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Original article

# Intracellular bacteriolysis triggers a massive apoptotic cell death in *Shigella*-infected epithelial cells

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**Abstract**

Infected epithelial cells, which act as a first barrier against pathogens, seldom undergo apoptosis. Rather, infected epithelial cells undergo a slow cell death that displays hallmarks of necrosis. Here, we demonstrate that rapid intracellular lysis of *Shigella flexneri*, provoked by either the use of a diaminopimelic acid auxotroph mutant or treatment of infected cells with antibiotics of the β-lactam family, resulted in a massive and rapid induction of apoptotic cell death. This intracellular bacteriolysis-mediated apoptotic death (IBAD) was characterized by the specific involvement of the mitochondrial-dependent cytochrome *c*/Apaf-1 axis that resulted in the activation of caspases-3, -6 and -9. Importantly, Bcl-2 family members and the NF-κB pathway seemed to be critical modulators of IBAD. Finally, we identified that IBAD was also triggered by *Salmonella enterica* serovar *Typhimurium* but not by the Gram-positive bacteria, *Listeria monocytogenes*. Together, our results demonstrate that, contrary to previous findings, epithelial cells are intrinsically able to mount an efficient apoptotic cell death response following infection. Indeed, apoptosis in normal circumstances is masked by powerful anti-apoptotic mechanisms, which are overcome in IBAD. Our results also uncover an unexpected consequence of the treatment of infected cells with certain classes of antibiotics.


*Keywords:* Shigella; Apoptosis; Antibiotics; Caspase

## 1. Introduction

The fate of cells infected by a bacterial pathogen is a complex response that is likely dependent on the nature of both the pathogen and the infected cell. In the case of enteroinvasive bacteria such as *Shigella* and *Salmonella*, studies have concentrated on defining the mechanism of bacteria-induced cell death in monocytes and macrophages. In these myeloid cells, both *Shigella* and *Salmonella* have been shown to trigger cell death that displays hallmarks of apoptosis. Moreover, an important feature of bacteria-induced cell death in macrophages is the implication of caspase-1, which results in inflammasome-dependent apoptosis associated with inflammation, through the secretion of IL-1β and IL-18 [1,2].

In contrast to macrophages, the cell death mechanisms in infected epithelial cells remain largely unknown. Other than mechanisms of toxin-induced apoptosis [3], the study of infection per se on epithelial cell death has not been studied in great

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detail. Infected epithelial cells appear resistant to infection-mediated cell death suggesting that either the host intracellular pattern recognition molecules are inefficient at triggering cell death pathways in response to *Shigella* infection, or that strong survival pathways are induced to counteract bacteria-induced cell death mechanisms [4]. In the case of *Salmonella* infection, a report identified that infected epithelial cells are resistant to cell death due to the activation of the pro-survival pathway Akt/PKB by the bacterial effector protein SopB [5].

In this study, our aim was to characterize *Shigella*-mediated cell death in infected epithelial cells. We describe a rapid apoptotic cell death process, by a novel mechanism we term "intracellular bacteriolysis-mediated apoptotic death or IBAD". This form of cell death is triggered during intracellular bacterial lysis, induced either by antibiotic treatment or by fragility of the cell wall of a peptidoglycan mutant. These results uncover an unexpected consequence of the treatment of infected cells with certain classes of antibiotics, which may prove to have important implications in anti-infectious clinical settings.

## 2. Materials and methods

### 2.1. Construction and growth of bacterial strains

*Shigella flexneri* 5 strain M90T streptomycin-resistant ($Sm^R$) and *Salmonella typhimurium* (wild-type strain DT104) were aerobically grown as previously described [6,7]. DNA manipulations were carried out by using standard methods and by following the manufacturers' suggestions. To obtain M90T Δ*dapB* (ZB401) a sequence between the nucleotide 243 and 720 of *S. flexneri dapB* locus, was amplified by using the primers 5′-CCCGGGCTGGAGCGTGAAGGAT-3′and 5′-CCCGGGGCCTTCACGACTGTAG-3′, digested with SmaI (underlined) and then ligated into the suicide plasmid vector pLAC1, obtaining pZB414. pZB414 was introduced into M90T to allow double allelic exchange between the wild-type *dapB* on M90T genome and the mutagenized *dapB* copy on pZB414. The M90T *dapB* gene was amplified with the primers: 5′CCCGGGCTGGAGCGTGAAGGAT-3′ and 5′-CCCGGGGCCTTCACGACTGTAG-3′, which yielded the 975-kb fragment that was cloned into pSTBlue-1 (Perfectly Blunt™ Cloning kit, Novagen) generating pZB415.

### 2.2. Bacterial infections

The HeLa cell invasion assay with *Shigella* was performed as previously described [8]. Briefly, cells were infected with an MOI of 50 and spun at room temperature for 10 min at 2000 rpm. Cells were then incubated for an additional 1 h before washing and adding gentamicin (60 μg/ml). After 1 h of gentamicin exposure, monolayers infected with M90T were treated as follows: either D-cycloserine (ICN, Biomedicals) or fosfomycin (Fluka, Bio-chemika) or imipenem (Merck-Sharp & Dhome), added at the concentration of 200, 20, and 60 μg/ml, respectively, and incubations were prolonged for 2 or 3 h. Cells infected with M90T and incubated with gentamicin alone were used as a control. For ZB401, incubation with gentamicin lasted 3 h and no antibiotic were added. For *Salmonella* infection, the MOI was 50 and the incubation with gentamicin (100 μg/ml) lasted 2 h before treatment with imipenem (200 μg/ml). For *Listeria*, the MOI was 10 and antibiotic concentrations were: 100 μg/ml for gentamicin, 40–300 μg/ml for imipenem, 100 μg/ml for vancomycin. These MOIs were chosen as they gave 90% infection efficiency in preliminary experiments.

### 2.3. Expression plasmids and transfections

C285S, created by cloning a mutated form of caspase-1 into the BamHI–XhoI sites of pEGFP-N1 (Clonetech) and C360S, harboring the DN form of caspase-8, were gifts of Arturo Zychlinsky, Max Plank Institut, Berlin. The plasmids expressing GST-IκBα superrepressor [9] and the IKK2 mutant, catalytically inactive (IKK2), have been already described [8]. Transfection experiments were performed as described [9]. pEF-Bcl-2 pGKpuro [10] was transfected with lipofectamine 2000 in HeLa cells and cell line was single-cell cloned using limiting dilution culture. Vector plasmid (pGKpuro) was used as controls.

### 2.4. Microscopy and cell death analysis

Confocal and electron microscopy were carried out as described previously [8]. Apoptosis was assessed using TUNEL colorimetric staining (Apoptag, Intergen or DeadEnd, Promega) according to manufacturer's instructions. The activities of caspases-1, -3, -6, -8, and -9 were determined with Caspase Colorimetric Protease Assay kits (R&D Systems, Inc.) following manufacturers' suggestions. Apaf1−/− mice have been described previously [11].

For caspase inhibitor experiments, HeLa cells were preincubated with Z-WEHD-FMK (R&D Systems, Inc.) or Z-YVAD-FMK (Stratagene) (both for caspase-1), Z-DEVD-FMK (for caspase-3), Z-VEID-FMK (for caspase-6), Z-IETD-FMK (for caspase-8), and Z-LEHD-FMK (for caspase-9) (all from R&D Systems, Inc.) for 80 min at 37 °C at a concentration of 100 μM prior to *Shigella* exposure. After 1-h incubation p.i. the inhibitors were added again to cell medium and infection proceeded as described above. Cell monolayer incubated with either staurosporine (STP) or camptothecin (CMP) (both SIGMA) at the concentration of 2 μM for 7 h were used as a positive control for caspase-9 and -8, respectively.

### 2.5. Cell lysis and Western blot analysis

For cytochrome *c* analysis digitonin-soluble and insoluble fractions of infected and uninfected HeLa cells were prepared as described [12].

### 2.6. RT-PCR analysis

RNA was obtained from HeLa cells ($5 \times 10^6$ cells) infected as described above or using NucleoSpin® RNA II kit (Macherey-Nagel). Conditions of reverse transcription (RT) and cDNA PCR were as previously reported [13].