Salmonella Pathogenicity Island 1-Independent Induction of Apoptosis in Infected Macrophages by Salmonella enterica Serotype Typhimurium

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The enteric pathogen Salmonella enterica serotype Typhimurium induces apoptosis in infected macrophages. This process is rapid, specific, and depends on the type III protein secretion system encoded within Salmonella pathogenicity island 1 (SPI1). Here, we demonstrate that serotype Typhimurium can activate programmed macrophage cell death independently of SPI1. SPI1 independent induction of apoptosis in infected macrophages is observed as early as 12 to 13 h postinfection, even in the absence of intracellular bacterial replication. Delayed activation of programmed macrophage cell death is not observed with serotype Typhimurium strains mutated in ompR or SPI2. Even though SPI2 mutants have a defect in intracellular proliferation, our results indicate that long-term intracellular survival and growth are not required for delayed macrophage killing, since Salmonella mutants that are severely defective in intracellular growth still induce delayed apoptosis. Inactivation of genes required for either rapid or delayed induction of apoptosis results in a conditional noncytotoxic phenotype, whereas simultaneous inactivation of genes required for both rapid and delayed induction of apoptosis renders serotype Typhimurium noncytotoxic under all conditions tested. Our hypothesis is that differential activation of programmed macrophage cell death by serotype Typhimurium occurs under discrete physiological conditions at distinct locations within an infected host.

Salmonella enterica serotype Typhimurium is a facultative intracellular pathogen that causes a typhoid like disease in mice. Following oral infection, bacteria actively invade the intestinal mucosa and enter the bloodstream via the gut-associated lymphoid tissue (GALT). Subsequent residence within professional phagocytes of the liver and spleen is required for a persistent infection, which ultimately leads to the death of the mouse. Growth and survival of Salmonella within macrophages is supported by numerous studies, including the direct observation of Salmonella within hepatic phagocytes (45), comparative infection studies in genetic strains of mice that produce macrophages with varying resistance to Salmonella (38, 40), and the persistence of infection in mice treated with gentamicin, an antibiotic that primarily kills extracellular bacteria (10, 18). Finally, genetic studies indicate that Salmonella mutants that are attenuated for intramacrophage survival are also attenuated for systemic infection in mice (20). While all of these studies demonstrate that Salmonella survives and replicates within macrophages, several groups have recently shown that Salmonella is also able to kill these host cells (3, 13, 35, 39).

Contradictory results have been reported for Salmonella genes required for the induction of apoptosis as well as the timing at which it takes place. One study showed that serotype Typhimurium kills macrophages as late as 18 h postinfection (35). This process depends on the two-component regulatory system ompR-envZ, as ompR was the only gene identified in a stringent selection to find Salmonella mutants that are unable to kill macrophages. InvA is an essential structural component of the Salmonella pathogenicity island 1 (SPI1)-encoded type III export apparatus, whereas SipB is a SPI1-secreted effector molecule (22, 30). Null mutations in either invA or sipB, two genes within SPI1, had no effect on the ability of serotype Typhimurium to kill infected macrophages in this study (35). However, other studies appear to contradict these observations and demonstrate that within a few hours upon contact, serotype Typhimurium induces apoptosis in infected macrophages in an invA (and thus SPI1)-dependent process (13, 36, 39). SipB is both necessary and sufficient for the rapid activation of this apoptotic pathway (29).

Here, we resolve this apparent controversy by demonstrating that serotype Typhimurium kills macrophages via two independent processes. It is demonstrated that SPI1 gene expression accounts for rapid induction of apoptosis, whereas SPI1-independent, delayed induction of apoptosis is abrogated in strains mutated in ompR and SPI2. These results have important implications for understanding Salmonella pathogenesis, which are discussed.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and recombinant DNA techniques. Bacteria were grown overnight in Luria-Bertani (LB) broth at 37°C. Antibiotics, when required, were used at the following concentrations: nalidixic acid (Nal), 50 µg/ml; chloramphenicol (Cam), 30 µg/ml; kanamycin (Kan), 60 µg/ml; and ampicillin (Amp), 100 µg/ml. Recombinant DNA techniques and Southern hybridizations were performed using standard protocols (4, 37). Analytical-grade chemicals were purchased from Sigma (St. Louis, Mo.) or Roche Biochemicals/Boehringer Mannheim (Indianapolis, Ind.).

Mutations in the ompR, invA, sipB, and invC genes have been described previously (20, 23, 35, 49) and were used to construct a set of isogenic serotype Typhimurium mutants (Table 1). Bacteriophage KBIint was used to transduce the ompR::MadF allele of SWL350 (35) into SR-11 x 3804 (wild type [wt]), yielding strain AWM405 (ompR). Bacteriophage P22Hinf was used to transduce the invA::Tpho allele of AJB75 (7) into AWM501 (sipB, see below) and AWM527 (swrB, see below), yielding AWM544 (invA sipB) and AWM545 (swrB...
immA), respectively. Bacteriophage P22Hinf was used to transduce the sprB::mTn5 allele of STN19 (49) into SR-11 x 3041 (wt), yielding strain AW568 (sprB). Bacteriophage P22Hinf was also used to transduce the prc::Tn10 allele of MS4290 (20) into SR-11 x 3041 (wt), yielding strain AW564 (prc).

Allelic exchange was performed to disrupt the serotype Typhimurium immA gene. An internal fragment of the immA gene was amplified from serotype Typhimurium ATCC14028 (wt) using primers 5'-CACGATATCTGAGCAGACGCGTCCG-3' and 5'-GGTCTTCAAGCTTCCATTTCT for an EcoRI and 3' XbaI sites, respectively. This PCR product was ligated into the EcoRV site of pBluescript II SK(+) and sequenced. Subsequently, the immA allele was inactivated by insertion of a chloramphenicol resistance gene (a 1.2-kb Smal fragment from pCMXX [7]) into a unique internal SmaI site and cloned into suicide plasmid pKAS32 (48). The resulting plasmid was electroporated into Escherichia coli SM10pir and conjugated to serotype Typhimurium ATCC 14028 derivative BA715 (pir+) (1). A double crossover at the immA allele was obtained via homologous recombination. A chloramphenicol- and streptomycin-resistant exconjugant was selected and named SWL2020 (immA). Bacteriophage KB1inf was used to transduce the sprB::mTn5 allele into SR-11 x 3041 (wt) and AW5405 (ompR), yielding strains AWM527 (sprB) and AWM543 (ompR sprB), respectively.

Macrophage assays. The murine derived macrophage cell lines J774 (American Type Culture Collection [ATCC], Manassas, Va.) and RAW264.7 (ATCC) were cultured for 6 days (37°C, 5% CO2) in DMEM supplemented with 10% FBS, essential amino acids (Gibco-BRL). Bone marrow-derived macrophages were isolated from C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine) and cultured for 6 days (37°C, 5% CO2) in DMEM supplemented with 10% FBS, 20% L929 supernatant (a generous gift from H. G. A. Bouwer, Immunology Research, VAMC, Portland, Ore.), and glucose and sodium pyruvate (Gibco-BRL).

Macrophage survival assays (gentamicin protection assays) were performed as described by Fields et al. (20). In brief, 105 J774 macrophages were infected with stationary-phase cultures (below) at a multiplicity of infection (MOI) of 0.1. At 1 h postinfection, monolayers were washed three times with phosphate-buffered saline (PBS) and lysed with Triton X-100 (Sigma). Bacterial viability was determined by plating for viable intracellular CFU. Differences between strains were observed and taken into account by normalizing to the number of internalized bacteria (approximately 1% of input bacteria). At 6 and 18 h postinfection, the release of LDH was quantified colorimetrically using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, Wis.). The absorbance (A492) was determined on a microplate reader (Dynatech Laboratories, Inc., Chantilly, Va.), after which the percentage of cytotoxicity was calculated using the following formula: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). The spontaneous release is the amount of LDH released from the inoculated macrophages.

### TABLE 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td>E. coli DH5α</td>
<td>endA1 hisd17 supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argF)U169 deoR [680 diur Δ(lacZ)M15]</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>S17pXir</td>
<td>pro thi recA hisd17 chromosomal RP4-2 (Tn1::JSR1 tet::Mu Km::Tn7); pXir</td>
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</tr>
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<td>S. enterica serotype Typhimurium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AJB3</td>
<td>SR-11 yΔ252 (Kan')</td>
<td>51</td>
</tr>
<tr>
<td>AJB75</td>
<td>ATCC 14028 invA::Tphpho4 (Kan')</td>
<td>7</td>
</tr>
<tr>
<td>ATCC 14028</td>
<td>ATCC 14028 [pCMXX]</td>
<td>ATCC</td>
</tr>
<tr>
<td>BA715</td>
<td>ATCC 14028 ppsL (Str')</td>
<td>1</td>
</tr>
<tr>
<td>MW129</td>
<td>ATCC 14028 srrB::cat (Cam')</td>
<td>This study</td>
</tr>
<tr>
<td>MS4290</td>
<td>ATCC 14028 prec::Tn10 (Tet')</td>
<td>20</td>
</tr>
<tr>
<td>SR-11 x 3041</td>
<td>wt</td>
<td>R. Curtiss III</td>
</tr>
<tr>
<td>STN119</td>
<td>IR715 sprB::mTn5 (Kan')</td>
<td>49</td>
</tr>
<tr>
<td>SWL350</td>
<td>SR-11 ompR::MudJ (Kan')</td>
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<td>SWL2020</td>
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<tr>
<td>AWM664</td>
<td>SR-11 x 3041 prec::Tn10</td>
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Serovar Typhimurium kills macrophages independently of SPI1. Conflicting reports on macrophage killing (13, 35, 39) prompted us to investigate the effect of bacterial growth phase on the ability of serotype Typhimurium to kill macrophages. Throughout this study, two complementary methods were used to determine Salmonella-induced cell death in both J774 and RAW264.7 macrophages. In addition to measuring the release of cytoplasmic LDH, macrophage killing was calculated using a quantified macrophage cytotoxicity assay (data not shown) (35). Strikingly similar results were obtained with these two independent assays. Salmonella-induced macrophage cell death was determined by measuring the release of LDH at infection rates of about 0.7 and 1.5 bacteria per macrophage. Other MOIs were also tested, with identical results (data not shown).

Under SPI1-inducing conditions (see Materials and Methods) (13), rapid, SPI1-dependent macrophage killing was observed (Fig. 1A). In contrast, bacterial cultures grown to stationary phase, while unable to rapidly kill infected macrophages, induced a delayed cytotoxic effect (Fig. 1B). Delayed induction of macrophage cell death required neither invA nor sipB (Fig. 1B) and was observed as early as 12 to 13 h postinfection (Fig. 1C). These results suggest that serotype Typhimurium induces delayed macrophage cell death independently of SPI1.

SPI2 and ompR are required for delayed macrophage killing. Delayed cytotoxicity was dependent on a functional ompR locus, since ompR mutant bacteria were unable to kill infected macrophages (Fig. 2A). Recent evidence suggests that OmpR activates transcription of the SPI2 encoded regulon srrAB (32). This operon is essential for the transcription of SPI2 genes (14), which are highly induced inside macrophages (16, 50). To test whether, in addition to ompR, SPI2 is required for delayed induction of macrophage cell death, serotype Typhimurium strains mutated in srrB and sipB were tested. These genes encode a transcriptional activator and a structural component of the SPI2 encoded type III protein export apparatus, respectively (41). As shown in Fig. 2B, serotype Typhimurium strains mutated in ompR, srrB, or sipB were unable to kill infected macrophages when grown to stationary phase prior to infection. However, these strains were fully cytotoxic under SPI1 inducing conditions (Fig. 2C), indicating that ompR and SPI2 are not required for rapid induction of macrophage cell death. Cumulatively, these results suggest that delayed, SPI1-inde-
In agreement with the literature, we observed a defect (2- to 10-fold) in intracellular proliferation for SPI2 mutant strains at 15 h postinfection (14, 27, 28, 41, 46). However, long-term intracellular survival and proliferation is not required for delayed macrophage killing per se, since a prc mutant, encoding a periplasmic protease (6, 20) required for intracellular survival and growth (Fig. 3A) (11, 21), kills infected macrophages as efficiently as the wild type (Fig. 3B). Thus, despite a profound macrophage survival defect, the prc mutant was fully cytotoxic. In fact, the prc mutant strain was representative of a large panel of serotype Typhimurium mutants that are defective in intramacrophage survival and yet were still cytotoxic (data not shown). Collectively, these observations suggest that long-term intramacrophage survival and growth are not required for delayed, ompR- and SPI2-dependent macrophage killing. However, an indirect effect can not be ruled out until we have identified the SPI2 secreted effector(s) involved.

**Rapid and delayed macrophage killing processes are independent.** To determine whether rapid and delayed macrophage killing were independent of one another, doubly deficient mutant strains were constructed. Double mutants carried
null mutations in genes required for either rapid macrophage killing only (invA sipB), delayed macrophage killing only (ompR ssrB), or genes required for both rapid and delayed macrophage killing (ompR sipB, invA sipB, or ssrB invA double mutants). (A and B) Bacterial cultures were grown to either late-log phase (A) or stationary phase (B) prior to infection. Macrophage cell death was quantitated at 6 h (A) and 18 h (B) postinfection by measuring the release of LDH. The data from each graph are arithmetic means of at least three independent experiments. The error bars indicate the standard deviations of the mean.

Collectively, these results indicate that bacterial strains mutated in loci that affect both rapid and delayed macrophage killing are noncytotoxic under all conditions tested. These observations are evidence that rapid and delayed macrophage killing processes act independently of one another.

ompR and SPI2, but not SPI1, are required for delayed induction of apoptosis in infected macrophages. Next, we in-
investigated the nature of serotype Typhimurium-induced rapid and delayed macrophage cell death. Thus far, a nonspecific method, measuring the release of host cytoplasmic LDH, was used to calculate macrophage cytotoxicity. To determine whether macrophages were undergoing apoptosis upon infection with serotype Typhimurium, the amount of cytoplasmically located histones bound to fragmented DNA was quantified. Under SPI1 inducing conditions, serotype Typhimurium rapidly induced apoptosis via an SPI1-dependent process (data not shown). Under conditions that favored delayed macrophage cytotoxicity, killing was independent of SPI1 (Fig. 6). Delayed induction of apoptosis was abrogated in strains defective in either ompR or SPI2 (Fig. 6). These results indicate that serotype Typhimurium induces either rapid or delayed apoptosis in infected macrophages. Rapid activation of programmed cell death depends on SPI1, whereas delayed induction of apoptosis is SPI1 independent. Furthermore, our observations indicate that ompR and SPI2 are required for delayed activation of programmed macrophage cell death.

**DISCUSSION**

In this study, we demonstrate that macrophages undergo either rapid or delayed apoptosis upon infection with serotype Typhimurium. Delayed activation of programmed cell death is masked when SPI1 genes are expressed. Mutations that affect either rapid or delayed induction of apoptosis result in noncytotoxic phenotypes only under specific growth conditions. However, mutants defective in both rapid and delayed macrophage killing are unable to induce apoptosis under any condition tested, even at a high MOI (data not shown). Rapid activation of programmed macrophage cell death depends on SipB and the SPI1 encoded type III protein export machinery, whereas delayed induction of apoptosis is SPI1 independent. Our results indicate that ompR and a functional SPI2 encoded type III protein secretion system are required for delayed induction of apoptosis. However, a nonspecific effect cannot be excluded until we have identified an SPI2 effector(s) that is both necessary and sufficient for the activation of delayed programmed macrophage cell death.

In agreement with the literature, we observed a defect (2- to 10-fold) in intracellular proliferation for SPI2 mutants at 15 and 18 h postinfection (14, 27, 28, 41, 46). However, prc, htrA, and 11 other macrophage-sensitive mutants tested are fully cytotoxic and yet are more severely defective in their ability to survive and grow inside phagocytic cells (Fig. 3A) (11, 20). In fact, MS4290 (prc) was the most sensitive mutant isolated in an extensive search for Salmonella mutants that cannot survive inside macrophages (11, 20). Despite this substantial defect, prc mutant bacteria, as well as a large panel of other macrophage-sensitive serotype Typhimurium mutants, induced both rapid (data not shown) and delayed apoptosis in infected macrophages (Fig. 3B and Fig. 6). These results strongly support an additional role for SPI2 in delayed induction of apoptosis in infected macrophages.

Our observations indicate that rapid and delayed activation of programmed macrophage cell death are independent of one another, since mutations in SPI1 do not affect delayed induction of apoptosis and mutations in SPI2 do not affect rapid induction of apoptosis. Recent studies support this view by demonstrating that these two specialized protein secretion systems are controlled by distinct regulatory circuits. For example, substrates for the SPI1 encoded type III protein export apparatus are secreted under mildly alkaline conditions (15), whereas substrates for the type III protein export system encoded within SPI2 are secreted at pH 5.0 (9). Furthermore, numerous studies suggest that, once inside a phagocytic host, serotype Typhimurium represses SPI1 gene expression and turns on genes that are important for long-term residence, growth, and survival inside these host cells (2, 5, 8, 14, 16, 19, 24, 25, 33, 34, 43, 44, 50). It is therefore unlikely that substrates for SPI1 and SPI2 encoded type III protein export systems are secreted simultaneously.

Our hypothesis is that serotype Typhimurium induces rapid and delayed apoptosis in infected macrophages under discrete physiological conditions at distinct times and locations during the natural course of infection in the host (Fig. 7). Accumulating evidence suggests that the SPI1 encoded type III protein secretion system is important primarily during the intestinal phase of infection, since SPI1 mutants are significantly attenuated only when administered to mice orally (reference 22 and references therein and reference 23). In contrast, ompR and SPI2 are absolutely required during the systemic phase of infection (12, 16, 17, 41, 47, 50). In fact, SPI2 has been implicated in growth inside phagocytic cells at systemic sites of infection (12, 16, 17, 41, 47, 50). A possible consequence of the rapid, SPI1-dependent induction of apoptosis in macrophages of the GALT is that additional phagocytic cells are attracted to the site of inflammation. Our model suggests that Salmonella represses the SPI1-dependent killing mechanism upon internalization by macrophages, allowing continued proliferation and systemic spread prior to ompR- and SPI2-dependent induction of delayed apoptosis at systemic sites of infection. Because apoptotic cells are ingested by neighboring phagocytes, we propose that delayed induction of apoptosis in infected macrophages may allow Salmonella to spread intercellularly within apoptotic bodies. This model is supported by a recent study in which it was demonstrated that serotype Typhimurium is transported from the intestine, via the bloodstream, to the liver and spleen by CD18-expressing monocytes in an SPI1-independent process (52), as well as by studies in which it was demonstrated that Salmonella virulence was un-
affected by treatment with antibiotics that kill extracellular bacteria (10, 18).

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