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Macrophage apoptosis associated with *Salmonella enterica* serovar Typhi plasmid

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The present study was undertaken to investigate the relationship between plasmid isolated from *S. enterica* serovar Typhi (pR_{ST98}) and macrophage apoptosis. pR_{ST98} was transferred into an attenuated *S. enterica* serovar Typhimurium strain RIA to create a transconjugant pR_{ST98}/RIA. Standard *S. enterica* serovar Typhimurium virulence strain SR-11 was used as a positive control, and RIA as a negative one. Murine macrophage-like cell line (J774A.1) was used as an infectious cell model *in vitro.* In order to determine the inhibition and bactericidal effect of amikacin (AMK) to extracellular bacteria and the best optimization co-culture ratio between *Salmonella* and J774A.1, the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of AMK to strains SR-11, pR_{ST98}/RIA and RIA and multiplicity of infection (MOI) were detected first, and then J774A.1 was infected by the above three serovar Typhimurium strains. Apoptosis of J774A.1 was examined with electron microscopy and flow cytometry after annexin-V/propidium iodide labeling at 0, 1, 3, 6, 12 and 24 h. Mitochondrial membrane potential was detected by JC-1 staining method. It was demonstrated that MIC of AMK to the three strains was 10μg/ml, MBC was 80μg/ml, and optimal MOI was 100:1. pR_{ST98}/RIA resulted in a higher apoptosis of J774A.1 than RIA, apoptotic features such as chromatin margination could be observed after 3 h, and death of J774A.1 cells was associated with the loss of mitochondrial membrane potential. These results indicated that pR_{ST98} could enhance the virulence of its host bacteria, evidenced by increased macrophage apoptosis.

Keywords: *Salmonella enterica* serovar Typhi, Plasmid, Macrophage, Apoptosis

Typhoid fever, an acute and life-threatening infectious disease caused by *Salmonella enterica* subspecies *enterica* serovar Typhi (hereafter referred to as *S.* Typhi), frequently occurs in developing countries. Annual worldwide incidence of typhoid fever is estimated at 21 million cases, with 200,000 related deaths[1]. Although the mortality rate of typhoid fever displays a descending trend all over the world, the incidence rate of this disease still keeps rising. In China, typhoid fever and paratyphoid fever have been under control after 1990. However, the incidence rate of these diseases still remains high with occasional outbreaks at some places. pR_{ST98} is a large 98.6Mdal (159 kb) plasmid isolated from a survey of multidrug-resistant *S.* Typhi strains by our lab in late 1980s. This plasmid encodes resistance to over 10 different drugs and is classified to incompatibility group C (Inc C)[2]. Using PCR, Southern Blot and DNA sequence analysis, our lab first reported that *spv* (*Salmonella* plasmid virulence gene) homologous genetic sequence in all pathogenic *Salmonella* serovars except *S.* Typhi, where it was represented by pR_{ST98}[3]. Although isolated from several sources including camels[4], *S.* Typhi is pahogenic exclusively for humans. Thus, there are no suitable animal model and effective genetic tools. We have been using *S. enterica* serovar Typhimurium (hereafter referred to as *S.* Typhimurium) to study the function of plasmid pR_{ST98}. Our results indicated that pR_{ST98} is a chimeric plasmid that mediates both drug-resistance and virulence. *Salmonella* Typhi is intracellular bacteria, mainly residing in mononuclear phagocytes of host. There are reports suggesting that *S.* Typhi can mediate macrophage apoptosis[5-7], but the underlying mechanisms have not been fully elucidated. In this study the relationship between plasmid pR_{ST98} and macrophage apoptosis was investigated.

Materials and Methods

Bacteria Strains — Three *S.* Typhimurium strains were used. *Salmonella* Typhimurium strain SR-11 was used as a positive control, it carried a 100 kb virulence plasmid[8]. *Salmonella* Typhimurium strain RIA is attenuated for mice[9,10]. pR_{ST98}/RIA was a

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transconjugant by conjugal transferring pR_{ST98} into strain RIA. Strain SR-11, pR_{ST98}/RIA, and RIA were grown to mid-logarithmic phase at 37°C in Luria-Bertani (LB) broth, and quantified spectrophotometrically by determining at OD_{600} nm. Then they were centrifuged at 2,300×*g* for 5 min and resuspended in RPMI 1640 medium without antibiotics before adding to cells.

Cell Culture—Murine macrophage-like cell line J774A.1 was propagated in RPMI 1640 medium with 10% fetal calf serum and 5 m*M* L-glutamine at 37°C in a humidified incubator containing 5% CO2 and 95% air. Cells from exponentially growing cultures were used in all the experiments, and they were seeded in 24-well tissue culture plates at 5×10^5 cells per well 16-24 h before use.

Measurement of MIC, MBC and MOI—In order to determine the inhibition and bactericidal effect of amikacin (AMK) on extracellular bacteria and the best optimization co-culture ratio, the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of AMK to SR-11, pR_{ST98}/RIA and RIA and multiplicity of infection (MOI) were detected. MIC and MBC of AMK to bacteria were determined by serial dilution method. For determination of MOI, J774A.1 cells were seeded overnight into 24-well tissue culture plates with cover slips, then cells were infected with *S.* Typhimurium strains at MOI of 10:1, 50:1 and 100:1, respectively. And the cover slips were stained with Giemsa stain.

FCM analysis of macrophage apoptosis— Mid-logarithmic phase grown cultures of *S.* Typhimurium strain SR-11, pR_{ST98}/RIA, and RIA were added to J774A.1 macrophage monolayers at a MOI of 100:1. After incubation at 37°C for 3 h (0-h time point), infected cells were washed three times with phosphate buffered saline (PBS), then RPMI complete containing 100 µg of AMK per ml was added to kill remaining extracellular bacteria. After 2 h of further incubation at 37°C, the medium in the 24-well plates was replaced again with RPMI containing 10 µg AMK per ml. Host cells remained in this medium for the remainder of the infection to prevent extracellular growth of released bacteria. At different time points (0, 1, 3, 6, 12, 24 h), J774A.1 cells were collected for Flow Cytometer analysis (FC500, Beckman Coulter, USA) with Annexin V-FITC apoptosis detection kit (JinMei Biotech, Shanghai, China). Percentage of apoptosis was determined based on three independent experiments.

*Examination of J*774A.1 *mitochondrial membrane potential*—J774A.1 cells were cultured and infected as described above. At different time points following infection with *Salmonellae*, J774A.1 macrophages were washed twice with PBS and dyed with JC-1 stain (Biyuntian Biotech, Beijing, China) as per the manufacturer's instructions. JC-1 is a cationic dye that fluoresces differently in apoptotic and nonapoptotic cells. Briefly, in living cells with normal mitochondrial membrane potential ($\Delta\psi_m$) the dye is taken up into the mitochondria to form red fluorescent aggregates. However, in apoptotic cells with compromised mitochondrial membrane potential, it remains in monomeric form in the cytosol and fluoresces green. In this study the change of mitochondrial $\Delta\psi_m$ was monitored dynamically with a laser scanning cytometer by JC-1 staining method. Laser scanning cytometer, one of the most advanced instruments in the current cell biology field, enables us to analyze cells with multi-parameters. It functions as both a flow cytometer and a static image cytometer, and can automatically calculate the mitrochondial membrane potential according to the red and green fluorescence by its software. iCysTM Research Imaging Laser Scanning Cytometer (CompuCyte Corporation, USA) was set at an original magnification ×200.

Sample processing for transmission electron microscopy—Cells were rinsed with D-Hank's solution and digested with trypsin, followed by centrifugation at 370 *g* for 5 min. The precipitate was fixed in 2.5% glutaraldehyde for 2 h, followed by fixing in 1% osmium tetroxide for 2 h, then dehydrated in acetone series, embedded in epoxy resin, processed with UC6 Ultramicrotomes, stained with lead citrate and uranyl-acetate, and observed under the transmission electron microscope (H600, Hitachi Co., Japan).

Statistical analysis-The statistical software package SPSS 15.0 (SPSS Inc., Chicago, IL) was used for all the data analysis.

Results

MIC of AMK to *S.* Typhimurium strains SR-11, pR_{ST98}/RIA and RIA was 10µg/ml. MBC of AMK to all the three strains was 80µg/ml. Bacteria were effectively killed by 100 µg/ml of AMK within 2 h. Giemsa staining of J774A.1 macrophage cells infected with *S.* Typhimurium strains SR-11, pR_{ST98}/RIA and RIA suggested a cytotoxic effect

by the bacteria on infected macrophage cells over the infection time period. The invasion of J774A.1 cells by *S.* Typhimurium strains were apparent after 3 h of incubation with an MOI of 100:1. The total number of J774A.1 cells, measured microscopically by Giemsa stain, decreased at 12 h following infection and the majority of cells underwent necrosis after 24 h of infection. The results were similar, but not so significant once the MOI was decreased to 10:1 or 50:1(Fig. 1).

Flow cytometry results revealed that the percentage of apoptosis induced by SR-11, pR_{ST98}/RIA and RIA at 3 h after infection was 21.3±1.56, 18.6±1.02 and 10.2±0.96%, respectively. Amount of apoptotic cells displayed an increasing trend over the incubation time. At 6 h of infection apoptosis induced by SR-11, pR_{ST98}/RIA and RIA was 45.6±5.12, 33.5 ± 3.57 and 25.8 ± 2.89%, respectively and difference among the three groups was statistically significant ($P < 0.05$).

Results from laser scanning cytometer demonstrated that strain SR-11 induced the largest proportion of J774A.1 cells with decreased mitochondrial Δψm at 1 h after infection. Infection with pR_{ST98}/RIA resulted in a higher number of J774A.1 with decreased Δψm than RIA strain. For all three strains the proportion of J774A.1 with decreased mitochondrial Δψm increased over the incubation time (Fig. 2).

At 3 h of infection, apoptotic changes such as chromatin margination and pycnosis were apparent in cells infected with all three *S.* Typhimurium strains. These changes were most obvious in SR-11 infected cells followed by pR_{ST98}/RIA strain infection, significantly more than caused by RIA strain. At 24 h of infection most of the J774A.1 cells underwent necrosis with all three strains. These results indicated that plasmid pR_{ST98} enhanced the ability of its host bacteria to induce macrophage apoptosis (Fig. 3).

Discussion

A pandemic of multidrug resistant *S.* Typhi occurred in Mexico in 1972, leading to 15,000 deaths. Patients infected with *S.* Typhi had severe symptoms,

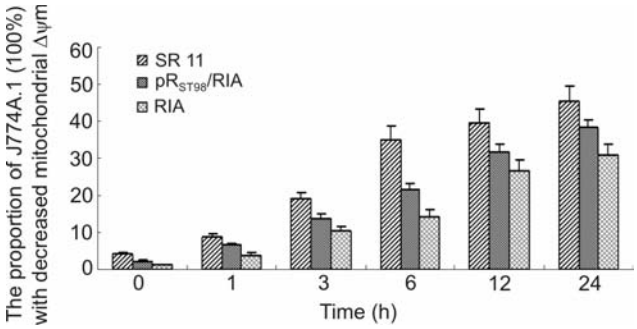

Fig. 2—The proportion of J774A.1 with decreased mitochondrial Δψm□

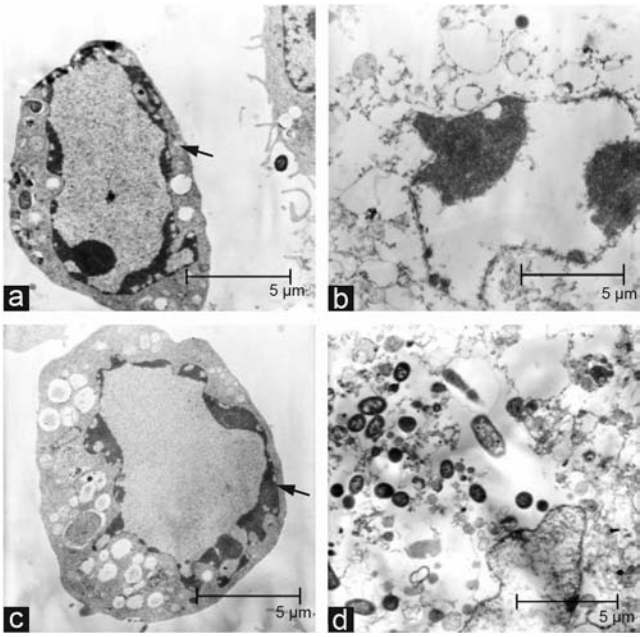

Fig. 3—Ultrastructure of J774A.1 infected with (a) pR_{ST98}/RIA for 3h; (b) pR_{ST98}/RIA for 24h; (c) RIA for 3h; and (d) RIA for 24h, detected by TEM (chromatin margination indicated by arrows)

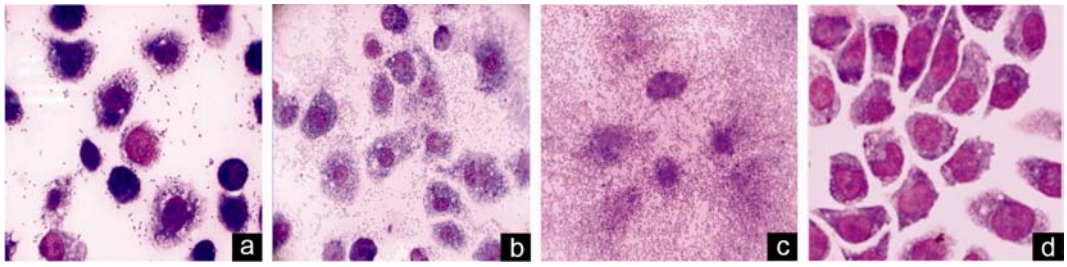

Fig. 1—Giemsa staining of J774A.1 infected with pR_{ST98}/RIA at different times (a) 3h; (b) 12h; (c) 24h; and (d) control (MOI =100:1; 1000×)

complications and high mortality rates suggesting that virulence of the pathogen had changed[11]. Thomas *et al*.[12] investigated a *S.* Typhi incompatibility group H1 R plasmid isolated in Vietnam, and found no association between R plasmid and bacteria virulence. An epidemic of multidrug resistant *S.* Typhi affected 13 provinces and cities in P. R. China in the mid to late 1980s and of the 591 strains of *S.* Typhi isolated in Suzhou, P. R. China during 1987-1992 more than 80% had multidrug resistance carried on a large conjugative 98.6 Mdal plasmid (pR_{ST98}). Patients infected with *S.* Typhi having pR_{ST98} had more severe disease with high rates of complication and mortality. It was postulated that pR_{ST98} might present a mosaic-like structure responsible for drug resistance and also for some virulence. Kurita *et al*. identified a highly conserved region of 8 kb, designated as *spv* genes, presenting on the plasmids of all other pathogenic *Salmonella* spp. except *S.* Typhi. In 2005, virulence genes on pR_{ST98} were identified using *spv*-specific polymerase chain reaction, Southern Blot and DNA sequence analysis[3]. Although *spv* genes existed on pR_{ST98}, their association with virulent phenotype could be established in the present study.

Salmonellae have been reported to effectively escape killing from macrophage with a number of defense mechanisms, such as resistance to lysosome, prevention of the fusion of lysosome, phagosome, ability to interfere with active oxygen species[13] and induction of macrophage apoptosis. In this study using three *S.* Typhimurium strains (SR-11, RIA and pR_{ST98}/RIA) to infect murine macrophage cell line J774A.1 to determine relationship between pR_{ST98} and the virulence, it was evident that pR_{ST98}/RIA strain induced significantly more macrophage apoptosis than RIA strain. Mitochondrial permeability transition due to decreased mitochondrial $\Delta\psi m$, is thought be an important and early indication in the apoptotic process[14]. Ultra-microstructure studies under transmission electron microscope indicated that infection with pR_{ST98}/RIA was associated with early reduction in $\Delta\psi$m than RIA strain ($P < 0.05$) to cause chromatin margination and pycnosis, in cells within 3 h of infection. Although *spvB* gene responsible for the positive regulation of *phoPQ* system is located on a virulence plasmid in most of the *Salmonella* serovars, homologue of *spv* has been found on pR_{ST98}. However, it is still not clear whether the macrophage apoptosis enhanced by pR_{ST98} is directly related to *spvB* gene or is a result of interplay of several genes. Moreover, mitochondrial permeability transition is not only related to apoptosis, but also with autophagy[15]. Decrease of mitochondrial membrane potential observed in this study could be a result from the increased autophagy mediated by *spv* gene on pR_{ST98}. Therefore, further studies on the relationship between autophagy and apoptosis are necessary to understand the role of pR_{ST98} in virulence of *S.* Typhi.

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