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Salmonella enterica Serovar Typhimurium Induces Cell Death in Bovine Monocyte-Derived Macrophages by Early *sipB*-Dependent and Delayed *sipB*-Independent Mechanisms

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It was previously demonstrated that *Salmonella enterica* serovar Typhimurium induces cell death with features of apoptosis in murine macrophages. Mice infected with *Salmonella* serovar Typhimurium develop systemic disease without diarrhea, whereas the infection in cattle and in humans is localized and characterized by diarrhea. Considering these clinical disease expression differences between mice and cattle, we investigated whether serovar Typhimurium is cytotoxic for bovine macrophages. Macrophages infected with serovar Typhimurium grown in the logarithmic phase quickly underwent cell death. Macrophages infected with stationary-phase cultures or with a mutant lacking *sipB* underwent no immediate cell death but did develop delayed cytotoxicity, undergoing cell death between 12 and 18 h postinfection. Both pathways were temporarily blocked by the general caspase inhibitor Z-VAD-Fmk and by the caspase 1 inhibitor Z-YVAD-Fmk. Comparisons of macrophages from cattle naturally resistant or susceptible to intracellular pathogens indicated no differences between these two genetic backgrounds in terms of susceptibility to serovar Typhimurium-induced cell death. We conclude that *Salmonella* serovar Typhimurium induces cell death in bovine macrophages by two distinct mechanisms, early *sipB*-mediated and delayed *sipB*-independent mechanisms.

Salmonellosis is one of the most important human enteric diseases worldwide. It is the most prevalent food-borne infection in the United States, where the number of infections has been estimated to range from 800,000 to 3,700,000 annually (4). *Salmonella* infections display a broad range of clinical manifestations that are dependent on both the host species and the serotype causing the infections (8). Murine infection by *Salmonella enterica* serovar Typhimurium has been used extensively as a model for human salmonellosis. However, the clinical disease caused by *Salmonella* serovar Typhimurium in mice is more similar to the nondiarrheal human systemic typhoid fever caused by *S. enterica* serovar Typhi than to the diarrheal syndrome in humans infected with serovar Typhimurium (32). In contrast, in cattle, serovar Typhimurium causes an enteric disease, characterized by diarrhea and dehydration, which infrequently progresses toward a systemic infection (8, 13, 35, 42). The pathogenesis of salmonellosis in mice has been linked to the ability of the organism to invade intestinal epithelial cells, preferentially M cells, and the ability to survive inside phagocytic cells (11, 12, 14, 19). Although it has also been demonstrated that serovar Typhimurium invades the intestinal epithelium in cattle, initially through M cells, and then undergoes phagocytosis by macrophages (13), the role of intracellular survival in the pathogenesis of diarrhea is not clear. On the other hand, a functional *Salmonella* pathogenicity island (SPI) 1 (SPI-1) is required for virulence and diarrhea in cattle (35).

A large number of the virulence genes of *Salmonella* are located in restricted regions of the genome called SPIs. Five SPIs have been identified so far (3, 15, 25, 40, 41). SPI-1, located at 63 min on the *Salmonella* serovar Typhimurium chromosome map, is a 40-kb segment that encodes a type III secretion system. Proteins secreted by SPI-1 are involved in cell invasion and in the induction of apoptosis in murine macrophages (reviewed in reference 7). SPI-2 at 31 min on the chromosome map is 40 kb long and encodes a type III secretion system that plays a role in intracellular survival (6, 25).

In vitro infection with virulent *Salmonella* serovar Typhimurium induces apoptosis in mouse macrophages and macrophage cell lines, such as J774 and RAW264.7 (5, 21, 23). The cytotoxicity of serovar Typhimurium observed at 2 h postinfection is related to the capacity of this organism to invade, but not with intracellular replication (23). Mutants lacking invasion proteins encoded by SPI-1 failed to induce apoptosis in murine macrophages at 2 h postinfection (5, 23). This cytotoxic phenotype is dependent on the stage of bacterial growth, since cultures in the logarithmic phase of growth are cytotoxic, whereas stationary-phase cultures are not (22). The ability of logarithmically growing *Salmonella* to induce apoptosis correlates with the expression of invasion proteins encoded by SPI-1, such as the secreted protein, SipB, the regulator of SPI-1 expression, HilA, and a structural protein of the type III secretion apparatus, PrgH. On the other hand, cultures in the stationary phase of growth do not express these proteins (22). Furthermore, the cytotoxicity observed at 2 h after infection of macrophages is dependent specifically on SipB which, after

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translocation to the macrophage cytoplasm, binds to and activates caspase 1, triggering apoptotic cell death. Activated caspase 1 cleaves the interleukin-1β precursor to give rise to the active proinflammatory cytokine, which may be released after cell death. This proposed mechanism of pathogenicity may be important in vivo for the induction of an inflammatory response (16). A similar mechanism had been previously proposed for *Shigella*-induced apoptosis. Here, IpaB, which is orthologous to the *Salmonella* invasion protein SipB, also binds to caspase 1, thereby triggering the release of inflammatory cytokines (17).

In addition to the cell death induced by the SPI-1 gene products, which occurs soon after infection, another pathway of cell death has been described for a mouse macrophage cell line. In this pathway, the cytotoxicity is delayed compared to that induced by SPI-1 and is not dependent on the expression of invasion genes. However, mutants lacking *ompR* do not have the late cytotoxic phenotype (21). The *ompR* gene is a regulator for the expression of the type III secretion system encoded by SPI-2 (20).

Considering the differences in clinical manifestations between *Salmonella* serovar Typhimurium infection in mice, a typhoid fever model, and the diarrheal disease caused in cattle, it is important to determine whether or not bovine macrophages are susceptible to the cytotoxic mechanisms of serovar Typhimurium. The variability in the susceptibility of host cells to bacterial infection is illustrated by *Shigella* infection, in which apoptosis induced in mouse macrophages is mediated by IpaB but in which cell death in human macrophages is induced by a nonapoptotic pathway (10).

Since SPI-1 invasion genes are required for enteropathogenicity in cattle (2, 35, 36, 38) and SipB, an SPI-1-encoded protein, can induce in murine macrophages apoptosis that is followed by the release of inflammatory mediators, it is possible that the induction of cell death in bovine macrophages by *Salmonella* serovar Typhimurium infection is involved in the pathogenesis of diarrhea. Thus, as a first step in addressing this question, this study was aimed at determining whether bovine monocyte-derived macrophages undergo cell death after serovar Typhimurium infection and whether SipB and caspases are involved in such a mechanism.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Salmonella* serovar Typhimurium strain IR715 (31), a spontaneous nalidixic acid-resistant derivative of strain ATCC 14028, was used in this study. A derivative of ATCC 14028 carrying a nonpolar *sipB* deletion has been described by Tsolis et al. (34).

Bacteria were grown in 5 ml of Luria-Bertani (LB) broth for 20 h at 37°C under agitation (230 rpm). Then, 50 μl of the bacterial suspension was reinoculated into 5 ml of fresh LB broth and incubated under the same conditions as those described above for 5 h to obtain a logarithmic-phase inoculum and for 20 h to obtain a stationary-phase inoculum.

Animals. Six crossbred cattle (one bull and five cows) ranging in age from 6 to 15 years were used. They were kept in U.S. Department of Agriculture-approved facilities and received hay, 10 lb of commercial food daily, mineral and vitamin supplements, and water ad libitum. The cattle were divided into two groups—naturally resistant ($n = 3$) and susceptible ($n = 3$) to intracellular pathogens—according to criteria previously reported (9, 26, 27). Except for the comparison between resistant and susceptible animals, all of the experiments were conducted using cells from a resistant cow.

Peripheral blood monocyte-derived macrophage isolation, culturing, and infection. The protocol used for monocyte isolation was described previously (27). Briefly, venous blood was collected into anticoagulant (acid-citrate-dextrose), diluted 1:2 in phosphate-buffered saline (PBS)–citrate (pH 7.4), layered over a Percoll (Amersham Pharmacia Biotech AB, Uppsala, Sweden) solution with a specific density of 1.0770 (mixture of the following solutions: 10:1 Percoll and 1.5 M NaCl in 1.2% NaH_2PO_4; 130 mM trisodium citrate; 5% bovine serum albumin; and PBS [adjusted for a final refractive index of 1.3460]), and centrifuged at $1,000 \times g$ for 30 min. The coat containing white blood cells was collected, washed in PBS-citrate, resuspended in supplemented RPMI medium (Gibco BRL, Life Technologies, Inc., Grand Island, N.Y.) with 4% autologous serum, and incubated at 37°C with 5% CO_2 overnight in Teflon flasks. Then, the medium containing the nonadherent cells was removed and replaced with supplemented RPMI medium with 12.5% autologous serum. The medium was changed every 3 days. The monocytes differentiated into macrophages after 7 to 10 days in culture. All the experiments were conducted with cells kept in cultures for 10 to 11 days.

For inoculation, the bacterial suspension was diluted in supplemented RPMI medium. A multiplicity of infection (MOI) of 50:1 was used for all experiments, since preliminary experiments showed that with MOIs of 10:1 and 100:1, high percentages of cells (mean and standard deviation, 83.65% ± 3.23% and 97.02% ± 3.24%, respectively) were infected in our system. The inoculation was followed by centrifugation ($500 \times g$, 5 min) and incubation at 37°C in 5% CO_2 for 30 min. Subsequently, gentamicin (Gibco BRL) was added to the medium to a final concentration of 25 μg/ml in order to kill extracellular bacteria.

Cytotoxic assay. Macrophages were harvested from Teflon flasks by placing the flasks on ice and then were resuspended in supplemented RPMI medium with 12.5% heat-inactivated autologous serum to make a suspension of 5×10^5 cells/ml. The macrophages were seeded in 96-well plates (50,000 cells/well), centrifuged ($500 \times g$, 5 min), and incubated overnight at 37°C in 5% CO_2. At 1, 6, 12, or 18 h after inoculation, the cells were fixed with 1.85% formaldehyde in PBS for 15 min, stained with 0.13% crystal violet for 2.5 h, and washed extensively. Absorption was measured by use of a microplate reader with a 630-nm filter (Dynatech Laboratories, Inc., Chantilly, Va.). The readings obtained for the uninfected wells were considered to represent 100% survival, and the survival of the infected cells was calculated based on the reading for the uninfected control [(A_{630} for infected cells/A_{630} for uninfected control) × 100]. For some experiments, cells were incubated with either a general caspase inhibitor, Z-VAD-Fmk, or the caspase 1 inhibitor Z-YVAD-Fmk or Z-WEHD-Fmk (Enzyme System Products, Dublin, Calif.) (33) for 1 h prior to inoculation.

TUNEL analysis of DNA. For terminal deoxyribonucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assays, macrophages were inoculated with *Salmonella* serovar Typhimurium in Teflon flasks (2×10^6 cells/flask). TUNEL analysis was performed using a commercial kit (Pharmingen, San Diego, Calif.) in accordance with the manufacturer's instructions, except for an additional incubation with purified mouse immunoglobulin G (Sigma, St. Louis, Mo.). The macrophages were harvested by placement on ice at 0, 20, 60, or 180 min after inoculation and incubation for 30 min as described above. The cells were fixed in 1% paraformaldehyde in PBS for 15 min on ice, washed, and stored in 70% ethanol at −20°C for 2 to 4 days. The cells were incubated with a labeling solution containing terminal deoxyribonucleotidyltransferase and bromo-dUTP, followed by washes and incubation with purified mouse immunoglobulin G, fluorescein-labeled antibody to bromo-dUTP, and finally propidium iodide. The cells were then analyzed by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, Calif.). Flow cytometric data were analyzed with Flow Jo (Tree Star, Inc., Palo Alto, Calif.).

Assessment of bacterial uptake and intracellular survival. Bacterial uptake and intracellular survival were assessed following a protocol previously described but with modifications (27). Macrophages were seeded in 96-well plates (40,000 cells/well) and incubated overnight (37°C, 5% CO_2). After inoculation, centrifugation, and incubation for 30 min (37°C, 5% CO_2), gentamicin was added to the medium to a final concentration of 25 μg/ml. The cells were incubated for 1 h and washed four times with 100 μl of fresh medium per well. At 1 and 6 h after inoculation, the macrophages were lysed by the addition of 0.5% Tween 20 (Sigma). After the wells were washed three times, samples were diluted and plated on LB agar plates to enumerate CFU. As a control, the inoculum was grown in the absence of macrophages under the same conditions, except for the addition of gentamicin, to ensure that the bacteria survived and grew. Each inoculum was also incubated with medium containing gentamicin for 1 h to confirm the activity of the antibiotic.

Statistical analysis. The quantitative data were submitted to analysis of variance, and the averages were compared by using the Duncan test. Percentage data underwent angular transformation before statistical analysis. Differences were considered significant when P was <0.05 (30).

TABLE 1. Percentages of TUNEL-positive bovine macrophages (apoptotic cells) infected with logarithmic- or stationary-phase *Salmonella* serovar Typhimurium at 0, 20, 60, and 180 min after infection

Time (min)	% of TUNEL-positive macrophages in the following samples[a]:		
	Uninfected control	Logarithmic phase	Stationary phase
0[b]	14.47 ± 15.42 x	47.75 ± 34.45 Ay	38.51 ± 39.49 xy
20	5.40 ± 6.20 x	75.54 ± 28.34 ABy	42.51 ± 28.88 z
60	6.65 ± 5.36 x	81.32 ± 26.97 By	35.57 ± 31.92 x
180	2.78 ± 1.88 x	93.41 ± 5.53 By	71.22 ± 13.39 y

[a] Values are means and standard deviations from five independent experiments. Different uppercase letters in the same column indicate significant difference ($P < 0.05$). Different lowercase letters in the same row indicate significant difference ($P < 0.05$).

[b] Time zero corresponds to macrophages incubated for 30 min at 37°C after challenge with *Salmonella* serovar Typhimurium (see Materials and Methods).

RESULTS

***Salmonella* serovar Typhimurium induces DNA fragmentation (TUNEL) in bovine macrophages infected with both logarithmic- and stationary-phase cultures.** Logarithmic-phase cultures of *Salmonella* serovar Typhimurium express SPI-1 genes (22), which have previously been shown to be necessary for diarrhea in calves (34, 35). In murine macrophages, stationary-phase cultures of *Salmonella* serovar Typhimurium induce a late form of cell death in a *sipB*-independent manner, while bacteria grown logarithmically cause cell death by an early, *sipB*-dependent pathway (37). Therefore, we addressed the questions of whether *Salmonella* serovar Typhimurium is cytotoxic for bovine monocyte-derived macrophages and whether the cell death in this system is dependent on the stage of bacterial growth.

Macrophages were infected with wild-type *Salmonella* serovar Typhimurium grown to logarithmic or stationary phase and processed for TUNEL and flow cytometric analysis. Macrophages were harvested at 0, 20, 60, and 180 min after infection. A relative increase in the numbers of TUNEL-positive cells was observed in infected samples compared to the low background observed in uninfected controls. Although both logarithmic- and stationary-phase bacteria induced DNA fragmentation (TUNEL), the percentages of labeled cells were higher in the samples infected with logarithmically growing bacteria at all times studied. This difference was statistically significant at 20 and 60 min after infection. Table 1 summarizes the results (means and standard deviations) for five independent experiments.

A mutation in *sipB* reduces early *Salmonella* serovar Typhimurium-induced DNA fragmentation in bovine macrophages. In the mouse model, it has been demonstrated that SipB is directly responsible for the induction of apoptosis by binding to caspase 1 (16). We therefore determined whether *sipB* is involved in the DNA fragmentation observed after infection of bovine macrophages with logarithmic-phase *Salmonella* serovar Typhimurium. Bovine macrophages were infected with either wild-type *Salmonella* serovar Typhimurium or a nonpolar mutant lacking *sipB*, both grown to logarithmic phase. One hour after infection, the cells were fixed, processed for TUNEL, and analyzed by flow cytometry. Significant differences in the percentages of cells showing TUNEL were observed between cultures infected with the wild type and those infected with the *sipB* mutant ($P < 0.05$). Higher percentages (means and standard deviations) of wild-type-infected macrophages (92.24% ± 2.71%; $n = 3$) than of macrophages infected with the *sipB* mutant (36.69% ± 20.58%; $n = 3$) were TUNEL positive. However, the *sipB* mutant was still able to induce DNA fragmentation in a fraction of cells that was significantly larger ($P < 0.05$) than the fraction of TUNEL-positive cells in uninfected controls (2.74% ± 0.48%; $n = 3$). These data indicate that early DNA fragmentation is at least partially associated with the secretion of SipB, although a low level of DNA fragmentation still can be observed in a mutant lacking *sipB* (Fig. 1).

The early cytotoxic effect of *Salmonella* serovar Typhimurium is *sipB* dependent, whereas delayed cytotoxicity is *sipB* independent, and both pathways require caspase activity. To determine whether *sipB*-mediated cell death involves caspase activity, the ability of a general caspase inhibitor (Z-VAD-Fmk) and two specific caspase 1 inhibitors (Z-YVAD-Fmk and Z-WEHD-Fmk) to block cell death measured by crystal violet staining was tested. The dose-response curve of Z-VAD-Fmk is shown in Fig. 2. The optimal concentration of the inhibitor was between 25 and 50 μM. Paradoxically, high concentrations of the inhibitor (100 μM) had no inhibitory effect at 1 h postinfection, and potentiation of the cytotoxic effect was observed at 6 h postinfection. A similar effect has been reported for cells undergoing tumor necrosis factor alpha (TNF-α)-induced apoptosis: Z-VAD-Fmk inhibited cell death at moderate concentrations but had the opposite effect at high concentrations (29). By 1 h after infection, 31.18% of untreated macrophages infected with the wild type grown logarithmically had died, and this cytotoxic effect was completely blocked by prior incubation of the cells with medium containing 25 μM general caspase inhibitor Z-VAD-Fmk. In contrast, macrophages infected with stationary-phase wild type or *sipB* mutant exhibited no cytotoxicity at 1 h after infection, and these cells even had an increase in the A_{630} reading. At 6 h after infection, the pattern of cytotoxicity was still the same; i.e., macrophages infected with stationary-phase wild type or *sipB* mutant showed no cytotoxicity (Fig. 3 and 4). Incubation of macrophages with a filter-sterilized supernatant from infected cells also caused an increase in the A_{630} reading (data not shown). In contrast, at 12 h after infection, all of the strains produced a cytotoxic effect (Fig. 4A) which could be inhibited partially by Z-VAD-Fmk (Fig. 4B). At 18 h after infection, most of the macrophages were dead regardless of the strain used (Fig. 4A), and the addition of Z-VAD-Fmk did not markedly inhibit cytotoxicity (Fig. 4B).

Approximately 92% of the cells were TUNEL positive at 1 h after infection with logarithmically growing *Salmonella* serovar Typhimurium (Fig. 1), while at this time the percentage of dead cells measured by crystal violet staining was considerably lower (Fig. 4). Thus, DNA fragmentation detected by TUNEL staining apparently preceded cell death detected by crystal violet staining during *Salmonella* serovar Typhimurium-induced cytotoxicity in bovine macrophages. However, significant differences were observed between stationary-phase wild type and the *sipB* mutant on the one hand and logarithmic-phase wild type on the other hand at 1 and 6 h postinfection (Fig. 4A), suggesting that crystal violet staining measured the

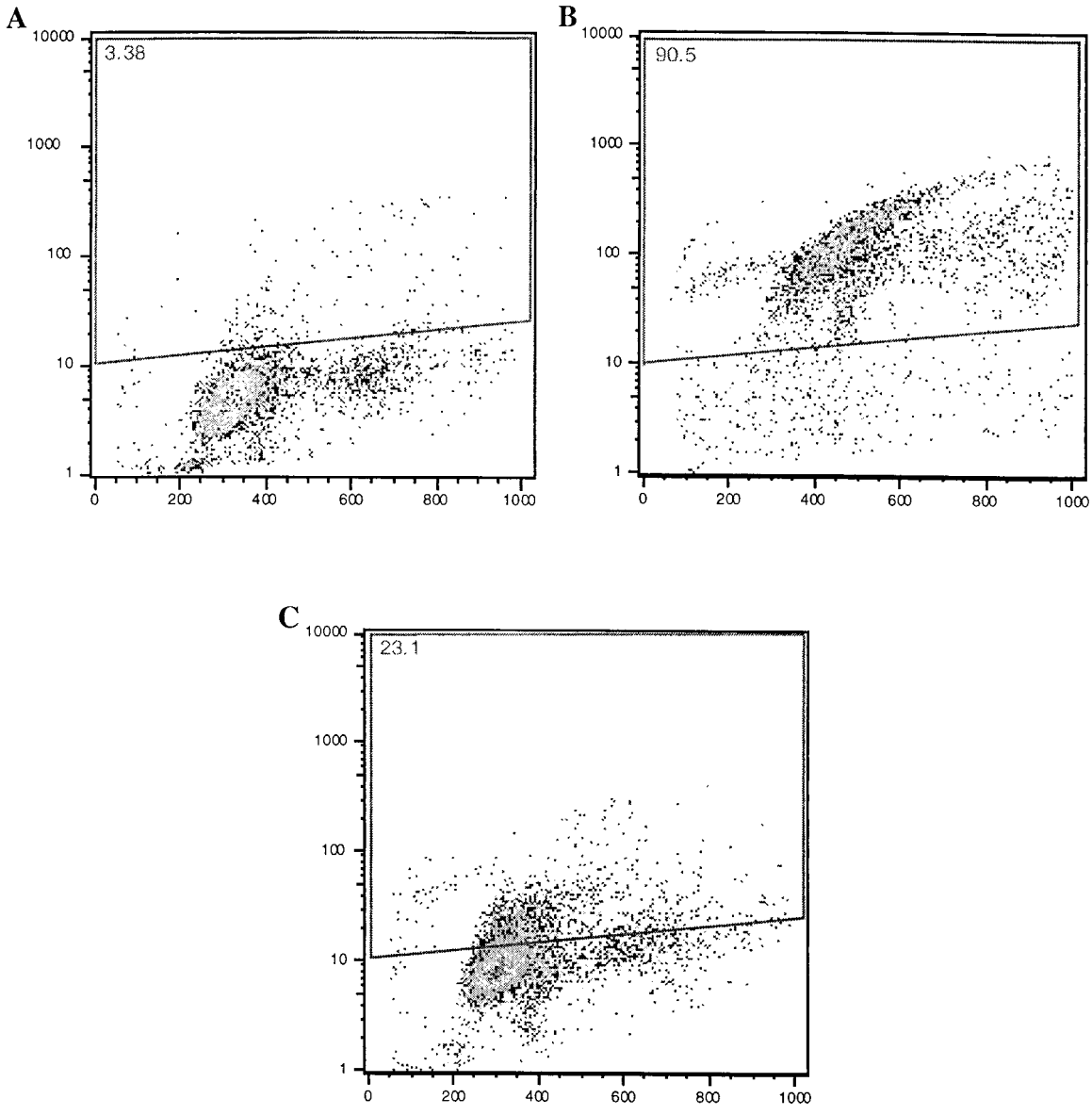

FIG. 1. Flow cytometric analysis of bovine macrophages infected with wild-type *Salmonella* serovar Typhimurium or a *sipB* mutant. All of the strains were grown to the logarithmic phase. Macrophages were infected in Teflon flasks with an MOI of approximately 50:1, harvested 1 h after infection, processed for TUNEL staining (see Materials and Methods), and analyzed by flow cytometry. In each dot plot, the *x* axis corresponds to propidium iodide staining and the *y* axis corresponds to bromo-dUTP incorporation. Apoptotic cells are within the area indicated by the quadrilateral, and the percentage of apoptotic cells is indicated at the top left corner of each panel. These data are from a representative experiment showing uninfected macrophages (A) with a low background of TUNEL-positive cells, macrophages infected with the wild type (B) and containing a high percentage of apoptotic cells, and macrophages infected with a mutant lacking *sipB* (C) and having a rate of apoptosis that was low but higher than that of the control.

sipB-dependent mechanism of cell death. Macrophages infected with the *sipB* mutant showed large numbers of TUNEL-positive cells at 6, 12, and 18 h postinfection (data not shown) which were similar to the numbers of TUNEL-positive cells infected with logarithmic-phase wild type at 60 and 180 min postinfection (Table 1). This result indicates a correlation between the cell death measured by these two methods.

The caspase 1 inhibitor Z-YVAD-Fmk was effective at 25 and 50 μM, blocking *sipB*-dependent cell death at 1 h after challenge and partially inhibiting the *sipB*-independent mechanism (Fig. 5). In contrast, when macrophages were preincubated with the specific caspase 1 inhibitor (Z-WEHD-Fmk) at concentrations ranging from 3.12 to 100 μM prior to infection with logarithmic-phase *Salmonella* serovar Typhimurium, no significant difference in the rate of survival compared to that for macrophages without inhibitor or with vehicle only was observed ($P > 0.05$; data not shown). Preincubation with 25 μM Z-WEHD-Fmk also had no effect on the delayed mechanism of cell death (data not shown).

To test the contribution of soluble factors to cell death, we

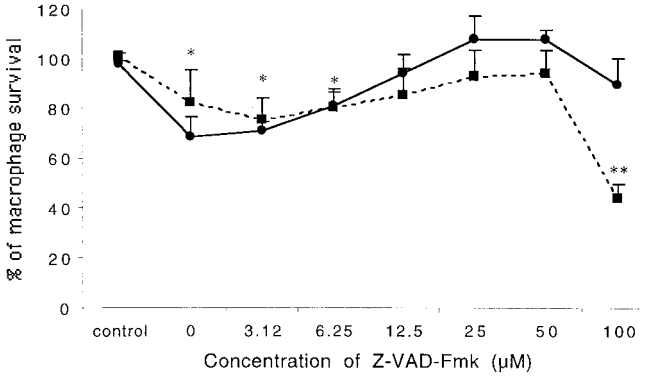

FIG. 2. Inhibition of *Salmonella* serovar Typhimurium-induced cytotoxicity by the general caspase inhibitor Z-VAD-Fmk. Macrophages were incubated with concentrations of Z-VAD-Fmk ranging from 0 to 100 μM, infected in 96-well plates with wild-type *Salmonella* serovar Typhimurium grown to the logarithmic phase, fixed at 1 h (solid line) and 6 h (broken line) after infection, stained with crystal violet, and analyzed by use of a microplate reader with a 630-nm filter; 100% survival corresponds to the average A_{630} reading of the uninfected control without the inhibitor. The values corresponding to the control value indicate the survival of uninfected macrophages treated with Z-VAD-Fmk (100 μM). Values are means and standard deviations ($n = 4$). Single asterisks indicate that the values at both 1 and 6 h postinfection were significantly lower than those of the control ($P < 0.05$). Double asterisks indicate that the value was significantly lower than all of the other values ($P < 0.05$).

collected medium from macrophages infected with both logarithmic- and stationary-phase wild-type *Salmonella* serovar Typhimurium from 0 min up to 12 h after infection. The medium was centrifuged, and the supernatant was filtered (0.2-μm pore size) and stored at −70°C. Macrophages were incubated with the filter-sterilized supernatant for various times, and cell death was evaluated by crystal violet staining. No cytotoxicity was observed when the macrophages were incubated with the supernatant for up to 18 h. Indeed, there was an increase in the A_{630} reading in the wells incubated with the supernatant; this increase was associated with morphological changes characterized by cells spreading out on the bottom of the well and rough cellular boundaries in comparison to the appearance of the control (data not shown). Macrophages inoculated with heat-inactivated organisms (65°C for 20 min) were not killed at 1, 6, and 12 h after challenge. Surprisingly, there was a slight but significant decrease in survival after 18 h of incubation when the macrophages were challenged with heat-inactivated *Salmonella* serovar Typhimurium; these cells showed 78.44% survival compared to the control ($P < 0.05$; $n = 4$).

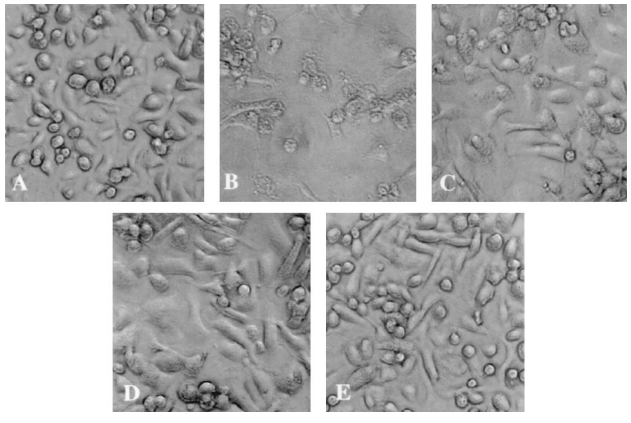

FIG. 3. *Salmonella* serovar Typhimurium-induced cytotoxicity on monocyte-derived bovine macrophages at 6 h postinfection. A cytotoxic effect was observed in logarithmic-phase *Salmonella* serovar Typhimurium-infected macrophages, whereas macrophages inoculated with stationary-phase wild type, *sipB* mutant, and heat-inactivated *Salmonella* serovar Typhimurium remained intact. (A) Uninfected control. (B to E) Macrophages inoculated with wild-type *Salmonella* serovar Typhimurium grown to logarithmic phase (B), wild-type *Salmonella* serovar Typhimurium grown to stationary phase (C), *sipB* mutant (D), and heat-inactivated *Salmonella* serovar Typhimurium (E).

The cytotoxic effects of *Salmonella* serovar Typhimurium are similar in macrophages from cattle with naturally resistant and susceptible genetic backgrounds. The experiments described above were performed with macrophages obtained from a single cow. To address whether this cow was representative of the general population, we compared cytotoxic responses produced by *Salmonella* serovar Typhimurium in macrophages isolated from different animals. In addition, the naturally resistant genetic background is important for in vitro resistance against intracellular infectious agents such as *Brucella abortus*, *Mycobacterium bovis*, and *S. enterica* serovar Dublin but not for resistance against *Salmonella* serovar Typhimurium (27). These experiments were designed to address the question of whether or not there is any difference in the cytotoxic effects of *Salmonella* serovar Typhimurium between resistant and susceptible individuals. No statistically significant difference was observed at 1 and 6 h postinfection in the A_{630} readings between macrophages from resistant and susceptible cattle infected with wild-type or *sipB* mutant *Salmonella* serovar Typhimurium (Table 2). The profile of cytotoxicity observed with these cattle was similar to that observed in the previous experiments, suggesting that the resistant animal used in the previous experiments was representative of the response in this host species.

Bacterial uptake and intracellular survival. To ensure that the differences in cytotoxicity were not due to more efficient uptake or intracellular survival of the logarithmic-phase wild type than of bacteria grown to stationary phase or *sipB* mutant bacteria, the numbers of intracellular bacteria were quantified at 1 and 6 h after infection. The percentages (means and standard deviations) of organisms phagocytosed [(number of intracellular organisms/number of organisms in the inoculum) × 100] were 36.81 ± 0.71 and 21.7 ± 1.44 for stationary-phase wild type and *sipB* mutant, respectively. Both strains survived intracellularly for up to 6 h postinfection (Table 3). The rapid killing of macrophages inoculated with logarithmic-phase IR715 made it impossible to quantify intracellular bacteria with this method since, due to the early cytotoxicity, only 39.72% of the macrophages remained attached after washing. Since cytotoxicity resulted in the detachment of macrophages infected with the logarithmically growing wild type, the bacterial numbers recovered from these wells could not be directly compared to the bacterial numbers recovered from wells infected with noncytotoxic mutants. These results suggest that although there are slight differences in uptake and intracellular survival, all of the strains were able to survive in macrophages.

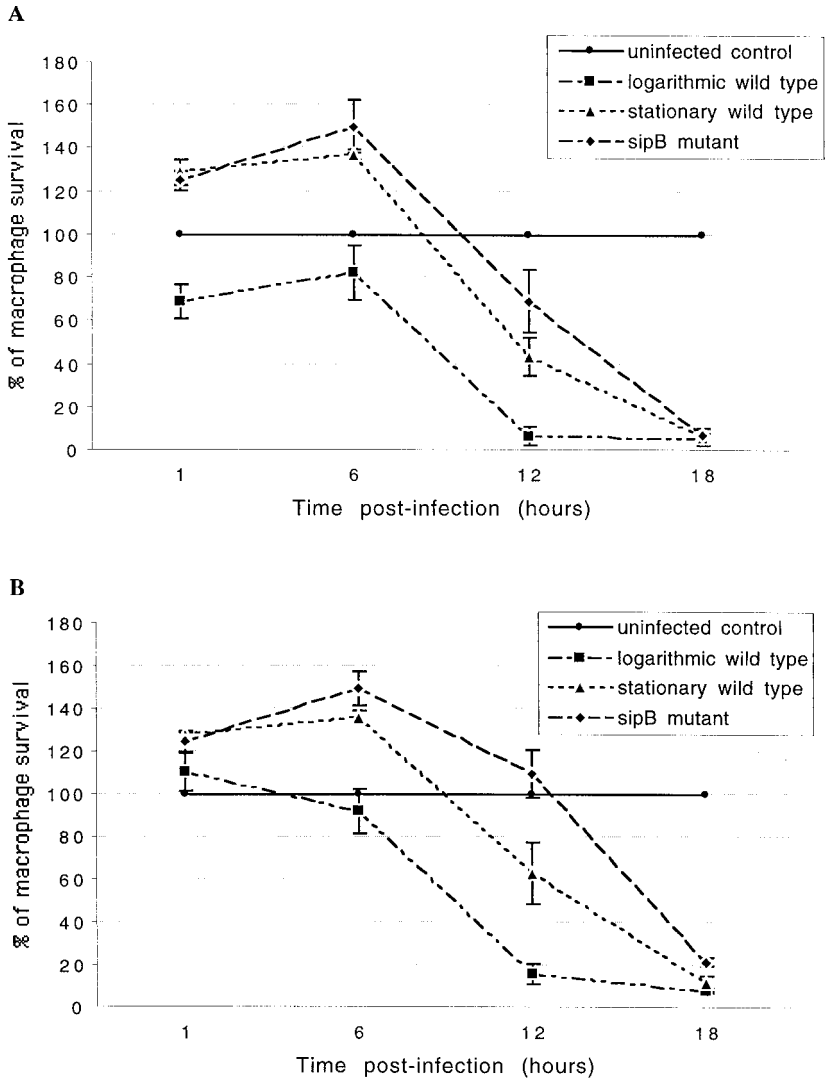

FIG. 4. Time course of bovine macrophage survival. (A) Survival after infection with wild-type *Salmonella* serovar Typhimurium grown to the logarithmic or stationary phase and a *sipB* mutant grown to the logarithmic phase. (B) Survival in the presence of a caspase inhibitor. Macrophages were incubated with the general caspase inhibitor Z-VAD-Fmk (25 μM) for 1 h prior to infection. To determine survival, macrophages were infected in 96-well plates, fixed at different times, stained with crystal violet, and analyzed by use of a microplate reader with a 630-nm filter. Values are means and standard deviations ($n = 4$).

DISCUSSION

Since the disease caused by *Salmonella* serovar Typhimurium in mice is systemic while that in cattle and humans is localized, bovine infection is a useful model for studying the pathogenesis of diarrhea. SPI-1 genes, including *sipB*, are required for the development of diarrhea in calves (34, 35). Recent reports indicated that SipB directly triggers apoptosis in murine macrophages. After SPI-1-dependent translocation of SipB into murine macrophage cytoplasm, the effector protein binds to caspase 1, which cleaves and activates interleukin-1β. This mechanism has been proposed to be a link between apoptosis and inflammation (16). Furthermore, caspase 1 knockout mice have an oral *Salmonella* serovar Typhimurium 50% lethal dose 1,000-fold higher than that for the wild type (24). In order to understand the role of SipB-mediated cytotoxicity during diarrheal disease in cattle, we investigated its contribution to eliciting cell death in bovine macrophages in vitro.

While this work was in progress, Watson et al. reported that *Salmonella* serovar Typhimurium kills bovine alveolar macrophages by a *sipB*-dependent pathway (39). Here, we demonstrated that in vitro infection of bovine monocyte-derived macrophages with *Salmonella* serovar Typhimurium induced cell death. There were two distinct mechanisms of cell death: the first, early cell death, which occurred very rapidly after infection and which depended on the presence of *sipB*, and the second, a delayed type of cell death, which occurred within 12 h after infection and which was *sipB* independent. DNA fragmentation occurred very early after infection, but DNA labeling was more intense when macrophages were infected with the logarithmically growing wild-type organism. Both mechanisms of cell death induced by *Salmonella* serovar Ty-

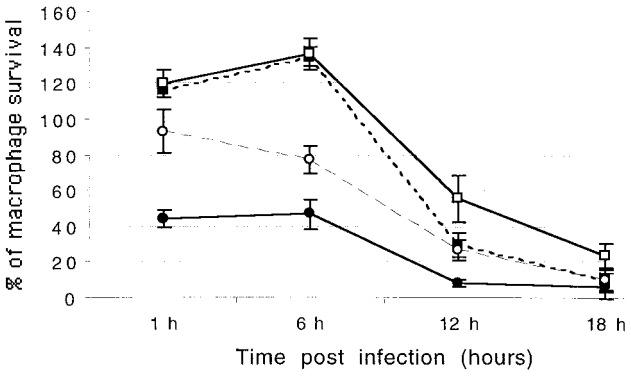

FIG. 5. Time course of bovine macrophage survival after infection with wild-type or *sipB* mutant *Salmonella* serovar Typhimurium grown to logarithmic phase in the presence or absence of a caspase 1 inhibitor (Z-YVAD-Fmk). Macrophages were incubated with Z-YVAD-Fmk (50 μM) for 1 h prior to infection. To determine survival, macrophages were infected in 96-well plates, fixed at different times, stained with crystal violet, and analyzed by use of a microplate reader with a 630-nm filter. Values are means and standard deviations ($n = 4$). Symbols: ●, wild type, 0 μM; ■, *sipB* mutant, 0 μm; ○, wild type, 50 μM; □, *sipB* mutant, 50 ìM.

TABLE 3. Uptake by bovine macrophages and intracellular survival of *Salmonella* serovar Typhimurium (wild type grown to the stationary phase and a *sipB* mutant) at 1 and 6 h postinfection

Strain	CFU (10^6)/well at the following hour postinfection[a]:		Ratio of 1-h to 6-h values (%[b])
	1	6	
Wild type	1.583 ± 0.03	2.403 ± 0.582	1.518 (151.8)
sipB mutant	1.063 ± 0.070	1.061 ± 0.238	0.998 (99.81)

[a] Values are means and standard deviations ($n = 3$).
[b] Rate of intracellular survival from 1 h to 6 h postinfection.

phimurium were temporarily blocked by incubation with a general caspase inhibitor (Z-VAD-Fmk) or a caspase 1 inhibitor (Z-YVAD-Vmk) prior to infection. Surprisingly, Z-WEHD-Fmk, which is the optimal target sequence for caspase 1 (33), did not significantly affect the rate of cell death induced by *Salmonella* serovar Typhimurium in bovine macrophages. The *sipB* mutant was fully able to infect and survive intracellularly within macrophages, a result which indicated that the differences in cytotoxicity between the wild type and the *sipB* mutant were not due to differences in uptake or survival.

After infection with logarithmic-phase *Salmonella* serovar Typhimurium, there was a rapid increase in the percentage of apoptotic cells, as detected by TUNEL, during the first hour of infection (Table 1). The DNA fragmentation observed by TUNEL preceded the cytolysis observed by crystal violet staining, indicating that cytolysis was delayed in relation to DNA fragmentation. Based on the mouse model, the extremely fast mechanism of cell death triggered by logarithmic-phase organisms can be explained by the direct action of SipB binding to and activating caspase 1 (16). A longer period required for cell death in *sipB*-independent cytotoxicity might suggest an indirect mechanism. It has been demonstrated with the mouse model that stationary-phase *Salmonella* serovar Typhimurium lacking the expression of the type III secretion system encoded by SPI-2 (20, 21) is not cytotoxic for J774 cells (37). Why these SPI-2 genes are required for delayed cytotoxicity is not clear. Recently, a SipB-mediated, caspase 1-independent mechanism of cell death, which involves caspase 2 activation, was reported for murine macrophages (18).

Macrophages infected with stationary-phase wild type or *sipB* mutant showed increases in A_{630} readings at 1 and 6 h after infection (Fig. 4) that may have been related to the staining of infecting bacteria, as previously reported (22). However, macrophages incubated with supernatant from infected cells also showed an increase in the A_{630} reading that may have been due to activation of the macrophages.

A homologue of the natural resistance-associated macrophage protein 1 (NRAMP1), initially identified in mice, has been described for bovine species (9). This protein has been implicated as a putative mediator of natural resistance for intracellular pathogens (1). A previous report on the cytotoxicity of *Salmonella* serovar Typhimurium for bovine alveolar macrophages did not specify whether cells were derived from resistant or susceptible animals (39). To investigate whether NRAMP1 may affect the ability of *Salmonella* serovar Typhimurium to kill bovine macrophages, we compared its cytotoxicities for macrophages from genetically susceptible and resistant animals. No differences in the rates of apoptosis were observed for *Salmonella* serovar Typhimurium-infected macrophages from resistant and susceptible animals. These results were consistent with previous reports indicating that macrophages from cattle naturally resistant to intracellular pathogens are more efficient at killing or preventing the growth of *B. abortus*, *M. bovis*, and *Salmonella* serovar Dublin but not *Salmonella* serovar Typhimurium (27). Our results suggest that

TABLE 2. Cytotoxicity of *Salmonella* serovar Typhimurium grown to logarithmic or stationary phase and a *sipB* mutant for macrophages from cattle naturally resistant or susceptible to *B. abortus* at 1 and 6 h after infection

Strain	% Macrophage survival at the following hour postinfection for the indicated cattle[a]:			
	1		6	
	Resistant	Susceptible	Resistant	Susceptible
Logarithmic phase	81.17 ± 6.74	88.71 ± 37.92	61.35 ± 12.38	65.01 ± 33.32
Stationary phase	130.61 ± 8.14	134.79 ± 42.31	132.44 ± 7.37	140.92 ± 37.95
sipB mutant	125.56 ± 10.67	132.22 ± 46.05	153.09 ± 2.06	153.46 ± 50.49

[a] Values are means and standard deviations for the percentage of macrophage survival in comparison to that in the uninfected control (see Materials and Methods). The experiments were performed in quadruplicate with three animals in each group. There was no significant difference between resistant and susceptible cattle ($P > 0.05$).

the cytotoxicity of *Salmonella* serovar Typhimurium may not be related to NRAMP1 in bovine species.

According to our results, both early and delayed mechanisms of cell death involve caspase activity, since the cytotoxic effect was either blocked or decreased when the macrophages were previously incubated with the general caspase inhibitor Z-VAD-Fmk (Fig. 4). The dose-response curve demonstrated maximal inhibition at concentrations of between 25 and 50 μM. The supernatant from infected bovine macrophages did not have a cytotoxic effect, suggesting that probably TNF-α and other soluble factors did not play an important role in the cell death induced by *Salmonella* serovar Typhimurium infection. In contrast, murine macrophages infected with *Mycobacterium tuberculosis* undergo apoptosis, and TNF-α plays a major role in this system (28). As previously proposed for *Salmonella*-induced murine macrophage apoptosis (16) and bovine alveolar macrophages (39), our results suggested that caspase 1 plays a role in the *sipB*-dependent mechanism of cell death in monocyte-derived bovine macrophages and apparently may have some function in the *sipB*-independent mechanism as well. The lack of activity of Z-WEHD-Fmk was not addressed in this study. These results suggest that *Salmonella*-mediated macrophage cell death is a proinflammatory mechanism that plays a significant role in the pathogenesis of enteritis and diarrhea in cattle. In vivo ligated ileal loop experiments with calves are the obvious next step to validate the implications of *Salmonella*-induced cell death in the pathogenesis of enteritis and diarrhea.

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