Effects of *Salmonella enteritidis* serovar *typhimurium* Infection in Adenocarcinomic Human Alveolar Basal Epithelial Cells A549 *In vitro*: Bacteria Induce Apoptosis in Adenocarcinomic Cell

Izidoro MS Jr¹, Varela JN1, Alves DA¹, Pereira RFC¹, Brocchi M², Lancellotti M²* and Hollanda LM¹

¹Labiotec Biotechnology Laboratory, IB, Biology institute, UNICAMP-State University of Campinas, São Paulo, Brazil
²Department of Genetics, Evolution and Bioagents, Institute of Biology, State University of Campinas (UNICAMP), Brazil

**Abstract**

*Salmonella enterica* serovar *typhimurium* is a facultative anaerobic bacterium, gram-negative, flagellated which generally develops and grows preferably in a wide variety of tumor cells. Until now, several factors for this predilection have been described, such as: an increase in number of nutrients favorable for bacterial growth due to rapid growth of the tumor cells, adaptation of *S. typhimurium*, loss of macrophages and neutrophils bactericidal activity in areas of hypoxia, the absence of antibodies and complement factors around the tumor. This study analyzed the infection *in vitro* of *Salmonella enteritidis* serovar *typhimurium* in A549 cell lineage, in three different aspects: morphological characterization of the infection, analysis of cellular DNA degradation and production of cytokines such as TNFα. The morphological assay showed tumor cell shrinkage, nuclear fragmentation with DNA degradation and chromatin condensation suggesting that these bacteria were inducing apoptosis on these cells. The cytokines production showed great variations among the strains analyzed and some predictable results, for example, bacterial strains isolated from diarhoea which was more virulent against the tumor cells than others. In conclusion, we have shown some data proving that *Salmonella enteritidis* serovar *typhimurium* is able to adhere and infect adenocarcinomic cells causing programmed cellular death. A pattern was established, the more virulent the strain, the higher the quantity of cytokines produced in the inflammatory process.

**Keywords:** Adhesion; Invasion; Apoptosis; Adenocarcinomic alveolar cells; A549; *Salmonella*

**Introduction**

*Salmonella enteritidis* is a rod-shaped, gram-negative, non-spore forming, predominantly motile *Enterobacteria* with diameters around 0.7 to 1.5 μm, lengths from 2 to 5 μm, and flagella which grade in all directions (i.e. peritrichous). They obtain their energy from oxidation and reduction reactions using organic sources, and are facultative anaerobes [1]. The nomenclature of this microorganism is used in conjunction with molecular biochemical assays for a more accurate analysis of its serovars that are ultimately divided into two types: *Salmonella enterica* (S. enterica), which is subdivided into six sub-species or groups (enteric-Group I, *salamae-Group II, arizonae-Group IIIa, diarizonae-Group IIIb, houtenae-Group IV, indica-Group V), and *Salmonella bongori* (S. bongori) [2]. Although this is the current classification, the division into species and subspecies has little practical importance for clinical veterinary and medical epidemiology. In the routine identification, the scheme described by Kauffman and White is still used. This scheme divides the genus *Salmonella* in serological types (serotypes or serovars) defined by the identification of somatic antigens (O), flagellar (H) and capsular (Vi). The capsular antigens are characteristic of a few samples and when present define a serovar. Also, 2541 have been described taking as a basis this scheme [2-4].

The majority of the virulence genes of the *S. enterica* are located on its “pathogenicity islands (PI)” [5]. Ten of them (IP) have been described in *S. enterica* and other regions of the genome that have similar characteristics of IP which were also studied [6]. SPI-1 was the first to be described [7,8] and encodes a type III secretion system involved in the reorganization of the cytoskeleton and invasion of host cells, as well as in induction of inflammation in the intestinal mucosa [9]. SPI-2 [10], SPI-3 [11] and two-component regulatory system PhoP/Q [12] are involved in intracellular survival and multiplication.

**The S. enterica expresses a number of different types of fimbriae or adhesins that are variable according to the bacterial serovar. These fimbriae are involved with the initial adhesion of bacteria and cells or the extracellular matrix, mediating the colonization of the intestinal tract and participating in the process of tissue invasion [5,13]. In addition, another striking feature is the inflammation of the intestinal mucosa mediated by the secretion of flagellin monomers that reach the cells and initiate the inflammatory process through recognition by Toll-like receptor 5 (TLR 5) [14-16]. The flagellin is also recognized intracellularly by a process involving the activation of caspase-1 and IL-1β in infected macrophages [17,18].

Some *S. enterica* serovars such as *typhimurium*, exhibit the ability to change the type of flagellin expressed, a phenomenon known as flagellar phase variation [19]. *S. enterica serovar typhimurium*, for example, expresses two types of flagellar antigenic proteins, encoded by *flIC* and *fjiB* genes, which are denominated in the serological classification as “1” and “1.2” respectively [20]. This explains the serological name of *S. enterica serovar typhimurium*.

The biological function of the flagellar phase variation in *S. enterica* is yet to be completely understood. The flagellar phase variation suggests

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*Corresponding author: Marcello Lancellotti, Labiotec Biotechnology Laboratory, IB, Biology institute, UNICAMP-State University of Campinas, Sao Paulo, Brazil, E-mail: mlancelot@unicamp.br*

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an escape function of the immune system by increasing the repertoire of antigens expressed by the bacterial cell [21], but its importance on the bacteria’s pathogenicity is still unknown. Some observations have suggested new roles for flagellar phase variation in S. enterica serovar typhimurium [14-16,22-25].

Ikedo et al. [24] showed that mutants of S. enterica, expressing only the antigen phase 2 (fljB) were attenuated, while mutants that expressed only the antigen phase 1 (flic) were as virulent as the wild type strain. Consistent with these results, in vivo studies with the wild type strain suggested a selective advantage to organisms that express flic, thus suggesting that the phase variation is a mechanism that influences the virulence of S. enterica in mice.

It was not yet verified whether different lines with flagellar phase variation differ in the process of adhesion and invasion in tumor cells, as well as in mRNA expression related to the inflammatory process in the adjacent tissues during the first stages of infection (TNF-a and IL-6), variation in one of the main mediators of the inflammatory response (IL-8) and variation in the activity of the enzyme caspase-3 responsible for activation of the apoptotic pathway of the infected cells. Thus, this is the major aim of this work.

The line of research using bacteria to treat tumors began in the second half of the nineteenth century when doctors W. Busch and F. Fehleisen observed that certain inoperable cancers regressed when patients were infected with Streptococcus pyogenes and developed erysipelas [26-28] and remained stagnant until 1976, when Morales, and Bruce Eidinger demonstrated that the Bacillus Calmette-Guerin (BCG) has been successfully used in the treatment of superficial bladder cancer [29]. Today BCG has become the treatment of choice for high risk, superficial bladder cancer in most countries, with an increasing rate of treating approximately one million patients per year [28].

In recent studies, it was shown that attenuated strains of S. enterica typhimurium served as antitumor agents, preferably growing within tumors in a ratio of 1000:1 when compared to normal tissues [30-35]. The preference for tumor tissues can be explained for some facts such as: There is the adaptation of S. enterica typhimurium to grow in areas with low oxygen concentration; increased availability of nutrients released by necrotic cells and decreased bactericidal activity of macrophages and granulocytic neutrophils in regions with low oxygen concentration. Positive pressure and irregularities in vascularityization of the tumor mass also inhibit, but not entirely, the entry of antibodies and complement factors favoring the growth of S. enterica typhimurium [36].

Due to their easy genetic manipulation [37], the attenuation of this specie in order to create antitumor carrier molecules have been widely used, for example, genetic alteration of the lipid A have been produced to reduce septic shock and msl6B gene disruption reducing the induction of TNFα, thereby increasing the LD50 causing shrinkage of melanoma in mice that received such attenuated strain [30]. Furthermore, attenuated strains which produce cytokines with the potential to stimulate the host immune response against tumors [35,38] and ultimately production of a promoter induced by hypoxia (HIP-1) enabling modulation of genes with their transcription increased in the areas of hypoxia in the tumor mass [39].

This study analyzed some strains of Salmonella enterica serovar typhimurium and its ability to cause apoptosis in adenocarcinomic human alveolar basal epithelial cells (A549) and its inflammatory response triggered by bacterial infection.

Material and Methods

Bacterial growth and colony-forming units (CFU) determination

The S. enteritidis strains used in this work (Table 1, Figure 1) were grown in Luria Bertani Agar at 37°C with 5% CO2. For CFU determination, several colonies grown were picked up with a sterile loop and re-suspended in saline solution 0.9 % (w/v) to a final optical density of 1.0.

Maintenance of A549 cell culture

A549 cells were grown in plastic flasks (25 cm2) with RPMI 1640 medium (Cultilab, Campinas, SP, Brazil), supplemented with 2% L-glutamine, 120 μg/mL kanamycin and 13% inactivated fetal bovine serum (complete medium). The cultures were incubated at 37°C in an atmosphere containing 5% of CO2. Medium was changed every 48 hours and the culture reached confluence, the subculture was performed by treatment with trypsin (Cultilab, Campinas, SP, Brazil).

Adhesion assay of S. enterica typhimurium

S. enteritidis growth obtained in Luria Bertani Agar plates was scraped under sterile conditions, resuspended in RPMI1640 medium to an optic density of 1.00 at 600 nm and 1:100 dilutions made in the same medium. Aliquots of 50 μl of the bacterial suspensions containing approximately 1.107 CFU were submitted to the adhesion tests in the A549 monolayer cells per well. After incubation during five hours at 37°C, the monolayers were washed five times with phosphate-buffered saline, pH 7.2. After that Giemsa staining was performed.

Caspase-3 activity assay

A549 cells were seeded in 24-well tissue culture plates and were allowed to grow until confluence and after that; the cells were infected in the conditions described on the adhesion assay. The Caspase-3 activity assay followed the design described by R & D Systems (Minneapolis, MN). After five hours of incubation, the cells were scraped and equivalent protein aliquots of each bacterial strain were determined for caspase-3 activation. All the assays were performed in triplicate. As a negative control, non-infected A549 cells were used.

DNA degradation by gel electrophoresis assay

Following the same conditions as described in the caspase-3 activity assay, after 4 hours of incubation the cells infected with bacteria were scraped and stained with WCLB (White Cell Lysis Buffer) solution (1% SDS, 0.01% bromophenol blue, 7% sucrose, 10 mM EDTA and 10 mM Tris HCl pH=8) and the DNA degradation was analyzed through a gel electrophoresis where the smaller pieces of damaged DNA is agglomerated.

Inflammatory cytokines expression by qRT-PCR

The analysis of the production of inflammatory cytokines such as TNFα, IL-06 and IL-08 were performed by Real Time PCR for those strains that showed morphological alterations when in contact with A549 cells in order to verify the apoptosis induction by bacteria. The cells were infected as described before and incubated for 5 hours. Then, the total RNA was extracted using the Trizol Reagent (Invitrogen, Calsbag, CA, USA). RNA yield was estimated by Nanodrop (Thermo Scientific). A minimum of 0.2 mg RNA was submitted to reverse transcriptase followed by detection reaction by qRT PCR. Real-time PCR primers are listed in table 2. qRT-PCR was performed using a StepONE Plus thermocycler (Applied Biosystems). Each 10 μl reaction
Comparisons Test was done and P<0.05 was considered significant. All experiments were performed in triplicate and the data shown in the graphs and in the table represent the mean standard errors.

Results

The results obtained with the adhesion assay using different strains of S. enteritidis demonstrate a significant adherence and suggests that the bacteria were causing programmed cellular death on the cell line. The optical microscopy analysis (Figure 1) of the adhesion and morphological alterations caused by the bacteria infection detected cellular stress, nuclear fragmentation and chromatin condensation suggesting that the bacteria were inducing apoptosis on the adenocarcinomic epithelial cells.

The DNA fragmentation (Figure 2) and the caspase-3 activation (Figure 3) were also observed among the strains proving that the bacteria triggered apoptosis in A459 cells. Furthermore, the major activator of the caspase-3 was the strain ST666 which was the bacteria that caused higher production of cytokines due to the inflammatory process developed after infection.

| Strain | Description | Origin |
|--------|-------------|--------|
| UK-1   | Salmo...<s>lerella enterica</s> serovar Typhimurium standard strain | ATCC   |
| LT-2   | Salmo...<s>lerella enterica</s> serovar Typhimurium standard strain | ATCC   |
| STi691 | Salmo...<s>lerella enterica</s> | Hemoculture |
| ST692  | Salmo...<s>lerella enterica</s> | Hemoculture |
| ST666  | Salmo...<s>lerella enterica</s> Typhimurium | Faeces |
| ST662  | Salmo...<s>lerella enterica</s> Typhimurium | Faeces |

Table 1: Strains used in this work and adhesion characteristics.

| Chemokine or endog-enous gene | Primers | Reference |
|-------------------------------|---------|-----------|
| IL6  | FW GAGGATAACCCTCCCAACAGACC [40] | RV AAGTGCATCATCGTTGTTCATA |
| IL8  | FW ATGACCTCAGAAGCTGGCTGTGGCT [40] | RV TTGAAATCTCGAGCCCTCTTCAAAA |
| TNFα | FW TCCTTGCAACCACCGAGTGA [40] | RV CCTCTGATGGCCGACCCAG |
| GAPDH | FW TGACACCAAGACCTCCTAGGC [40] | RV GGGATGAGCCTGTGGTCATGAG |

Table 2: Oligonucleotides used in this work for qRT-PCR.

Figure 1: Optical microscopy of non-infected cells 10x (A); A549 infected with ST662 demonstrating the cellular stress, 10x (B); A549 after infection with the strain LT-2 showing both cytoplasmic fragmentation and chromatin condensation, 100x (C); A549 infected with Uk-1, showing cellular death 100x (D).

Figure 2: DNA fragmentation assay using the white cell lysis buffer, the square shows where the fragmented DNA appears: (1) Negative control, (2) UK-1, (3) LT-2, (4) ST662, (5) ST666, (6) ST692, (7) STi691, (8) Ladder 1 Kb.

Figure 3: Caspase-3 colorimetric assay (405nm). P-value<0.05 except for ST662 (P<0.5) and STi691 (P<0.1).

Statistical analysis

The data from each assay were statistically analyzed by the t-student test compared with a control sample, and in the cytokines production assay which was performed, a Student-Newman-Keuls Multiple

Comparison
Discussion

It is well known that *Salmonella enteritidis* can adhere and invade different mammalian cell lines such as HeLa, Hep-2 and Henle [42] but its capacity to harm different tumor cells are yet to be studied. In this work, we highlighted the possibility to use bacteria in the treatment for cancer as a promising delivery system.

*Salmonella* infections affect generally the digestive tract. In mice, *S. typhimurium* causes a systemic disease with symptoms similar to typhoid fever caused by *S. typhi* in humans, regardless of the pathway of infection. Classically, the kinetics of infection in mice is characterized by four phases. The first results in the rapid elimination of bacteria from the serum. During the week following infection, *Salmonella* rapidly replicates within phagocytic cells. This phase precedes a plateau phase, characterized by the recognition of certain pathogens. This results in production of several cytokines (TNF-α, IL-1, IL-6, IL-12, IFN-γ) and a massive infiltration of monocytes and polymuclear neutrophils at sites of inflammation. In the fourth phase, the infection sets acquired inflammatory defense, acting B and T cells, as well as humoral factors that derived from it [43].

The pathogenic mechanisms in salmonellosis are quite complex and is yet to be completely understood. It is known that the bacteria has a great number of virulence factors such as adhesions, exoenzymes and enterotoxins [44] and the ability to invade the gastrointestinal mucosa, multiply, spread and survive within the cells of the reticuloendothelial system.

As a gram-negative bacterium, *Salmonella* expresses in its bacterial wall LPS, a highly immunogenic molecule. LBP (LPS-binding protein), is a serum protein whose main characteristic is to present the LPS on its monomeric form to the CD14 receptor. The first studies described the role of LBP as being necessary for an effective inflammatory reaction to eliminate the invasive pathogen. The CD14 receptor, present mainly in monocytes and macrophages, is the major receptor for LPS. The receptor is presented in two distinct forms, in the membrane (mCD14) and soluble (sCD14) [43]. The attachment of the LPS to the cell does not cause cell activation immediately. The time interval necessary for activation (15-30 minutes) is explained by the need to internalize the complex LPS/CD14. Some studies show that blocking the endosome fusion or its internalization cause a break in the signaling induced by LPS [45].

The complex LPS/LBP may generate a message via the intracellular CD14 receptor, through a protein with a transmembrane domain, the TLR-4 receptor, which specifically recognizes the LPS. This receptor has an extracellular domain rich in leucine and an intracellular domain homologous to the intracellular domain of IL-1 receptor (IRR domain) [46].

The fixing of the complex LPS/LBP to the CD14/TLR-4 complex (in cooperation with a per membrane protein called MD2) produces the activation of two distinct signaling pathways inducing the production of inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8. IL-12.

*Salmonella* is also recognized by TLR-5 receptor. In contrast to the TLR-4, which has several different ligands, TLR-5 presents a unique affinity for flagellin. This protein, presented in bacterial flagella, presents a high immunogenic capacity, for example, it is able to activate the mononuclear phagocyte system [47] and intestinal mucosal epithelial cells [14]. TLR-5 induces a similar message to the TLR-4, once it also produces the inflammatory cytokines TNF-α and IL-6 [47].

In contact with the host cell, *Salmonella* stimulates its own uptake by the epithelial cells. The ability to be internalized into the host cell is utterly important for the success of the infection. For this purpose, it makes use of an apparatus known as type 3 protein secretion (TTSS "type three secretion system"), where it introduces into the host cell, a number of effector proteins that are able to mimic the functions of many proteins in the host cell [48]. The entry mechanism is characterized by a profound rearrangement of the actin cytoskeleton where the bacteria come in contact with the host cell [49]. This remodeling of the cytoskeleton of the cell induces the cell membrane to form extensions which envelop the bacteria, leading to the entry of it into membrane-bound vacuoles. This entry into non-phagocytic cells is absolutely dependent on the release of specific effector proteins introduced into the host cell via the TTSS encoded by a pathogenicity island of *Salmonella* (SPI-1, *Salmonella* pathogenicity island 1”). This virulence factors work together in order to use the host cell proteins for its own behalf and culminate in the entry of the bacteria into the host cell [50].

As stated, the infection of *Salmonella* in the host cell is dependent on several factors that culminate on the host cell death. In this work, we managed to measure some important points of this process such as, damaged cells morphology, DNA degradation, caspase-3 activation due to apoptosis and some important inflammatory cytokines produced by the interaction between host and pathogen.

The cell morphology, DNA degradation and the activation of the caspase-3 (Figure 3) are enough to come to the conclusion that the pathogen is inducing apoptosis on the adenocarcinomous cell. Furthermore, the easy genetic manipulation of this microorganism when added to the fact that it causes programmed cellular death on the tumor makes this bacteria an important and promising candidate for the development of a new therapy based on living organisms.

The inflammatory profile of cytokines demonstrated that the more virulent the bacteria, higher the expression of these molecules by the host cells in comparison with the standard strains. According to figure 4, UK-1 and LT-2, which are standard strains, were not as aggressive as the strains isolated from diarrhoea; supposedly the ones isolated straight from the organism with the pathology are more virulent and instigate the host cells to a higher production of inflammatory molecules.
In conclusion, we can imply that for the purpose of becoming carriers of any chemotherapy, the more virulent the strain of Salmonella, the better it is. The methodology for treatment of adenocarcinomic cells based on living organisms is a promising alternative, although more research is required. This study corroborates with previous data in based on living organisms is a promising alternative, although more research is required. This study corroborates with previous data in

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