sigma) and the intensity of the bands was quantified using ImageJ software. The relative expression of cleaved caspases was corrected against histone 3. The ratios between caspase 3 and histone 3 for each lane quantified were means for three independent experiments.