Salmonella typhimurium and its host : host-pathogen cross-talk, immune evasion, and persistence

Diepen, A. van

chapter four

An *rmlC Salmonella enterica* serovar Typhimurium mutant is attenuated in vivo but is able to persist in RAW264.7 macrophages

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Abstract

Salmonella is a facultative intracellular pathogen that can invade and replicate within several cells, including epithelial cells and macrophages. To be able to spread from the intestines into the body and to the liver and spleen, *S. enterica* serovar Typhimurium has to go from cell to cell and it can do so by inducing cell death, although it is currently unknown what the in vivo relevance of *Salmonella*-induced cell death is. To gain more insight into the strategy that is used by *Salmonella* to survive within macrophages and to induce apoptosis, we have created and selected *S. enterica* serovar Typhimurium mutants with increased ability to survive within macrophages without causing enhanced cell-death and analyzed the in vitro cytotoxicity and its in vivo virulence.

In this way, we have selected for an *rmlC* mutant that has truncated LPS chains. This mutant as well as a rough (Ra chemotype) variant displayed increased bacterial intracellular numbers in RAW264.7 macrophages and persisted even after 48 h, while inducing cell death to a similar extent as the wild-type strain. However, despite the increased ability to survive within cells, these LPS mutants were strongly attenuated in mice.

Introduction

Salmonella is a facultative intracellular pathogen that can invade and replicate within several cells, including epithelial cells and macrophages. *Salmonella* is able to infect both humans and animals and depending on the type of *Salmonella* strain and host it can cause a range of diseases. *S. enterica* serovar Typhimurium causes gastroenteritis in humans, but causes typhoid fever-like disease in mice. *S. enterica* serovar Typhimurium is most widely studied in vitro and in vivo and serves a good model for human typhoid fever caused by *S. enterica* serovar Typhi.

Natural infection with *S. enterica* serovar Typhimurium occurs through the ingestion of contaminated food or water. Those bacteria that have survived the acidic environment of the stomach and have reached the intestine will eventually encounter membranous epithelial (M) cells overlying the Peyer's patches. *S. enterica* serovar Typhimurium uses these M cells to pass the intestinal lining and to invade the body by inducing its own uptake through a mechanisms known as *Salmonella*-induced membrane ruffling mediated by type three secretion system proteins that are encoded by genes of *Salmonella*-pathogenicity island 1 (SPI-1) (reviewed in (16)). *S. enterica* serovar typhimurium is transported through the cytoplasm to the underlying lymphoid cells where it predominantly infects the macrophages. *S. enterica* serovar Typhimurium then becomes systemic and spreads to the liver and spleen and causes the chronic inflammatory response that is typical for typhoid fever.

To be able to spread from the intestines into the body and to the organs, *S. enterica* serovar Typhimurium has to go from cell to cell. *S. enterica* serovar Typhimurium can do so by inducing cell death. Two types of *Salmonella*-induced cell death have been described. The first one involves a rapid, caspase-1-dependent, induction and the second one a slower, caspase-1-independent, induction of cell death. The rapid *Salmonella*-induced cell death leads to a strong pro-inflammatory response that is mediated by IL-1β and IL-18 and therefore differs from the classical apoptotic mechanisms. It has therefore been stated that *Salmonella* induces programmed necrosis in a caspase 1-dependent manner (2). This type of *Salmonella*-induced necrosis is dependent upon the production and secretion of the SPI1 encoded protein SipB and requires the presence and activation of caspase-1 (5, 10). The second type of *Salmonella*-induced cell death is slower than the rapid caspase-1 induced cell death (at 12-13 h post infection) and is not dependent upon SipB and caspase-1 as *Salmonella* can still induce cell death in the absence of these proteins (8, 15). This type of induced cell death is reminiscent of apoptosis and occurs even in the absence of bacterial replication, is SPI-1 independent and requires a functional SPI-2 and *ompR* (15). It is currently unknown which other mechanisms, besides SPI-2-encoded genes and *ompR* might play a role in this second type of *Salmonella*-induced cell death.

Van der Velden et al. have proposed a model in which the rapid and delayed *Salmonella*-induced apoptosis in infected macrophages is induced under different physiological conditions at distinct time and location during a natural infection. The rapid

SPI-1-dependent induction of cell death resulting in inflammation may be required for the recruitment of phagocytes and for systemic dissemination, while the delayed, caspase-1 independent, apoptosis is required during the systemic phase of infection and is used to spread intercellularly within apoptotic bodies (15). In this model, *Salmonella*-induced cell death is generally thought to reflect a bacterial strategy to promote disease (1) and seen as a virulence mechanism.

Alternatively, cell death upon *S. enterica* serovar Typhimurium infection might also been seen as a host response to infection that is beneficial to the host as cell death exposes *S. enterica* serovar Typhimurium to immune defense mechanisms of the host such as antibodies, complement, and neutrophils. By inducing cell death of infected macrophages, the bacteria are released into the host tissues and blood and can be rapidly killed by complement or can be opsonized and then killed by granulocytes.

Further research on the relevance of *Salmonella*-induced cell death is necessary since it is currently unknown what the in vivo relevance of *Salmonella*-induced cell death is. Although it has been shown that several *Salmonella* mutants are less cytotoxic and cannot induce apoptosis in vitro (11, 13), it is not known whether such mutants are attenuated or not. Therefore, we have selected for *S. enterica* serovar Typhimurium mutants that reside in macrophages and that are still viable after prolonged times of infection when most of the cells have undergone cell death. Analysis of in vitro cytotoxicity and in vivo virulence of such mutants might give more insights into the role of *Salmonella* induced cell-death in in vivo virulence.

Materials and Methods

Mice. Six- to 8-week-old female *Salmonella*-resistant (Ity^r) C3H/HeN mice were purchased from Harlan (Horst, The Netherlands). Mice were maintained under standard conditions according to the institutional guidelines. Water and food were given ad libitum. All experiments were approved by the local Animal Ethical Committee.

Bacterial strains and growth conditions. The bacterial strains used in this study are enlisted in Table 1. Single colonies of the different strains were grown in Luria-Bertani (LB) medium (10 mg of tryptone, 5 mg of yeast extract, and 10 mg of NaCl/ml) at 37°C while being shaken (225 rpm). For the in vivo experiments, the overnight cultures were diluted in fresh LB medium and grown to the end of log phase and were then washed and diluted in sterile PBS. The CFU in the inoculum were determined by plating serial dilutions.

Generation of *S. enterica* serovar Typhimurium mutants. Wild type *S. enterica* serovar Typhimurium 14028s was used as the parental strain to isolate mutants that displayed reduced ability to induce cell death in RAW264.7 cells. Mutants were made by random MudJ transposon insertion. Phage lysate containing the MudJ was made using TT10289 (7). Briefly, P22 phages were added to a 1:10 diluted ON culture of TT10289 and

incubated at 37°C ON while being shaken. After adding 10% chloroform and another incubation at 37°C for 30 minutes, the lysate was centrifuged for 2 minutes to remove the cell debris. This cell lysate containing phages with the MudJ transposon (10 μl) was added to100 μl ON culture of the recipient strain 14028s. After incubation at 37°C for 5 hours, the bacteria were plated on LB agar containing 50 μg/ml kanamycin (Sigma) and 0.1% sodium citrate (Merck) to select for bacteria in which the MudJ transposon had been inserted.

Table 1. *Salmonella* strains and plasmids used in this study

Salmonella Strain	Characteristics	Origin or reference
S. enterica serovar Typhimurium		
14028s	Wild type	ATCC
14028r	Ra chemotype (rough)	This study
SF1398Re	Re chemotype (deep rough)	This study
TT10289	LT2 *hisD*9953::MudJ *hisA*9949::Mud1	{370}
AVD16703	14028s *rmlC*::MudJ	This study

Selection of *S. enterica* serovar Typhimurium mutants. To select for *S. enterica* serovar Typhimurium mutants we pooled the random MudJ transposon insertion mutants by scraping the plates. The pooled bacteria were washed with PBS and were used for in vitro infection of RAW264.7 cells as described. Only now the cells were seeded in 150 cm^2 flasks at a density of 1×10^7 cells per flask and were allowed to adhere ON at 37°C and 5% CO_2 and the pooled bacteria were used to infect the cells at a 10:1 multiplicity of infection. Endocytosis was allowed to proceed for 30 minutes and gentamicin treatment was performed as described for the in vitro infection experiment. At 72 hours after infection, the cells were washed and lysed in milliQ and the lysate was plated on agar plates. The bacteria were again scraped off the plates, washed with PBS and again used for infection of RAW264.7 cells. After this second passage, single colonies were made phage-free by repetetive plating on EBU agar and were tested in a regular in vitro infection experiment.

Inverse PCR. To identify the gene in which the MudJ transposon had inserted, the DNA flanking the left end of the transposon was amplified and sequenced using inverse PCR. Genomic DNA of strain AVD16703 was isolated and digested with *HaeIII* (Gibco-BRL) for 4 hours. After inactivating the *HaeIII* enzyme, the sample was treated with T4 DNA ligase (Invitrogen). The digested and ligated DNA was amplified using the following primers: 5'-CCGGGAGGACATTGGATTAT-3' (sense) and 5'-CGTACTTCAAGTGAATCAATAC-3'. The PCR product was purified using the QIAquick PCR purification kit (Qiagen) and was sequenced.

In vitro infection experiment. RAW264.7 cells were seeded in a 24-wells plate at a density of 1×10^5 cells per well and allowed to adhere ON at 37°C in RPMI medium supplemented with 10% fetal calf serum. Bacteria were washed in PBS and were added to the cells at a 10:1 multiplicity of infection. The bacteria were spun onto the cell by centrifugation for 10 min at 270 × g. Cells were incubated for 10 min at 37°C and 5% CO_2 to allow bacterial endocytosis. After washing the cells with PBS, medium containing 100 μg/ml gentamicin was added and the cells were incubated at 37°C for another 10 min to kill the extracellular bacteria. The cells were then washed again. This was designated time point zero. Medium containing 10 μg/ml gentamicin was added to the cells to kill the extracellular bacteria and to prevent reinfection. At 0, 24, and 48 hours the cells were lysed in 1 ml milliQ and serial dilutions were made to determine the number of bacteria.

Cytotoxicity test. *Salmonella*-induced cytotoxicity was determined using the Cytotox 96® Non-Radioactive Cytotoxicity Assay (Promega) that is based upon the release of lactate dehydrogenase (LDH). From each well, 50 μl supernatant was taken and transferred to a 96 wells plate. As a control for spontaneous release of LDH 50 μl supernatant was taken from non-infected cells on each timepoint. The maximum LDH release was determined by adding 0.9% Triton X-100 to the cells and thereby lysing them and transferring 50 μl to the 96-wells plate. Then 50 μl substrate mix was added to each well and the plate was incubated at room temperature in the dark for 30 min. The coloring reaction was stopped by adding 50 μl stop solution provided with the kit and the OD490 was determined using an ELISA plate reader (VICTOR^2 1420 multilabel counter, PerkinElmer Life and Analytical Sciences). The induced cytotoxicity (%) was calculated as follows:

$$\frac{OD_{490}(\textit{Salmonella}\text{-induced release}) - OD_{490}(\text{spontaneous release})}{OD_{490}(\text{maximum release}) - OD_{490}(\text{spontaneous release})} \times 100\%$$

In vivo infection experiment. Mice were inoculated subcutaneously in the flanks with 0.1 ml bacterial suspension containing 3×10^4 CFU *S. enterica* serovar Typhimurium 14028s, 14028r, or AVD16703. For each group on each time point 4 mice were used. Mice were sacrificed by carbon dioxide inhalation and blood was taken immediately by cardiac puncture. Part of the blood was used to obtain serum and the other part was prevented from coagulation by adding 40 U heparin/ml to count the number of leukocytes and to test the blood for the presence of *Salmonella* by culture in LB medium. To determine the bacterial load within spleens, livers, and inguinal lymph nodes, these organs were aseptically removed and single cell suspensions were prepared by using sterile 70-μm-mesh-size cell strainers (Falcon). Cells were pelleted by centrifugation for 10 min and were lysed in distilled water. The bacterial number per organ was determined bacteriologically by plating serial dilutions.

Preparation of cell envelopes followed by Proteinase K digestion. Single colonies were grown ON in 10 ml LB medium at 37°C while being shaken. The ON culture was pelleted for 10 min at 3,000 rpm and cell envelopes were isolated as described in (3). Briefly, the pellets were washed once in ice-cold sonicationbuffer (50 mM Tris-HCl, 2 mM EDTA, pH 8.5) and were then resuspended in 4 ml of this buffer. The suspension was then sonicated with 8 pulses of 20 sec with an amplitude of 20-24 μm and with cooling. The sonicates were pelleted and the pellets containing cell debris and the cell envelopes were resuspended in 100 μl of 2 mM Tris-HCl pH7.8. Protein concentrations in the samples were measured using a BCA (Pierce) protein assay according to the manufacturers recommendations to standardize the samples at 1 mg/ml. The samples were then treated with Proteinase K (250 μg/ml) for 2 hours at 60°C to degrade the proteins present in the cell envelopes and equal volumes of sample buffer were added.

Electrophoretic separation. For the separation of the LPS fragments was done by SDS-PAGE by loading 35 μl of the samples on a 16% acryl amide separation gel and a 5% acryl amide stackinggel.

Silver-staining. To visualize the LPS fragments, the acryl amide gel was stained using a silver staining method according to Heukeshoven and Dernick (6) with a few modifications. Briefly, the acryl amide gel was incubated in fixing solution (40% ethanol, 10% acetic acid) for 30 min. The gel was then placed ON in incubation solution (30% ethanol, 0.5 M sodium acetate, 0.13% glutardialdehyde, 0.2% sodium thiosulphate). After washing with distilled water, the gel was stained in silver solution (0.1% silver nitrate, containing 0.007% formaldehyde) for 40 min and developed in 0.24 M sodium carbonate containing 0.0035% formaldehyde for 15 min until the bands become intensively dark. The coloring reaction was stopped by placing the gel in 0.04 M EDTA-NA_2·2 H_2O and was washed with distilled water. The gel was then preserved in 10% glycerol.

Statistics. Statistical analysis was performed using Student's *t* tests and a *P* value <0.05 was considered significant.

Results

Isolation of *S. enterica* serovar Typhimurium mutants with reduced ability to induce cell damage. In a typical in vitro infection experiment of RAW264.7 macrophages with the wild-type strain 14028s, the bacteria start to replicate until they reach a maximum after 24 h. At later timepoints after infection, the bacterial numbers decline due to death of the macrophages and subsequent exposure of the bacteria to gentamicin that is present in the medium or due to eventual killing of the bacteria by the macrophages. To select for mutants with reduced capacity to induce death of the host cell, we have generated *S. enterica* serovar Typhimurium mutants by random MudJ transposon insertion in the wild-

type strain 14028s and pooled them for use in in vitro infection of RAW264.7 cells. As a control we infected cells with wild-type *S. enterica* serovar Typhimurium. At 72 h after infection, the cells were lysed and the lysate was plated onto agar in order to enrich for mutants with a reduced capacity to cause death of the macrophages. The bacteria were again scraped off the plates and were again used for infection of RAW264.7 cells. During this second passage we observed that many cells were no longer attached to the bottom of the tissue culture flask when wild-type bacteria were used for infection, while in the flasks used for infection with the pooled mutants cells remained attached. This suggested that this pool contained mutants with a reduced ability to induce cell damage. After the second passage, several single colonies were isolated and used in an in vitro infection experiment. Mutants H and J displayed increased survival compared to the wild-type strain 14028s at 48 h after infection in in vitro infection (Figure 1) and these were selected for further study.

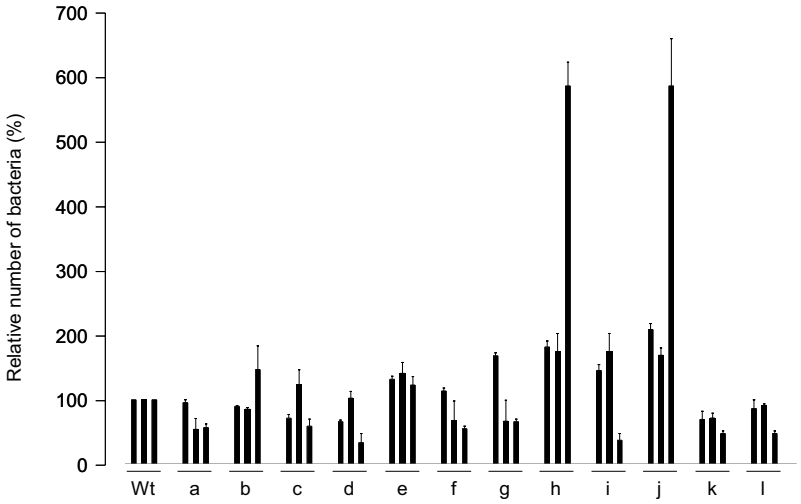

Figure 1. Relative number of random MudJ transposon insertion *S. enterica* serovar Typhimurium 14028s mutants in RAW264.7 macrophage-like cells. On t=0, 3, and 24 h the number of intracellular bacteria was determined bacteriologically and the relative number compared to the wild-type strain on each timepoint was calculated. Each first bar represents t=0 h, the second bar t=3 h, and the third bar t=24 h.

Identification of the gene inactivated by MudJ insertion. The gene that was inactivated by the MudJ insertion and rendered mutants H and J to be able to survive within RAW264.7 cells even after 48 was identified by inverse PCR. Part of the gene in which the MudJ had inserted (left flanking region) was sequenced and NCBI nucleotide BLAST search revealed homology of the MudJ flanking region to *rmlC*, a gene encoding dTDP-4-deoxyrhamnose 3,5-epimerase (complement 2175118-2175669, accession number NC_003197.1). Sequence analysis also revealed that mutants H and J were identical. The MudJ had been inserted at exactly the same position in the *rmlC* gene. Therefore, we continued our experiments with mutant J only and have named this strain

AVD16703. *RmlC* is part of the *rfb* gene cluster that encodes the enzymes for O-antigen biosynthesis. *RmlC* acts together with *rmlB*, *rmlD*, and *rmlA* to encode L-rhamnose, which is part of the repeating unit of the O-antigen polysaccharide of LPS.

LPS fragment analysis. The MudJ insertion in *rmlC* should in theory result in the lack of production of L-rhamnose and result in a lack of the O-antigen. To confirm that the isolated mutant AVD16703 was indeed an LPS mutant we isolated cell envelopes and analyzed the LPS fragments by gel electrophoresis and silver staining. As a control we used the LPS rough mutant 14028r (Ra chemotype) and the deep rough LPS mutant SF1398Re (Re chemotype). LPS of 14208r consists of the lipid A portion and the core region and lacks the O-antigen. The LPS chain of SF1398Re is even shorter as it consists only of lipid A glycosylated with two 3-deoxy-D-manno-octulosonic acid (Kdo) residues and thus lacks both the O-antigen and the core region. Figure 2 shows the LPS fragments of the wild-type strain 14028s show the typical ladder pattern. LPS from mutant SF1398Re is very small and gives only a smear at the bottom of the gel. LPS from our mutant AVD16703 appeared to be very much alike that of the 14028r strain. This 14082r strain is an LPS mutant that is known to lack the O-antigen and consists only of lipid A and the core region.

One striking feature was that mutant AVD16703, although very much alike 14028r in the LPS fragment analysis, showed different colony morphology on LB agar plates. Colony morphology of the Ra chemotype mutant 14028r was rough, while that of mutant AVD16703 was not. On blood agar plates, on the other hand, the colony morphology of AVD16703 was slightly rough, but less clearly compared to the 14028r strain (data not shown).

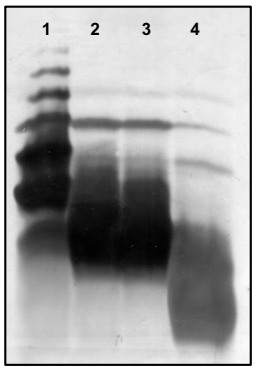

Figure 2. SDS-PAGE fractionation of LPS fragments from wild-type *S. enterica* serovar Typhimurium 14028s (lane 1), the *rmlC* mutant AVD16703 (lane 2), the Ra chemotype rough mutant 14028r (lane 3), and the Re chemotype mutant SF1398Re (lane 4). The gel was silver stained.

In vitro intracellular growth of *S. enterica* serovar Typhimurium mutant strains. Since LPS fragment analysis revealed that LPS from 14028r and mutant AVD16703 was very much alike, we tested both mutants in an in vitro infection experiment. Since mutant AVD16703 was originally isolated as a strain that was able to survive for a longer period of time and is thought to induce less cell death, we also assessed the intracellular

survival/outgrowth after 48 h. The wild-type strain grows out after 24 h, but after 48 h the number of viable bacteria is reduced (Fig. 3A). In contrast, mutant AVD16703 is able to survive after 48 h. The bacterial numbers are somewhat reduced compared to 24 h, but are still significantly higher than those of the wild-type strain. Remarkebly, the 14028r mutant showed even higher numbers of intracellular bacteria both at 24 and 48 h after infection.

***Salmonella*-induced cytotoxicity.** The reduction in the number of wild-type bacteria at 48 h after infection and the higher numbers of the LPS mutants could be explained in two ways. The intracellular replication of the mutants could have been faster than that of the wild-type, or the infection with the wild-type strain could have caused more cell death than the LPS mutants, resulting in a reduction in the number of viable bacteria. Therefore, we analyzed the induced cytotoxicity by measuring the LDH release in the infected wells. The induced cytotoxicity at 24 h was around 20% for all three strains and increased to

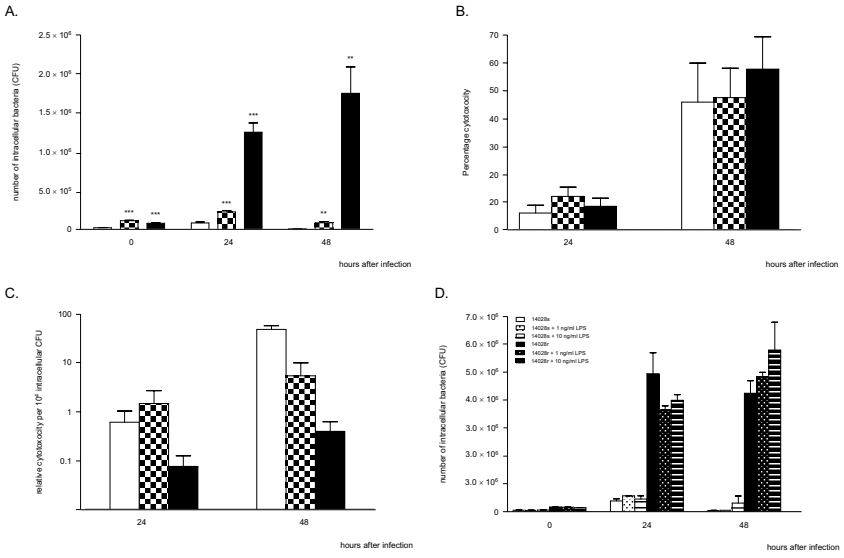

Figure 3. Intracellular *S. enterica* serovar Typhimurium in RAW264.7 mouse macrophage-like cells (A), percentage cytotoxicity induced by the infection (B), relative induced cytotoxicity per 10^4 intracellular CFU (C), and number of intracellular bacteria in the absence or presence of different concentrations of extracellular *E. coli* LPS (D). The cells were challenged with *S. enterica* serovar Typhimurium 14028s (white bars), AVD16703 (checkered bars), 14028s (black bars) as described in Materials and Methods. The numbers of intracellular bacteria were determined at 0, 24, and 48 h after infection and the percentage cytotoxicity 24 and 48 h after infection. Asterisks indicate that the number of intracellular bacteria is significantly different from that of wild-type *S. enterica* serovar Typhimurium 14028s. Mean data of two independently performed experiments ± standard errors of the means are shown.

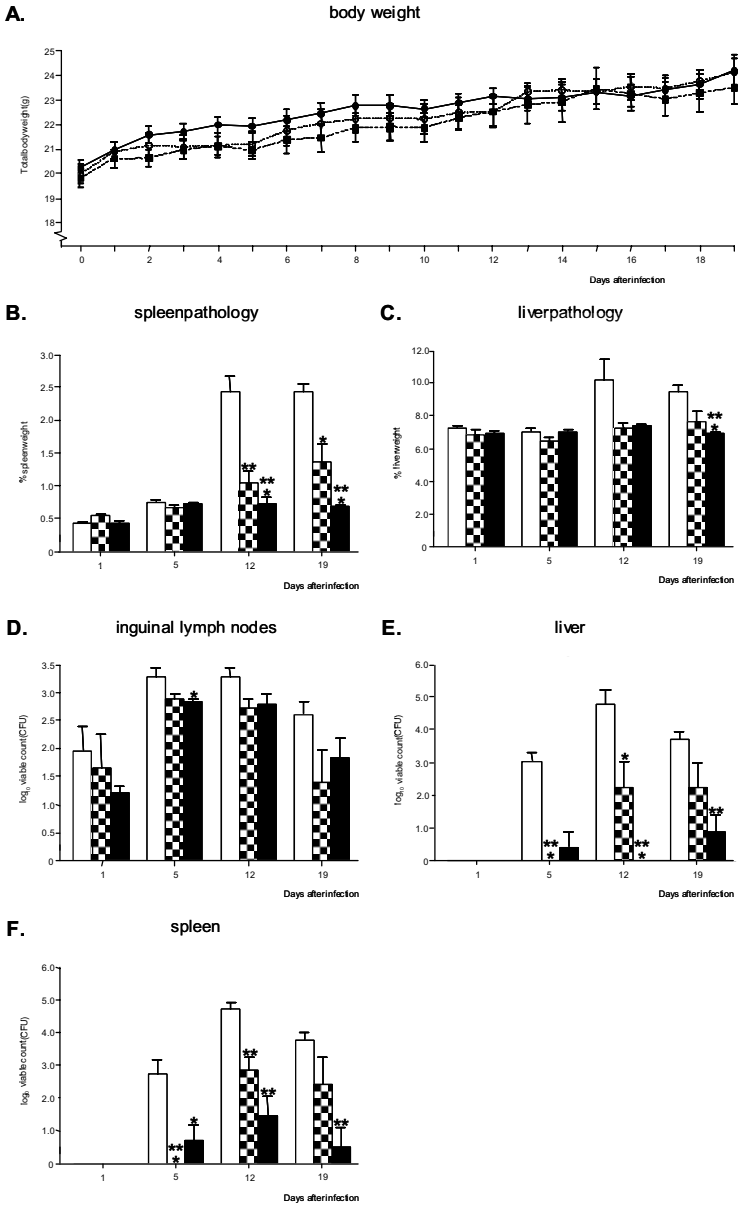

Figure 4. Total body weight (A), induced pathology in the spleen (B) and liver (C), and number of bacteria in the inguinal lymph nodes (D), liver (E), and spleen (F). Mice were infected subcutaneously in the flanks with 3×10^4 CFU of 14028s (white dots and white bars), AVD16703 (black squares and checkered bars), and 14208r (black dots and black bars). At the indicated time points, livers, spleens, and lymph nodes were aseptically removed and weighed. The viable counts within the organs were determined by making lysates and plating serial dilutions of the lysates and are expressed as $\log_{10}$ viable counts (means ± standard errors of the means). Averages from 4 mice per time point and per group are shown. Asterisks indicate statistically significant differences compared to the wild-type-infected mice (Student's *t* test) and the gray dashed lines represent the detection limit of the microbiological method (50 CFU for the livers and 30 CFU for the spleens and lymph nodes

~50% after 48 h (Fig. 3B). The induced cytotoxicity seemed slightly higher for the 14028r strain at 48 h, but this was not statistically significant. When the relative cytotoxicity was calculated as LDH release per 10^4 intracellular bacteria, it appeared that this was reduced for both mutant AVD16703 and the 14028r strain (Fig. 3C). This indicates that with similar cytotoxicity more mutant *S. enterica* serovar Typhimurium was present in the macrophages as reflected by the relative cytotoxicity (Fig. 3C). Since the mutants have truncated LPS that might have influenced the activation status of the macrophage, we added different concentrations of LPS during the in vitro infection. However, no effect of extracellular LPS was observed (Fig. 3D).

In vivo infection with AVD16703 and 14028r. To determine whether the mutant strains are capable of surviving for a longer period of time in mice, we performed an in vivo infection experiment in which C3H/HeN mice were infected subcutaneously in the flanks with ~3 $\times$ 10^4 CFU of wild-type 14028s, 14028r, or AVD16703. All the mice showed an increase in total body weight during the 19 days after infection. Only in the wild-type infected mice showed growth was halted between days 2 and 5 after infection (Fig. 4A) but the mice showed no reduction in body weight. The mice that were infected with the wild-type strain showed an increase in spleen and liver pathology (Fig. 4B and 4C) while the 14028r-infected mice showed no signs of hepatosplenomegaly. The AVD16703 infected mice showed no increase in liverweight, but did show a slight increase in spleenweight reaching intermediate spleenweights (Fig. 4B and 4C). All the strains tested showed detectable numbers of bacteria in the inguinal lymph nodes already on day 1 after infection and in all groups the numbers increased reaching a peak between days 5 and 12 (Fig. 4D). For the livers and spleen the strains behaved differently. The wild-type infected mice showed high bacterial numbers in the liver and spleen peaking on day 12 after infection, while in the 14028r and AVD16703-infected mice hardly any bacteria could be detected (Fig. 4E and 4F).

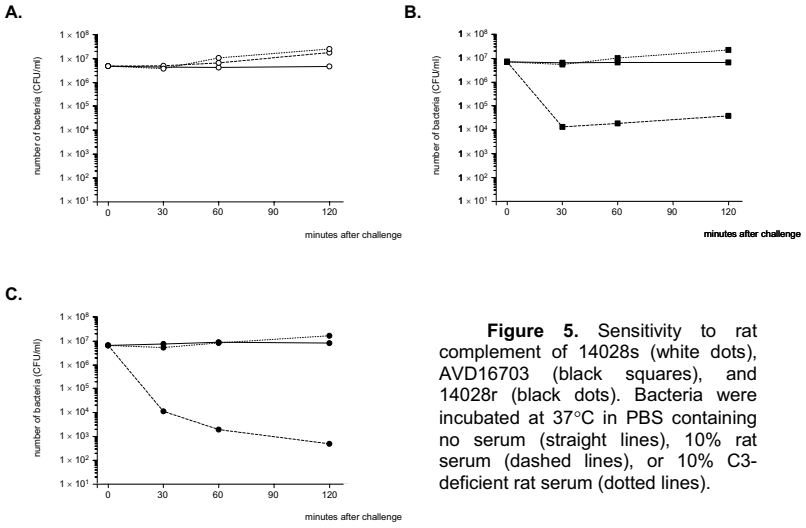

Figure 5. Sensitivity to rat complement of 14028s (white dots), AVD16703 (black squares), and 14028r (black dots). Bacteria were incubated at 37°C in PBS containing no serum (straight lines), 10% rat serum (dashed lines), or 10% C3-deficient rat serum (dotted lines).

In vitro sensitivity to complement. The in vivo attenuation could have been explained by increased sensitivity to complement-mediated killing as has been described for other LPS mutants (14). Therefore, we determined the rate of killing by complement of the wild-type strain and the two LPS mutant strains AVD16703 and 14028r. First, we determined the in vitro killing of rat serum that had not been heat inactivated. The wild-type strain is not killed when incubated in the presence of 10% rat serum and bacterial numbers are comparable to those bacteria that were incubated in the absence of serum (Fig. 5A). The LPS mutant strains, on the other hand, appeared to be more sensitive to rat complement since bacterial numbers declined already after 30 minutes (Fig. 5B and 5C). AVD16703 then stabilized, while the numbers of 14028r declined even further during the next 90 min (Fig. 5C). Comparable results were obtained when human serum was used (data not shown). When rat serum was used that was deficient for the C3 component of the complement system, the bacteria were not killed (Fig. 5A, B, and C), indicating that the C3-mediated complement is involved in the increased sensitivity to complement-mediated killing of the LPS mutants. However, when mouse serum was used in this experiment, no killing was observed even when the bacteria were incubated in 100% serum. Even when much lower numbers of bacteria were used, these mutants were not killed (data not shown). However, the outgrowth within 4 h of the LPS mutants was less than that of the wild-type strain, indicating that complement-mediated killing could play a role in vivo, when bacterial numbers are very low.

Discussion

To gain insight into the role of *Salmonella*-induced cell death in virulence, we have selected for mutants that survived for prolonged periods after infection in macrophages, when most of the cells infected with wild-type *S. enterica* serovar Typhimurium 14028s have undergone cell death. We reasoned that such mutants would be less cytotoxic. The relative cytotoxicity of the selected mutants was lower compared to the wild-type strain. These mutants reached higher intracellular numbers in the in vitro macrophage infection assay while inducing a similar cytotoxicity as the wild-type strain did at a much lower number of intracellular bacteria. Also, the LPS mutants were highly attenuated in vivo indicating that *Salmonella*-induced cell death should be regarded as a virulence determinant.

We have selected two mutants from the in vitro infection assay and sequence analysis revealed that these mutants were identical and the MudJ transposon had inserted in the *rmlC* gene encoding dTDP-4-deoxyrhamnose 3,5-epimerase (RmlC). RmlC is involved in the pathway of biosynthesis of dTDP-$_{L}$-rhamnose from glucose 1-phosphate and thymidine triphosphate. The *rmlC* gene is part of the *rfb* gene cluster that is involved in the LPS O-antigen biosynthesis of *S. enterica* serovar Typhimurium (9). RmlC catalyzes the third step in the biosynthesis of dTDP-$_{L}$-rhamnose, which requires three additional enzymes RmlA, B, and D. These enzymes act together to synthesize dTDP-$_{L}$-rhamnose from $_{D}$-glucose-1-

phosphate and dTTP. The sugar dTDP-$_L$-rhamnose is the precursor of $_L$-rhamnose, a major residue in the O-antigen of LPS. An intact LPS chain is essential for colonization and resistance to complement-mediated serum killing as the shorter the LPS chain, the more sensitive these mutants get to complement-mediated serum lysis and the lesser these *S. enterica* serovar Typhimurium mutants are able to colonize the intestines (12, 14). The biosynthesis of dTDP-$_L$-rhamnose by the four enzymes RmlABCD is a very important process for *Salmonella* since humans do not synthesize dTDP-$_L$-rhamnose and therefore cannot be taken up and has to be produced by *Salmonella* itself. It has therefore been stated by others that these four enzymes including RmlC might be very good targets against which new drugs might be designed (4). When looking at the data presented here, this might indeed be a good target for defense against *Salmonella* colonization since our mutant lacked the O-antigen of LPS and as a result showed an attenuated in vivo phenotype in mice and increased sensitivity to human complement, so targeting of the genes involved in $_L$-rhamnose biosynthesis might indeed be a good way of designing a potent drug against human *Salmonella* infection.

Since infection with the RmlC mutant and the 14028r strain resulted in decreased relative cytotoxicity in RAW264.7 macrophages and since SPI-1-inducing conditions had no effect on the early induction of cell death (data not shown), this would suggest that these LPS mutants induce cell death in a SPI-1-independent manner and that SipB and caspase-1 are not required. Therefore, it is most likely that the second, delayed type of *Salmonella*-induced cell death is induced at a higher rate for these mutants. In the model proposed by Van der Velden et al. (15) *Salmonella*-induced cell death is generally thought to reflect a bacterial strategy to promote disease (1) and seen as a virulence mechanism allowing the bacteria to spread from cell to cell within the host. The delayed, caspase-1 independent, type of *Salmonella*-induced cell death is proposed to be required during the systemic phase of infection and is used to spread intercellularly within apoptotic bodies (15). The LPS mutants displayed similar cytotoxicity to infected macrophages as the wild-type strain while showing increased intracellular numbers. This could be due to increased intracellular replication that could continue for a longer period because of the relatively lower induction of cytotoxicity. However, despite the increased numbers of intracellular bacteria, these mutants were highly attenuated in vivo. This suggests that these LPS mutants were killed at a higher rate or were not able spread from the inguinal lymph nodes. Increased killing could have been due to increased complement-mediated killing as the LPS mutants are highly susceptible to rat complement (Fig. 5). However, the LPS mutants were not killed when mouse serum was used and therefore we could not confirm the in vivo attenuation to be due to increased complement-mediated killing. Still, complement-mediated killing is a likely explanation for the in vivo attenuation since mouse complement should be able to kill, eliminate, or at least reduce bacterial loads when bacterial numbers are low and local concentrations of complement are high. Another explanation could be the increased opsonisation of the LPS mutants with mouse complement that does not result in killing by complement itself, but to increased uptake and killing of the bacteria by granulocytes, thereby preventing the bacteria form reaching the liver and spleen.

We have shown that LPS mutants display increased bacterial intracellular numbers in RAW264.7 macrophages that persist even after 48 h while inducing more cell death. This would suggest that the infected cells that were still intact contained more intracellular bacteria without being lysed. This could mean that the LPS mutants are able to grow very fast intracellularly and are able to reach high bacterial numbers inside macrophages. However, despite the increased ability to survive within cells, these LPS mutants are strongly attenuated in vivo due to mechanisms that need to be studied in more detail. Based on the data presented here, one would suggest that the relatively reduced induction of cell death of the LPS mutants is not beneficial to the in vivo virulence of *S. enterica* serovar Typhimurium, despite the increased ability to survive within macrophages. Apparently, *Salmonella*-induced cell death is a process that needs to be regulated and further research on the relevance of *Salmonella*-induced cell death is necessary before it is clear what the in vivo relevance of *Salmonella*-induced cell death is.

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