

Factors influences on the survival and death of macrophages infected with *Salmonella typhi* VMB-3

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Abstrak

Dalam penelitian ini biakan jaringan makrofag manusia THP-1 diinfeksi dengan *Salmonella typhi* dan *S. typhimurium*. Apa yang terjadi dengan makrofag tersebut setelah infeksi oleh kedua patogen tersebut ternyata berbeda. Makrofag yang diinfeksi oleh *S. typhi* tetap hidup lebih dari 24 jam, sedangkan 80% makrofag yang diinfeksi oleh *S. typhimurium* mati dalam waktu 16 jam. Karakter apoptosis makrofag yang diinfeksi oleh kedua patogen tersebut juga berbeda. Proses apoptosis pada makrofag yang diinfeksi oleh *S. typhimurium* terjadi lebih awal daripada infeksi oleh *S. typhi*. Untuk mengetahui mengapa makrofag yang diinfeksi oleh *S. typhi* dapat bertahan lama, dilakukan analisis protein yang disekresi oleh *S. typhi* dan produksi TNF- α yang dihasilkan oleh makrofag yang sudah diinfeksi. Galur *S. typhi* dengan kapsul Vi dan galur yang Vi antigennya delesi ternyata mensekresi protein dengan profil berbeda. Tingkat transkripsi mRNA TNF- α dari kedua kelompok makrofag inipun berbeda.

Abstract

Salmonella typhi and *S. typhimurium* were infected to human macrophage cell line THP-1. Fate of macrophage after infection of these two pathogenic species was different. Most of macrophages infected with *S. typhi* were survived even after 24hr, however, 80% of macrophages infected with *S. typhimurium* were dead after 16 hour. Apoptosis of macrophages infected with these two pathogens were also different. At early stage of infection with *S. typhimurium*, macrophages were in apoptotic process but *S. typhi* infected macrophages remained at normal stage. To find the reason why *S. typhi* infected macrophages can survive for a long time, we analyzed secreted proteins of *S. typhi* and TNF- α production of infected macrophages. Vi capsulated *S. typhi* strains and Vi deleted *S. typhi* strains had completely different secreted protein profiles. Level of transcribed mRNA of TNF- α was also remarkably different between macrophages infected with the two pathogens.

INTRODUCTION

Salmonella typhi is known as a facultative intracellular parasite and it can survive inside macrophages. But, the survival mechanism is still unclear. We previously reported that Vi capsular polysaccharide played an important role for *S. typhi* to survive inside macrophages, because Vi positive strains did not stimulate host macrophages and as a result, the macrophage did not produce TNF- α .

In this study, we constructed *rpoS* mutant of *S. typhi* which was derived from *S. typhi* Vi positive strain (wild type) and analyzed the survival under various stress. The death of THP-1 cells infected with *S. typhi* wild type, *S. typhi rpoS* mutant were also compared. *S. typhi rpoS* mutant could replicate inside THP-1 cells at the similar level with *S. typhi* wild type strain. However, *S. typhi rpoS* mutant caused cell death less

extent to infected macrophage than *S. typhi* wild type strain.

MATERIALS AND METHODS

Bacteria and cell line

Salmonella typhi wild type (GIFU 10007), *S. typhi* Vi negative mutant (GIFU 10388), *S. typhi rpoS* mutant (GIFU-PPP 330) and *S. typhimurium* (GIFU 12142) were used in this study. THP-1 cells, which derived from human monocytic leukemia, were used for infection assay.

Cell staining

Cell staining to distinguish live and dead cells was carried out with Live/Dead Eukolight Viability/cytotoxicity assay reagent provided from Molecular probe. Staining was performed according to the manufacture's instrument. After staining, cells were observed by a confocal laser scanning microscope. Live cells emitted green fluorescent, and dead cell showed red fluorescent after staining with this reagent.

Flowcytometric analysis

S. typhi and *S. typhimurium* were cultured in L-broth for indicated time. Bacteria were stained with Live/Dead Eukolight Viability/cytotoxicity assay reagent and analyzed by flow cytometry (Bio Rad Co, Japan). Live bacteria were carefully gated by forward scatter and lateral scatter to remove dead bacteria. Live bacteria were analyzed by lateral scatter and FITC intensity.

RT-PCR

Bacterial mRNA was extracted from *S. typhi* after cultured for indicated time. cDNA was synthesized from mRNA by reverse transcriptase. sigma32, sigma38 and sigma70 detected by RT-PCR using specific primers.

Phagocytosis assay

Bacteria and THP-1 cells were mixed in HBSS at the ratio of 50 bacteria to 1 cell, and then cells were incubated in 37°C for 1 hour. The cells were resuspended in complete medium with gentamicin to remove extracellular bacteria for 30 min.

Staining intracellular bacteria

Vi antigen of *S. typhi* inside THP-1 cells were detected by rabbit anti-Vi polyclonal antibody and goat FITC conjugated-anti-rabbit IgG. THP-1 cells were stained with PE-conjugated anti-CD14 monoclonal antibody.

RESULTS

S. typhi replication inside activated macrophages

S. typhi Vi positive strain and Vi negative strain internalized into resting THP-1 cells almost the same level (data not shown). *S. typhi* Vi positive strain survived into resting THP-1 cells, whereas Vi negative *S. typhi* could not survive (Figure 1). *S. typhimurium* internalized to a greater extent than *S. typhi* strains and survived inside resting THP-1 cells. However, in THP-1 cell which activated by IFN- γ , *S. typhi* strains and *S. typhimurium* did not survive in spite of the higher numbers of bacteria were internalized.

S. typhi expressed Vi antigen inside THP-1 cell after infection

We detected the Vi antigen of intracellular *S. typhi*. After invasion to THP-1 cells, *S. typhi* inside THP-1 cells were stained with rabbit anti Vi polyclonal anti-

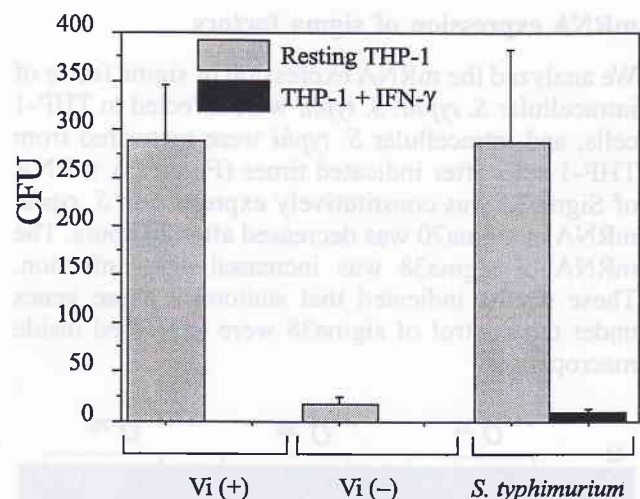


Figure 1. Replication of *S. typhi* and *S. typhimurium* inside resting and activated THP-1 cells.

Data were represented as mean \pm SEM.

body and FITC conjugated goat anti rabbit IgG. Intracellular *S. typhi* expressed Vi antigens even after infected into macrophages (Figure 2). *S. typhi* Vi positive strain recovered from THP-1 cells were only agglutinated with Vi antiserum but not with O9 antiserum. This means O9 antigen is completely masked with Vi antigen (data not shown). These data suggested that *S. typhi* inside macrophages continuously expressed Vi antigen after invasion into THP-1 cells.



Figure 2. *S. typhi* expressed Vi antigen inside THP-1 cells.

S. typhi wild type was infected to THP-1 cells. After infection, Vi antigen of *S. typhi* inside THP-1 cells was detected by rabbit anti-Vi polyclonal antibody and goat FITC conjugated-anti-rabbit IgG. THP-1 cells were stained with PE-conjugated anti-CD14 monoclonal antibody.

mRNA expression of sigma factors

We analyzed the mRNA expression of sigma factor of intracellular *S. typhi*. *S. typhi* were infected to THP-1 cells, and intracellular *S. typhi* were recovered from THP-1 cells after indicated times (Figure 3). mRNA of Sigma32 was constitutively expressed in *S. typhi*. mRNA of sigma70 was decreased after 20 hours. The mRNA of sigma38 was increased after infection. These results indicated that stationary phase genes under the control of sigma38 were expressed inside macrophages.

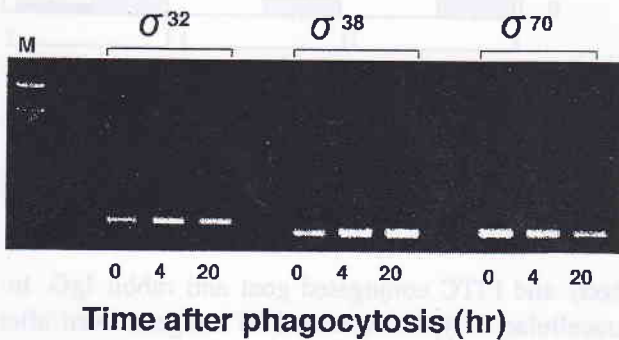


Figure 3. RT-PCR analysis of sigma factors of *S. typhi* in THP-1 cells.

***S. typhi* survival in low pH condition**

The *S. typhi* Vi positive strain cultured stationary phase and log phase were cultured in the L broth in pH 4 and the survival rate was compared (Figure 4). This low pH condition was expected to be similar to intracellular environment. Stationary phase bacteria were more resistant to intracellular low pH condition and log phase bacteria were sensitive and died within 60 min. These results indicated that stationary phase *S. typhi* cells were more resistant in intracellular condition.

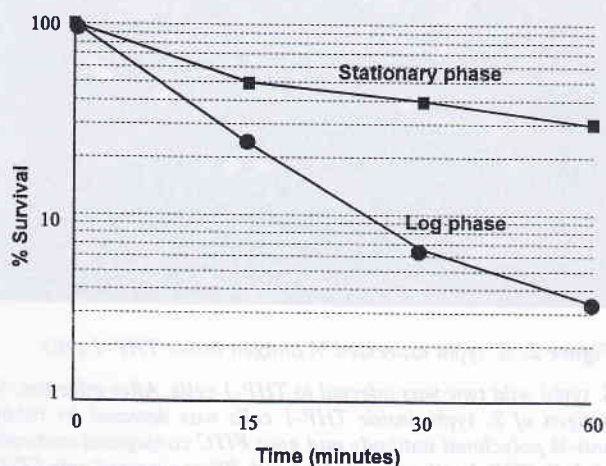


Figure 4. The survival of *S. typhi* cultured in pH 4.

Analysis of *S. typhi* viability by Flowcytometry

S. typhi Vi positive strain was cultured in L-broth for 4, 8 and 24 hours. The strain was stained with Live / dead cell eukolight viability assay reagent, and bacteria were analyzed by flowcytometer. After 8 hours culture, *S. typhi* cells were separated into two populations shown in Figure 5, however, *S. typhimurium* remained single population even after 24 hours. The upper population indicated brightly stained bacteria, and lower population indicated faintly stained bacteria. The bacteria brightly stained in green color means that these bacteria are still active. Another group of bacteria stained in faint green means that viability were decreased. These results suggested that *S. typhi* might change the metabolic phase and became stationary phase in 24 hours when cultured in ordinary laboratory media.

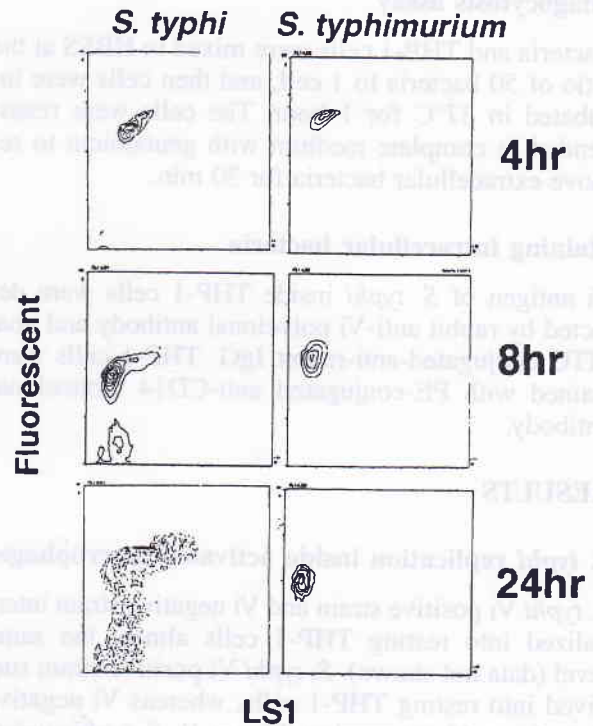


Figure 5. Flowcytometric analysis of *S. typhi* and *S. typhimurium* cultured in L-broth for 4, 8, and 24 hours. X axis represents the intensity of lateral scatter, and Y axis represents the intensity of FITC.

Host macrophage death after invasion of *S. typhi* rpoS mutant

We compared host cell death after *S. typhi* invasion into macrophages cell line THP-1 cells. *S. typhimurium*, *S. typhi* wild type and *S. typhi* rpoS mu-

tant were infected to THP-1 cells and the cells were incubated for 1, 4, 16 and 32 hours. After incubation, THP-1 cells were stained with Live/dead cell euk-olight viability assay reagent, then the cells were observed by confocal laser microscope and generated into images. The live cells and dead cells were counted. The percentages of dead THP-1 cells after invasion were shown in Figure 6. After 32 hours incubation, *S. typhimurium* killed about 90% of THP-1 cells. But, *S. typhi rpoS* mutant were significantly less than *S. typhi* wild type strain. These results indicated that *rpoS* gene expression played an important role for the pathogenesis of *S. typhi*.

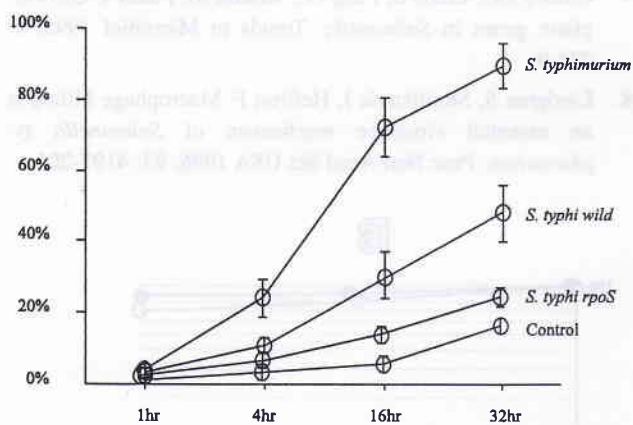


Figure 6. Host macrophage cell death after invasion of *S. typhi* strains and *S. typhimurium*.

Data were expressed as mean \pm SEM.

S. typhi survivals in various culture conditions

S. typhi wild type strain and *rpoS* mutant were compared on their survivals in various culture conditions. In Figure 7A, *S. typhi* wild type and *rpoS* mutant were cultured in L-broth. After culture transferring the cells into pH 4, viable bacteria numbers were counted by the plating 15, 30, 60 min later. More than 90% of *S. typhi rpoS* mutant were dead after 60 min in pH 4 medium. This result indicated that *S. typhi rpoS* mutant is sensitive to low pH conditions. In Figure 7B, we compared intracellular replication inside THP-1 cells. *S. typhi* were infected to THP-1 cells and incubated for further 4 or 20 hours in RPMI1640 with 10% FBS. After incubation, cells were lysed and intracellular viable bacteria were counted. After 4 hour or 20 hours incubation, intracellular viable bacteria in THP-1 cells were almost at the same level at each point. In Figure 7C, *S. typhi* wild type strain and *rpoS* mutant were cultured in M9 medium for 5 days. All of *S. typhi rpoS* mutant were dead on day 4 after

inoculation, whereas 20% of *S. typhi* wild type strain were still alive on day 5. This result means *S. typhi rpoS* mutant is sensitive to starvation. In Figure 7D, *S. typhi rpoS* mutant and wild type were cultured in the medium containing 15 mM of hydrogen peroxide. *S. typhi rpoS* mutant was rapidly died within 15 min., however, *S. typhi* wild type strain was resistant to 15 mM hydrogen peroxide and 70% of bacteria were still viable after 60 min. incubation. From these experiment, *S. typhi rpoS* mutant was sensitive to low pH, starvation and hydrogen peroxide. This means that ropes gene expression might be essential for *S. typhi* to survive long time inside macrophages.

DISCUSSION

We previously reported that Vi capsular polysaccharide played an important role for *S. typhi* to survive inside macrophages. The macrophages infected with *S. typhi* Vi mutant or *S. typhimurium* produced a large amount of TNF- α , whereas, Vi positive strains did not stimulate host macrophages to produce TNF- α , because Vi antigen covered the bacterial surface LPS and Vi antigen prevent the LPS from macrophage CD14.

In this study, we constructed *S. typhi rpoS* mutant which derived from *S. typhi* Vi positive strain (wild type) and compared the survival of *S. typhi* wild type, *S. typhi* Vi mutant, *S. typhi rpoS* mutant.

S. typhi could not survive in the activated THP-1 cells (Figure 1). The organisms, thus, internalizes into resting THP-1 cells to survive inside the cells, and intracellular *S. typhi* continuously expressed Vi antigen inside THP-1 cells (Figure 2). Once entered into macrophages, sigma38 of *S. typhi* is induced inside THP-1 cells, and *S. typhi* might changes their metabolic phase to stationary phase. Stationary phase *S. typhi* is resistant to low pH (Figure 4) condition which is similar to intracellular conditions. *S. typhi* cultured in L-broth for 24 hours separated into two groups of bacteria (Figure 5), one population is still log phase bacteria, another population is changed into stationary phase. *RpoS* mutant was less toxic to host cell, and was sensitive to low pH, starvation and hydrogen peroxide. *RpoS* gene might be required for the pathogenesis of *S. typhi*. From these data, we concluded that Vi positive *S. typhi* shift metabolic phase to stationary phase after invasion into macrophages to adapt to the intracellular environment, and continued to survive without stimulating host cell recognition systems.

We did not show another evidence that Vi positive *S. typhi* strain produced less amount of secretory proteins into cultured media. Most of the secreted proteins were flagellar antigens. However, Vi negative strains produced almost equal level of secreted proteins to *S. typhimurium*. Thus, we expected that Vi negative *S. typhi* stimulated host immune system through complement system, and CD14. *S. typhimurium* also stimulated host cells, but this organism was serum resistant and more resistant to oxygen radicals.

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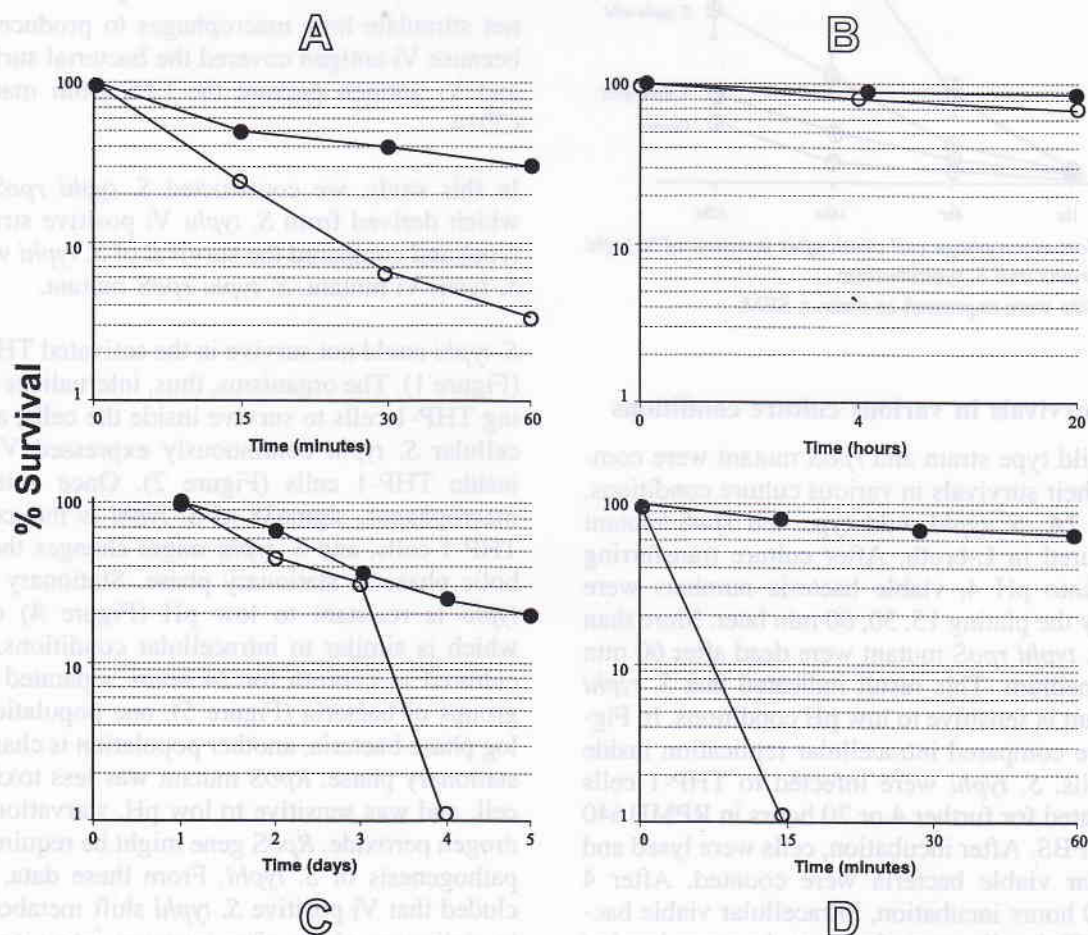


Figure 7. *S. typhi* survivals in various culture conditions. *S. typhi* wild type (-o-) and *rpoS* mutant (-o-) were cultured in low pH medium (pH 4) (A), cultured in M9 medium (C), and cultured in the medium containing 15 mM hydrogen peroxide (D). The survival inside THP-1 cells were compared between *S. typhi* wild type and *rpoS* mutant (B).