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In-Vitro Adhesion and Invasion Properties of Salmonella Typhimurium Competing with Bacteriophage in Epithelial Cells and Chicken Macrophages

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ABSTRACT

This study was designed to assess the role of bacteriophage P22 in the adhesion, invasion, intracellular survival of, and cellular immune response to *Salmonella* Typhimurium in intestinal epithelial INT-407 and chicken macrophage-like HD11 cells. The ability of *S*. Typhimurium to adhere, invade, and survive to INT-407 and HD11cells was evaluated under *Salmonella* infection alone (control), phage treatment followed by *Salmonella* infection (PS), *Salmonella* infection followed by phage treatment (SP), and a combination treatment with *Salmonella* and phage (S+P). The number of *S*. Typhimurium associated on INT-407 cells was reduced from 4.2 to 2.7 log cfu/cm^2 by phage treatment (SP). The number of intracellular *S*. Typhimurium within INT-407 cells was significantly reduced to below the detection limit (0.7 log cfu/cm^2) compared with the control (3.4 log cfu/cm^2). *S*. Typhimurium remained inside HD11 cells at 49% and 17% levels in the absence and presence of phages, respectively, at 24 h post-infection (hpi). The expression levels of IFN-g, IL-10, IL-1b, IL-6, IL-8, iNOS, and IL-12 increased in HD11 cells regardless the absence and presence of phages, while those of IL-16, TLR2-1, TLR3, and TLR7 were decreased at 0 and 24 hpi. This study sheds new light on our understanding of the role of phages in *Salmonella* adhesion, invasion, survival, and cellular immune responses.

INTRODUCTION

Salmonella enterica serovars are major causes of the human infectious disease known as salmonellosis, resulting from the ingestion of contaminated food (Vikram *et al.*, 2012).The ingested *Salmonella* undergoes the infection process, including bacterial adherence, colonization, invasion, and propagation in the intestinal epithelium and macrophage cells (Antunes *et al.*, 2010). Although innate immune responses are the first line of host defense against bacterial infection, *Salmonella* can effectively induce cytoskeletal rearrangement and membrane ruffling of host cells by several effector proteins that are responsible for *Salmonella* internalization into the epithelial cells (Kubori & Galan, 2002, Yano & Kurata, 2011).The intestinal adhesion and invasion of the epithelium is an important stage to initiate the *Salmonella* pathogenesis, depending on the type III secretion system (TTSS) encoded by *Salmonella* pathogenicity island (SPI) (Baxter *et al.*, 2003). Therefore, the effective control of *Salmonella* infection is a high priority worldwide.

Bacteriophages have recently been received great attention as an alternative biocontrol to antibiotics due to the increasing emergence of antibiotic-resistant bacteria (Lu and Koeris, 2011, Golkar *et al.*, 2014). From the viewpoint of phage therapy against extracellular and intracellular pathogens, however, there is a challenging question as whether phages can affect extracellular *S*. Typhimurium adhesion

and invasion of epithelium cells and evade the cellular immune responses in the host cells. Therefore, a systematic approach for studying the adherence and invasion properties of extracellular *S*. Typhimurium competing with phages, the intracellular survival of *S*. Typhimurium,and macrophage cellular immune responses to phages is essential to elucidate the pathogen-phage-macrophage interactions at cellular immune response levels and to design an effective phage therapeutic strategy.

Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs), including lipoteichoic acid (TLR2), double-stranded RNA (TLR3), lipopolysaccharide (LPS; TLR4), flagell in (TLR5), single-stranded RNA (TLR7), and CpG oligodeoxynucleotides (ODN; TLR9) (Ishii *et al.*, 2005). TLRs, as pattern recognition receptors (PRRs), can trigger inflammatory immune responses in macrophages, inducing the production of cytokines and chemokines (Iqbal *et al.*, 2005; Kawai & Akira, 2006). However, the potential role of phages in macrophage-mediated immune response to intracellular pathogens still remains unclear. Therefore, the aims of this study were to examine the effect of phage P22 on the adhesion and invasion of *S*. Typhimurium into the intestinal epithelium cell line INT-407, and assess the cellular immune response of *Salmonella*-infected macrophage cell line HD11 against phage P22.

MATERIALS AND METHODS

Bacterial strain and bacteriophage

The strain of *Salmonella enteric* subsp. *enteric* serovar Typhimurium LT2 (ATCC 19585) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultivated in Trypticase Soy broth (TSB) (Difco, Becton, Dickinson and Co., Sparks, MD, USA) at 37°C for 20 h. After cultivation, cultures were centrifuged at 3,000 × *g* for 20 min at 4°C and resuspended in 0.1% sterile buffered peptone water (BPW) at approximately 10^8 cfu/mL. *Salmonella* bacteriophage P22 (ATCC 97540) was purchased from ATCC and propagated with *S*. Typhimurium at 37°C for 24 h. The culture was centrifuged at 13,000 × *g* for 2 min, and the supernatant was filtered by using a 0.2-μm filter to eliminate bacterial lysates. The phages was purified according to the polyethylene glycol (PEG) precipitation method (Yamamoto *et al.*, 1970).

Phage plaque assay

Phagetiter was determined by using a soft-agar overlay method (Bielke *et al.*, 2007). The phages were serially (1:10) diluted with phosphate buffered saline (PBS, pH 7.2) buffer. Each dilution was mixed with *S*. Typhimurium cells in TSB (0.5% agar), pour-plated onto the surface of pre-warmed TSB (1.5% agar), and incubated 37°C for 24 h to enumerate lytic phages expressed as plaque-forming unit (pfu).

Cell lines and culture conditions

Human intestinal epithelium cell line, INT-407 CCL-6, and chicken macrophage-like cell line, HD11, were purchased from American Type Culture Collection (ATCC). INT-407 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; HyClone Laboratories Inc., Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) and 100 μg/mL gentamicin. HD11 cells were cultured in DMEM supplemented with 2 mM $_L$-glutamine, 10% heat-inactivated chicken serum (Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were incubated at 37°C with 5% CO_2.

Cell invasion assay

For the invasion assay, INT-407 and HD11 cells were seeded at 2 × 10^6 cells/mL into 24-well plates and 25 cm^2 T-flask, respectively, and incubated to approximately 90% confluence for 24-48 h at 37°C under 5% CO_2. The post confluent INT-407 or HD11 cultures were rinsed twice with PBS buffer and then stabilized in serum- and antibiotic-free DMEM for 1 h prior to bacterial infection. On the INT-407 cell monolayers, *S*. Typhimurium and phage P22 were infected simultaneously or sequentially at approximately 10^6 cfu/cm^2 and 10^7 pfu/cm^2, respectively: i) 1 h of pre-treatment of INT-407 cell monolayers with phage P22 followed by 1 h of *Salmonella* infection (PS), ii) 1 h of *Salmonella* infection of INT-407 cell monolayers followed by 1 h of phage treatment (SP), and iii) 1 h of combined treatment of phage and *S*. Typhimurium (S+P). INT-407 cells infected with *S*. Typhimurium or phage P22 alone were used as control. On the HD11 cell monolayers, *S*. Typhimurium was infected for 1 h at 37°C. The infected HD11 cells were treated with 100 μg/mL of gentamicin at 37°C for 1 h to eliminate adherent *S*. Typhimurium cells and then incubated with or without phage P22 at 37°C for 24 h.

Quantification of bacterial adhesion and invasion

The infected INT-407 and HD11 cell monolayers were rinsed 3 times with PBS to eliminate non-adherent *S*. Typhimurium cells. For associated bacterial count, the INT-407 and HD11 cell monolayers were lysed with

1% Triton X-100 for 15 min at 37°C. For intracellular bacteria count, the INT-407 and HD11 cell monolayers were treated with 100 µg/mL of gentamicin at 37°C for 1 h and then lysed with 1% Triton X-100 for 15 min at 37°C to release intracellular bacteria. The collected lysates were serially diluted with PBS to plate on TSA, incubated for 24 to 48 h at 37°C, and enumerate the adherent and intracellular.

RNA extraction and cDNA synthesis

HD11 cell monolayers at 0 and 24 h post-infection (hpi) were rinse with PBS, lysed with 1 mLof TRIzol reagent (Life Technologies Co. Carlsbad, CA, USA), and then collected using a cell scrapper. The cell lysates were mixed with 200 µL of chloroform and incubated for 3 min at 25°C. The mixtures were centrifuged at 12,000 × g for 15 min at 4°C to collect the upper aqueous phase (500 µL), which were mixed with 500 µL of isopropanol for 10 min at 25°C and centrifuged at 12,000 × g for 10 min at 4°C. The pellet was rinsed with 1 mL of 75% ethanol, air-dried to remove ethanol, and dissolved in 20 µL of RNase-free water at 40°C. According to the protocol of qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA), the RNA extract (1 µg) was mixed with 4 µL of 5X qScript cDNA SuperMix ($MgCl_2$, dNTPs, RNase inhibitor protein, qScript reverse transcriptase, and oligo(dT) primer). The mixture was incubated subsequently at 42°C for 30 min and 95°C for 3 min.

Quantitative RT-PCR assay

The reaction mixture containing 10 µL of 2× QuantiTect SYBR Green PCR Master, 2 µL of each primer, and 2 µL of cDNA, and 4 µL of RNase-free water was amplified using an iCycler iQ™ system (Bio-Rad Laboratories, Hemel Hempstead, UK). The PCR mixture was denatured at95 °C for 30 sec, followed by 40 cycles of 95 °C for 5 sec, 55 °C for 15 sec, and 72 °C for 10 sec. The relative expression levels of genes were estimated by the comparative method (Livak & Schmittgen, 2001). The C_T values of target genes in *Salmonella*-infected HD11 cells with and without phage P22 were compared to those in non-infected HD11 cells. The reference gene (GAPDH) was used for normalization of inflammatory mediator gene expression.

Statistical analysis

All experiments were conducted with three replicates. Data were analyzed using the Statistical Analysis System software. The general linear model and least significant difference (LSD) procedures were used to evaluate the treatment as a fixed effect. Significant mean differences were calculated by Fisher's LSD at $p< 0.05$.

RESULTS

Adhesion and invasion abilities of *S.* Typhimurium to INT-407 and HD11 cells

The adhesion ability of *S.* Typhimurium to INT-407 cells were evaluated under different treatment conditions: i) INT-407 infected with *Salmonella* or phage alone (control), ii) phage treatment followed by *Salmonella* infection (PS), iii) *Salmonella* infection followed by phage treatment (SP), and iv) combination treatment with *Salmonella* and phage (S+P) (Fig.

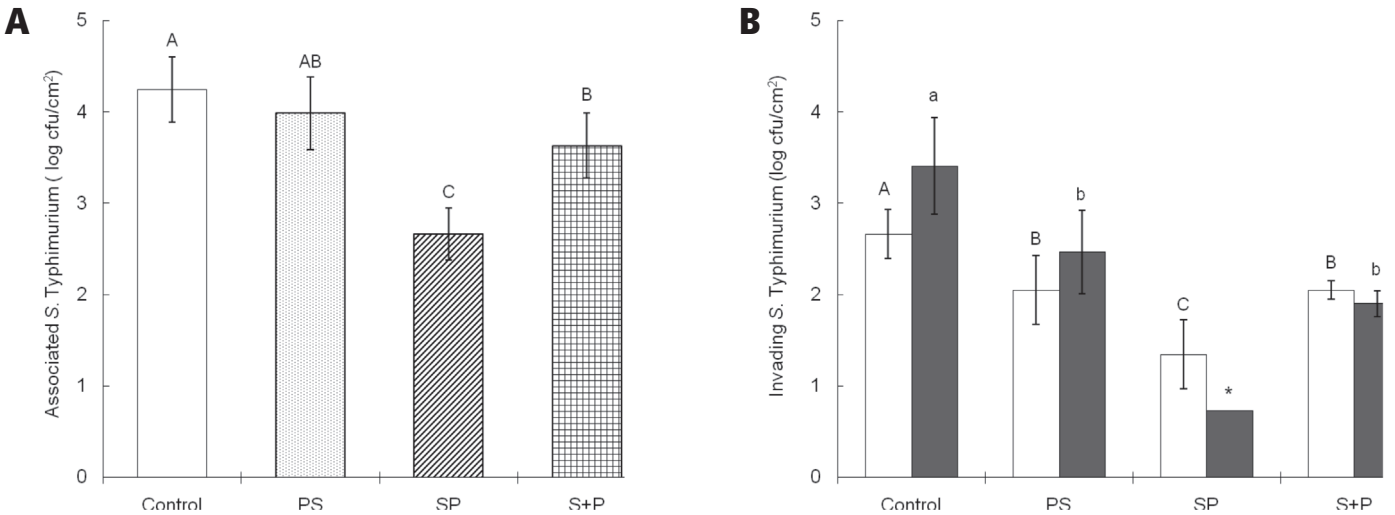

Figure 1 – Adhesion ability of *S.* Typhimurium (A) intracellular survival at 0 hpi (□) and 24 (▪) hpi (B) to INT-407 cells infected with *Salmonella* or phage alone (control), phage treatment followed by *Salmonella* infection (PS), *Salmonella* infection followed by phage treatment (SP), and combination treatment with *Salmonella* and phage (S+P). Means with different letters (A-C and a-b) are significantly different at $p < 0.05$. The asterisk (*) indicates the bacterial count below the detection limit (0.73 log cfu/cm²).

1A).Compared with the control, the ability of *S*. Typhimurium to adhere to INT-407 cells significantly decreased in SP treatment, followed by S+P treatment. The associated numbers of *S*. Typhimurium were 4.24, 3.99, 2.65, and 3.63 cfu/cm^2, respectively, for the control, PS, SP, and S+P.

The invasion and survival properties of *S*. Typhimurium were evaluated in INT-407 cells treated with PS, SP, and S+P at 0 and 24 hpi (Fig. 1B). *S*. Typhimurium cells were more invasive in the control than in the phage treatments, including PS, SP, and S+P. The invasive ability was significantly decreased in SP, corresponding to the adhesion ability. The numbers of intracellular *S*. Typhimurium were 2.66, 2.05, 1.34, and 2.05 log cfu/cm^2, respectively, at 0 hpi. The phage treatment after *Salmonella*-infection effectively blocked the internalization of *S*. Typhimurium in theINT-407 cells. Compared with the control, the intracellular survival of *S*. Typhimurium in INT-407 cells was slightly increased in the control and PS at 24 hpi, while that was marginally decreased in SP and S+P. The intracellular survival of *S*. Typhimurium was evaluated in HD11 treated with and without phage (Fig. 2). The number of intracellular *S*. Typhimurium in HD11 was 3.76 log cfu/cm^2 at 0 hpi, which was reduced to 3.44 log cfu/cm^2 (51% reduction) in the absence of phage and 2.93 log cfu/cm^2 (83% reduction) in the presence of phage at 24 hpi.

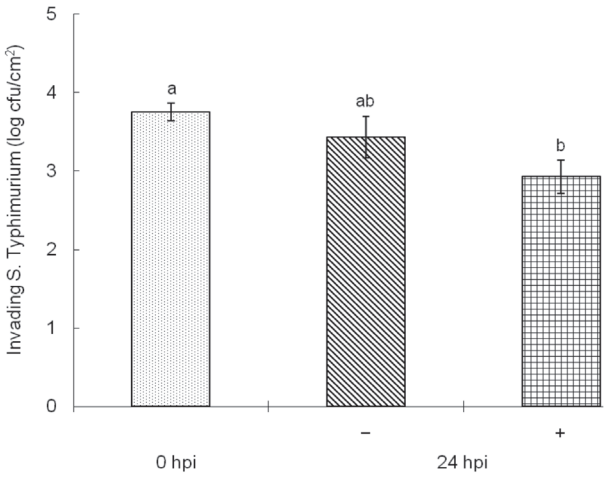

Figure2 – Survival of intracellular *S*. Typhimurium infected in HD11 cells treated without (–) and with (+) phage P22 for 24 h at 37ºC. Means with different letters (a-b) are significantly different at $p < 0.05$.

Inflammatory-mediated gene expression in *Salmonella*-infected HD11 cells

The relative expression profiles of pro-inflammatory and TLR genes were determined in HD11 cells cultured with and without phage at 24 hpi (Fig. 3). The relative expression levels IFN-g, IL-10, IL-1b, IL-6, IL-8, iNOS, and IL-12 mRNAs increased, while those of IL-16, TLR2-1, TLR3, and TLR7decreased in HD11 cells at 24 hpi. The levels of IL-13, IL-15, IL-17, IL-18, IL-3, TLR2-2, TLR4, and TLR5 mRNAs remained constant in HD11 cells at 24 hpi. The mRNA levels increased between 2 to 7-fold for IFN-g, IL-10, IL-1b, IL-6, IL-8, iNOS, and IL-12. Although no significant differences were observed in the expression levels of pro-inflammatory and TLR mRNAs between absence and presence of phages, the expression levels of pro-inflammatory cytokines were slightly higher in the presence of phages than the absence of phages.

DISCUSSION

Enteric bacteria can pass through the epithelial barrier, infect macrophage, and then develop survival strategies against the phagocytosis-associated cellular defense system. Phage can be a potential approach for controlling intracellular pathogens. This study describes the potential role of phage P22 in the adhesion and invasion of *S*. Typhimurium in the intestinal epithelial cells and the macrophage-mediated immune response to intracellular *S*. Typhimurium.

The phage treatment after *Salmonella* infection effectively excluded the attached *S*. Typhimurium from the INT-407 cells (Fig. 1A). Phage can be used as a possible approach to treat rather than prevent bacterial infections. This observation indicates that there were no significant changes in the lytic activity or binding affinity of phages against the adherent *S*. Typhimurium, resulting in the bacterial invasion of host cells.

There are two bacterial invasion mechanisms, including zippering (affinity binding of bacteria adhesins and host receptors) and triggering (injection of effector proteins into the host cells via TTSS) events (Ó Cróinín & Backert, 2012). Bacterial cells can internalize and even reproduce within the nonphagocytic epithelial cells (Kim *et al.*, 2011).

The numbers of intracellular *S*. Typhimurium were reduced to below detection limit in the SP treatment (Fig. 1B). The phage treatment after *Salmonella*-infection most successfully inhibited the growth of intracellular *S*. Typhimurium in INT-407 cells. This implies that the phage-infected *S*. Typhimurium entered and was then lysed within the INT-407 cells. The number of intracellular *S*. Typhimurium in HD11 was significantly reduced to 2.93 log cfu/cm^2 (83% reduction) in the presence of phage at 24 hpi (Fig. 2). The internalized *S*. Typhimurium cells undergo a series

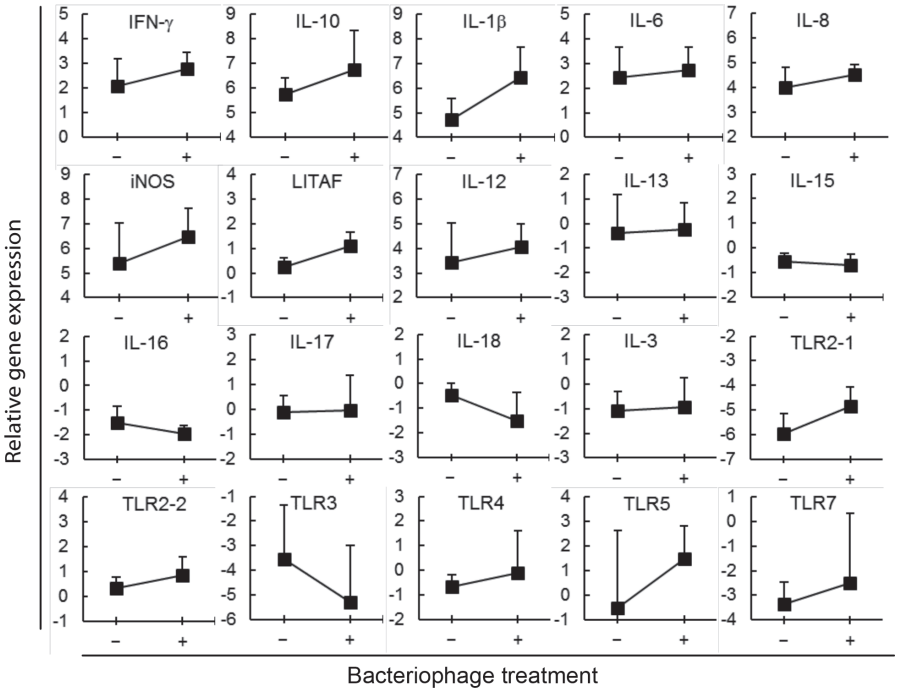

Figure3 – Relative expression of inflammatory-mediated genes in *Salmonella*-infected HD11 cells treated with (+) and without (–) phage P22 at 24 hpi.

of survival processes, SPI-1 and SPI-2 (Flannagan *et al.*, 2009). The significant reduction in the number of internalized *S*. Typhimurium in HD11 cells might be due to the inflammatory immune responses to the phage P22.

The phage P22 marginally induced immune responses in HD11 cells at 24 hpi (Fig. 3). The expression of IL-1β, IL-6, and IL-8 was up regulated in *Salmonella*-infected macrophages (Wigley, 2004, Withanage *et al.*, 2004). IL-1β is a pro-inflammatory cytokine and IL-6 is a pro- and anti-inflammatory cytokine (Lee *et al.*, 2010). The inflammatory cytokines were inhibited by IL-10 and IL-13, which were increased during the initial stage of bacterial infection (Rothwell *et al.*, 2004, Setta *et al.*, 2012). The expression level of iNOS mRNA in bacteria-infected HD11 cells was enhanced by IFN-g but not IL-18 (He *et al.*, 2011). IL-18 acts as an inducer of cellular immune responses against intracellular pathogens when combined with IL-12 (Biet *et al.*, 2002). Lipopolysaccharide-induced TNF-afactor (LITAF) is involved in the initiation of cytokine cascade in response to *Salmonella* infection (Ma *et al.*, 2010). TLR2, TLR4, and TLR5 are activated on the host cell surface, whereas TLR3 and TLR7 are activated within the host cells in response to bacterial infections (Ishii *et al.*, 2005). TLR2 was primarily involved in the induction of anti-inflammatory cytokine, IL-10 (Netea *et al.*, 2004). TLR2 was stimulated by TLR-4 which was not up-regulated after *Salmonella* infection (Higgs *et al.*, 2006). The TLR activation is mainly responsible for the cytokine induction in macrophages. However, the levels of TLR transcripts were decreased in this study. This result implies that the pro-inflammatory gene expression may be induced by a non-TLR-mediated recognition pathway (Bliss *et al.*, 2005).

In conclusion, the most significant findings of this *in-vitro*study were:1) the phage treatment after *Salmonella* infection effectively inhibited the invasion ability and intracellular survival of *S*. Typhimurium in epithelial INT-407 cells, and 2) although no significant macrophage-mediated inflammatory immune responses were observed in HD11 cells exposed to phage, the number of intracellular *S*. Typhimurium was significantly reduced in HD11 cells at 24 hpi. This *in-vitro* study provides useful information for understanding the role of phage in inhibiting extracellular and intracellular pathogens and also improving phage therapy at the cellular level. However, further *in-vivo* studies are needed to elucidate the complex gene regulatory networks of the inflammatory immune system, which is the ongoing investigation of our laboratory.

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