Shigella-induced necrosis and apoptosis of U937 cells and J774 macrophages

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It is currently unclear whether Shigella kills its phagocytic host cells by apoptosis or necrosis. This study shows that rapid necrosis ensues in macrophage-like cell lines (U937 cells differentiated by all-trans-retinoic acid and J774 cells) infected with the Shigella flexneri strain YSH6000. The infected cells rapidly lose membrane integrity, a typical feature of necrosis, as indicated by the release of the cytoplasmic lactate dehydrogenase and the exposure of phosphatidylserine (PS) associated with the rapid uptake of propidium iodide (PI). The infected cells exhibit DNA fragmentation without nuclear condensation, and substantial involvement of either caspase-3/-7 or caspase-1 was not detected, which is also contrary to what is normally observed in apoptosis. Cytochalasin D potently inhibited Shigella-induced cell death, indicating that only internalized Shigella can cause necrosis. Osmoprotectants such as polyethylene glycols could suppress cell death, suggesting that insertion of a pore by Shigella into the host cell membrane induces the necrosis. The pore was estimated to be 2.87 ± 0.4 nm in diameter.

Shigella was also found to be able to induce apoptosis but only in one of the lines tested and under specific conditions, namely U937 cells differentiated with interferon-γ (U937IFN). Caspase-3/-7 but not caspase-1 activation was observed in these infected cells and the exposure of PS occurred without the uptake of PI. An avirulent Shigella strain, wild-type Shigella killed with gentamicin, and even Escherichia coli strain JM109, could also induce apoptosis in U937IFN cells, and cytochalasin D could not prevent apoptosis. It appears therefore that Shigella-induced apoptosis of U937IFN cells is unrelated to Shigella pathogenicity and does not require bacterial internalization. Thus, Shigella can induce rapid necrosis of macrophage-like cells in a virulence-related manner by forming pores in the host cell membrane while some cells can be killed through apoptosis in a virulence-independent fashion.

INTRODUCTION

Apoptosis is an active process of cellular suicide that is triggered by a variety of physiological and stress stimuli. Apart from playing important roles in normal development and tissue homeostasis, apoptosis is also involved in the interaction between host cells and bacterial pathogens, as a number of bacterial pathogens appear to be capable of manipulating host cell apoptotic pathways (Gao & Abu Kwaik, 2000). Whether these manipulations are to the advantage of the host or the bacteria varies among pathogens. Obligate intracellular bacteria such as Chlamydia trachomatis and Rickettsia rickettsii inhibit host cell apoptosis and this may allow these organisms to grow...
and persist intracellularly (Fan et al., 1998; Clifton et al., 1998). In contrast, infection with many facultative intracellular bacteria, such as species of Shigella (Zychlinsky et al., 1992), Salmonella (Monack et al., 1996), Yersinia (Ruckdeschel et al., 1997) and Mycobacterium (Fratazzi et al., 1997), appears to cause the host cell apoptosis. The pathways involved in this vary between bacteria. Yersinia induces apoptosis by translocating soluble proteins, such as YopJ or YopP, via the type III secretion system into the host cell cytoplasm. These proteins then bind to the mitogen-activated protein kinase kinase and thus inhibit its activity (Orth et al., 1999), which serves to block the activation of nuclear factor kB, a central regulatory molecule involved in cell survival (Schesser et al., 1998).

With regard to intracellular Shigella and Salmonella, these bacteria have been suggested to be able to induce host cell apoptosis by secreting effector proteins into the host cytoplasm, via the type III secretion system, factors that directly bind and activate caspase-1 (Hilbi et al., 1998; Hersh et al., 1999). Shigella kills its host macrophage by releasing IpaB, a secreted bacterial protein that is associated with virulence and the ability to invade host cells (Chen et al., 1996). IpaB directly binds to and activates caspase-1 and is thus thought to be the key molecule in the induction of caspase-1-dependent apoptosis by Shigella infection (Hilbi et al., 1998; Chen et al., 1996). Similarly, Salmonella is believed to induce macrophage caspase-1-dependent apoptosis by secreting the SipB protein into the cytoplasm of its host macrophage. The SipB protein is thought to be the Salmonella homologue of IpaB and it also binds directly to and activates pro caspase-1 (Hersh et al., 1999).

Despite the involvement of caspase-1 in the apparent induction of apoptosis by Shigella and Salmonella, this molecule is usually not involved in most apoptotic processes. Kuida et al. (1995) and Li et al. (1995, 1997) demonstrated that caspase-1-deficient mice fail to produce interleukin (IL)-1β and thus could resist endotoxic shock, indicating that caspase-1 plays a major role in cytokine maturation, and the mouse cells did not exhibit significant defects in apoptosis. In contrast, caspase-3 and/or caspase-7 are known to play a central role in driving the classical apoptotic pathways triggered by a variety of stimuli. However, in Shigella-induced macrophage cell death, the activation of caspase-3/-7, or the cleavage of poly(ADP-ribose) polymerase (PARP), one of the specific substrates for these caspses during apoptosis, has not been detected (Hilbi et al., 1998; Chen et al., 1996). The lack of caspase-3/-7 involvement in Shigella-induced cell death thus casts doubt on whether the killing truly occurs through apoptosis. Supporting this is the finding by Fernandez-Prada et al. (1997, 2000) that human monocyte-derived macrophages infected in vitro with Shigella flexneri undergo a rapid cytolytic event that is similar to oncrosis (necrotic cell death) but not apoptosis. They observed that when human monocyte-derived macrophages were infected with virulent Shigella, this resulted in cell death that involved rupture of the plasma membrane, cell swelling, disintegration of the cellular ultrastructure, and generalized karyolysis. Other groups have also reported that the macrophage cell death induced by Salmonella infection does not resemble typical apoptosis (Brennan & Cookson, 2000; Watson et al., 2000), as Salmonella-infected macrophages appear to rapidly lose their membrane integrity in a manner similar to Shigella-infected macrophages (Fernandez-Prada et al., 1997, 2000; Nonaka et al., 1999). Thus, the mechanism by which Shigella and Salmonella kill their host macrophages is unclear and controversial (Boise & Collins, 2001).

In this study, we report that the wild-type S. flexneri strain YSH6000 can induce both types of cell death, necrosis and apoptosis, depending on the type of host cell. We used terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) staining and measurements of caspase activities to discriminate between apoptotic and necrotic cell death and show that Shigella can induce rapid necrotic cell death in specifically macrophage-like cells. We also found that Shigella can induce apoptosis of particular cells. The mechanism does not require phagocytosis of the bacteria and is not dependent on their virulence. Thus, Shigella can kill host cells through either necrosis or apoptosis depending on the host cell type.

**METHODS**

**Bacterial strains and cell cultures.** S. flexneri 2a strain YSH6000 is the wild-type and N1411 is an ipaB::Tn5-induced avirulent mutant of YSH6000 (Sasaki et al., 1986, 1988). S. flexneri-derived strains and the Escherichia coli strain JM109 were grown in brain-heart infusion (BHI; Difco) broth or L broth (Difco) at 37 °C. Human monoblastic U937, myeloid HL-60, monocytic THP-1, and murine macrophage-like J774 cells were cultured in RPMI1640 medium (Nissui Pharmaceutical) supplemented with 10% (v/v) fetal calf serum (JRH Biosciences), 2 mM Gln, 100 units penicillin G mL⁻¹ and 200 μg streptomycin mL⁻¹ at 37 °C in a humidified atmosphere of 5% (v/v) CO₂.

**Infection of host cells with Shigella.** U937 cells were pre-treated with 100 units interferon-γ mL⁻¹ (IFN; a gift from Shionogi Pharmaceuticals) or 3 μM all-trans-retinoic acid (RA; Sigma) for 48 h to induce them to differentiate into macrophage-like cells before infection as described previously (Nonaka et al., 1999; Kikuchi et al., 1996). The host cells (~10⁶ cells per well in 12- or 24-well plates) were cultured with antibiotic-free medium for 1 h before infection. To obtain highly invasive bacteria, cultures incubated overnight at 30 °C were diluted 1:50 with BHI broth and incubated at 37 °C for 2 h before use. The cells were then infected with bacteria at the indicated m.o.i., centrifuged at 700 g for 10 min, and incubated for 1 h at 37 °C in a CO₂ incubator. Subsequently, 100 μg gentamicin mL⁻¹ (Gm; Sigma) was added to the medium and the cell/bacteria mixture was further incubated at 37 °C in a CO₂ incubator for the indicated time to kill the extracellular bacteria.

**Measurement of Shigella-induced cytotoxicity.** Cytotoxicity induced by Shigella infection was analysed with the CytoTox 96 Cytotoxicity Assay Kit (Promega). The percentage cytotoxicity was calculated by quantifying the amount of cytoplasmic lactate...
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Measurement of caspase activity in Shigella-infected cells. J774 or undifferentiated and differentiated U937 cells were either infected with Shigella or incubated with 0-5 μM of the apoptosis-inducing agent staurosporine (stsp; Sigma) as a positive control for apoptosis. After the indicated period, ~10^6 cells were harvested and washed with 0-15 M NaCl. Cytosolic fractions were prepared as follows. Cells were resuspended in 200 μl lysis buffer (20 mM HEPES/KOH, pH 7-5, 1 mM EDTA), disrupted by sonication, and insoluble material was removed by centrifugation at 15,000 g for 20 min at 4 °C. The supernatant was tested for the measurement of caspase-3/-7 or caspase-1 activity by using the fluorescent synthetic peptide substrates Ac-DEVD-MCA and Ac-WEHD-MCA, respectively (Peptide Institute) according to the established method (Thornberry et al., 1997). In brief, the samples (5, 10, 20 μl) were mixed with 0-5 ml of assay buffer (20 mM HEPES/KOH, pH 7-5, 1 mM EDTA, 100 mM NaCl and 10 mM 2-mercaptoethanol) and water in a total volume of 1 ml. After pre-incubation at 37 °C for 15 min, 10 μl of 10 mM peptide substrate was added to the reaction mixtures and incubated at 37 °C for 15 min. One millilitre of 2% (v/v) acetic acid was added to stop the enzymatic reaction, and 7-amino-4-methylcoumarin (AMC) release was measured fluorometrically using an excitation wavelength of 365 nm and an emission wavelength of 460 nm. One unit was defined as the amount of enzyme capable of generating 1 nmol AMC in 1 h.

Detection of the cleavage of PARP by immunoblotting. Infected or stsp-treated cells (~10^6 cells) were harvested and washed with 0-15 M NaCl. Trichloroacetic acid was added to the cell suspension to a final concentration of 10% (v/v), and the sample was placed on ice for 20 min. Precipitates were recovered by centrifugation at 15,000 g for 5 min at 4 °C, dissolved in 100 μl SDS-sample buffer containing 2-3% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol and 10 μg bromophenol blue ml^-1 in 125 mM Tris/HCl, pH 6-8, and heated at 100 °C for 5 min. Samples were run on a 7-5% (w/v) polyacrylamide gel containing SDS and electroblotted onto a PVDF membrane (Immobilon, Millipore). The membrane was then soaked for 2 h at room temperature in Tris-buffered saline (TBS: 0-15 M NaCl in 20 mM Tris/HCl, pH 7-5) containing 20 mg bovine serum albumin (BSA) ml^-1. The membrane was subsequently incubated overnight at 4 °C with a polyclonal antibody specific for the amino-terminal region of the 85 kDa fragment of human PARP that is generated by caspase-3/-7 (Nonaka et al., 1999; Kato et al., 2000). The antibody was diluted 1:500 with TBS containing 20 mg BSA ml^-1. After washing three times in TBS containing 0-05% (v/v) Tween 20 (Wako Pure Chemical Industries) for 15 min, the membrane was incubated at room temperature for 1 h with 1:7000 diluted anti-rabbit IgG conjugated with alkaline phosphatase (Promega) and then again washed three times in TBS containing 0-05% Tween 20 for 15 min. Immune complexes were detected by the enzymatic reaction of alkaline phosphatase with 5-bromo-4-chloro-3-indolyl phosphate (Nacalai tesque) and nitro blue tetrazolium (Nacalai tesque).

Inhibition of caspase activity and phagocytosis. Host cells were pre-treated with 100 μM Ac-DEVD-CHO, which inhibits caspase-3/-7, Ac-YVKD-CHO, which inhibits caspase-1, or Z-VAD-fmk, a general caspase inhibitor (Peptide Institute, Osaka, Japan), or with 5 μg ml^-1 of the phagocytosis inhibitor cyclohexalin D (Sigma) 1 h prior to infection with Shigella. LDH assays were performed 2 and 5 h after infection at an m.o.i. of 50.

TUNEL staining and immunofluorescence microscopy. J774 cells were grown to ~10^6 cells on a coverslip (18 x 18 mm). In the case of U937IFN and U937RA, ~10^6 cells were incubated on a poly-l-lysine (Sigma) pre-treated coverslip before infection. Cells on the coverslips were infected with Shigella at an m.o.i. of 50, incubated for 30 min, and then 100 μg Gm ml^-1 was added to each sample. After incubation for the indicated times, the infected cells on the coverslips were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. The coverslips were then incubated in 50 mM NH_4Cl in PBS for 5 min and cell permeabilization was performed with 0-2% (v/v) Triton X-100 in PBS for 5 min. After blocking for 30 min in 40 mg BSA ml^-1 in TBS, the bacteria on the coverslips were stained by a rabbit anti-S. flexneri 2a lipopolysaccharide (LPS) antibody (Watarai et al., 1997) followed by the Cy5-labelled goat anti-rabbit IgG (Sigma) as the secondary antibody. After washing, the apoptotic cells were labelled by using the In Situ Cell Death Detection Kit (Roche Diagnostics), according to manufacturer’s protocol. The stained cells were observed by confocal laser scanning microscopy (Bio-Rad).

Flow cytometric analysis. Undifferentiated and differentiated U937 and J774 cells were infected with YSH6000 at an m.o.i. of 50 or treated with 0-5 μM stsp, and then harvested and washed with PBS. Adherent J774 cells were removed from 6-well plates with PBS-EDTA and pooled with the non-adherent cells in the culture medium. Cells were labelled with Annexin-V-FLUOS (Roche Diagnostics) according to the manufacturer’s protocol. In brief, ~10^6 cells were washed with incubation buffer (10 mM HEPES/NaOH, pH 7-4, 140 mM NaCl, 5 mM CaCl_2), resuspended in 100 μl labelling solution [10 μl Annexin-V-FLUOS and 10 μl 50 μg ml^-1 propidium iodide (PI) in 1 ml incubation buffer] and incubated at room temperature in the dark for 15 min. Cells were immediately analysed on a FACScan (Becton Dickinson).

Osmoprotection assay. U937RA and J774 cells were pre-incubated in RPMI1640 medium supplemented with 20 mM of various osmoprotectants, namely sucrose (Nacalai Tesque), PEG 600 (Tokyo Kasei), PEG 1000 (Nacalai Tesque), and PEG 2000 (Nacalai Tesque) made in PBS. The cells were then infected at an m.o.i. of 50 for 2 h, and LDH release by the infected cells was measured as described above. For the estimation of the functional diameter of inserted pores into host membrane, we used the values for hydrodynamic diameters of the non-electrolytes (Scherrer et al., 1971), where the hydrodynamic diameters were calculated on the viscosity of non-electrolyte solutions.

Measurement of apoptosis-inducing potential of Shigella. J774 or undifferentiated and differentiated U937 cells were incubated with either the wild-type YSH6000 strain, the avirulent mutant N1411 lacking ipaBCDA, the Gm-treated wild-type YSH6000, or E. coli JM109 at the indicated m.o.i.. The Gm-treated YSH6000 were obtained by resuspension in RPMI1640 with 100 μg Gm ml^-1 and incubation at 37 °C for 30 min. As a positive control for apoptosis, the cells were incubated with 0-5 μM stsp. The bacterial suspension was added to host cells at the indicated m.o.i., and incubated at 37 °C for 5 h. The cells were harvested and prepared for measurements of caspase activity or immunoblot analyses as described above.

Protein concentration. Protein concentrations were determined by the method of Bradford (1976) using BSA as the standard.

RESULTS

Shigella induces macrophage-like cell lines to undergo rapid cell death

We previously reported (Nonaka et al., 1999) that infection with the wild-type S. flexneri strain YSH6000 caused rapid...
and striking oncosis (necrosis) only in U937 cells that had been differentiated by treatment with RA (U937RA) and induced apoptosis in U937 cells that had been differentiated by IFN (U937IFN). U937RA appear to be more macrophage-like than U937IFN cells in that they have a greater capacity to generate superoxide (Kikuchi et al., 1994) and they express higher levels of CD11b (Kikuchi et al., 1996). To determine whether the rapid necrotic cell death induced by infection with S. flexneri YSH6000 occurs only with macrophage-like cells, we attempted to infect another macrophage-like cell line (murine J774 cells), the myeloid HL-60 line and the monocytic THP-1 line, as well as undifferentiated and RA- or IFN-differentiated U937 cells. Host cells were infected with S. flexneri YSH6000 at various m.o.i. After incubation for 1, 3 or 5 h, the viability of the infected cells was quantified by measuring the activity of LDH released due to loss of cell membrane integrity. As shown in Fig. 1(a), Shigella-induced death of the macrophage-like U937RA and J774 cells at an m.o.i. of 50 was more rapid and striking than that seen with the other cells. We also observed a dose-dependent effect of m.o.i. (data not shown). The morphology of the infected U937RA and J774 cells revealed plasma membrane damage and swelling of the cytoplasm but not nuclear condensation (Nonaka et al., 1999, and data not shown). Wild-type Shigella could not effectively induce cell death in the less phagocytic cell lines HL-60, THP-1, and undifferentiated U937 cells (U937UD) (Fig. 1a). The U937IFN cells were lysed by Shigella to a lesser degree than U937RA or J774 cells.

Similar experiments were performed with the N1411 strain, which is an avirulent mutant of S. flexneri YSH6000 that lacks ipaBCDA. While this strain was phagocytosed by U937RA and J774 cells at levels similar to the wild-type bacteria (data not shown), it failed to cause the rapid cell death observed with the wild-type (Fig. 1b). These observations suggest that S. flexneri YSH6000 causes rapid cell death accompanied by membrane damage only in macrophage-like cell lines and that this cell death is dependent on the virulence of S. flexneri and its expression of the IpaB, C, D and/or A proteins.

**Cell death of macrophage-like cells infected with Shigella is not accompanied by caspase activation**

Hilbi et al. (1998) reported that Shigella infection induces macrophage apoptosis accompanied by caspase-1 but not caspase-3 activation. This is unusual because caspase-1 is not involved in most apoptotic processes, being more prominent in cytokine maturation. We thus characterized Shigella-induced cell death in macrophage-like cell lines further by directly examining the caspase activities in the infected cells. Host cells (U937UD, U937IFN, U937RA and J774) were infected with wild-type Shigella at an m.o.i. of 50. After subsequent incubation in the presence of Gm for several hours, the cytosolic fractions of the infected cells were prepared and assayed for caspase-1 and caspase-3/-7 activity using the synthetic peptide substrates Ac-WEHD-MCA and Ac-DEVDE-MCA, respectively. As shown in Fig. 2(a), no cleavage of either Ac-DEVDE-MCA or Ac-WEHD-MCA by the cytosolic fractions of infected U937RA and J774 cells was detected. In contrast, strong activity against Ac-DEVDE-MCA, but not Ac-WEHD-MCA, was seen with the infected U937IFN cells (Fig. 2a) as well as the stsp-induced apoptotic cells that were used as a positive control for apoptosis (Fig. 2b).

Poly(ADP-ribose) polymerase (PARP) is one of the target substrates cleaved by caspase-3/-7 during apoptosis. The cleavage of this 116 kDa protein occurs at Asp-214 (Lazebnik et al., 1994) and generates the regulatory 30 kDa and the catalytic 85 kDa fragments. To confirm the lack of caspase-3/-7 activity in S. flexneri YSH6000-infected U937RA and

**Fig. 1.** Infection with S. flexneri strain YSH6000 predominantly kills macrophage-like cell lines. Undifferentiated U937 (U937UD) ( ), U937 differentiated with IFN (U937IFN) ( ) or RA (U937RA) ( ), J774 ( ), HL-60 ( ) and THP-1 ( ) cells were infected at an m.o.i. of 50 with either the wild-type S. flexneri strain YSH6000 (a) or the avirulent N1411 mutant of YSH6000 that lacks the ipaBCDA genes (b). Supernatants were taken at the indicated time points and LDH released in the supernatants was assayed to measure cytotoxicity. The percentage cytotoxicity was calculated as described in Methods. Each data point represents the mean±SD from triplicate measurements.
J774 cells, we analysed the proteolysis of PARP by immuno-blotting with an anti-PARP antibody that recognizes the 85 kDa fragment. Undifferentiated and differentiated U937 and J774 cells were infected with the YSH6000 strain at an m.o.i. of 50 and samples for immunoblot analyses were prepared at various time points. PARP was not cleaved in wild-type Shigella-infected U937RA and J774 cells (Fig. 3a). In contrast, time-dependent PARP cleavage was observed in U937IFN cells infected with wild-type Shigella (Fig. 3a) and stsp-treated cells as a positive control for apoptosis (Fig. 3c).

To further assess the role of caspase(s) in Shigella-induced cell death of macrophage-like cells, we examined the effects of synthetic caspase inhibitors on the mortality of U937RA cells infected with wild-type Shigella. The inhibitors used were the caspase-3/-7 inhibitor Ac-DEVD-CHO, the caspase-1 inhibitor Ac-YVKD-CHO, and the general caspase inhibitor Z-VAD-fmk. Ac-DEVD-CHO and Z-VAD-fmk are potent inhibitors of apoptosis (Kato et al., 2000), and these inhibitors had no effects on viabilities of cells used here (data not shown). U937RA cells were pre-treated with the caspase inhibitors followed by infection with YSH6000 at an m.o.i. of 50. After incubation for 2 and 5 h, the viabilities of the infected cells were examined by measuring their release of LDH. Z-VAD-fmk suppressed Shigella-mediated U937RA cell death by 45 %, 2 h post-infection, while its inhibitory effect was almost undetectable at 5 h after infection (Fig. 4). The caspase-1-specific inhibitor also decreased Shigella-induced U937RA and J774 cell death 2 h post-infection by 24 %, but after 5 h of incubation, its inhibitory effect completely disappeared (Fig. 4). In contrast, the caspase-3/-7 inhibitor did not suppress Shigella-induced cell death at either time point. The suppression of Shigella-induced cell death by Z-VAD-fmk at 2 h post-infection indicates that caspase(s) other than caspase-3/-7 may be involved in the early phase of Shigella-induced cell death. However, the observation that even the general caspase inhibitor could only partially delay

Fig. 2. Neither caspase-3/-7 nor caspase-1 is activated during Shigella-induced macrophage-like cell death. U937UD (○), U937IFN (△), U937RA (■) and J774 (●) cells were either infected with wild-type Shigella at an m.o.i. of 50 (a) or treated with the apoptosis-inducing agent stsp (b). At the time points indicated, cytosolic fractions of the cells were prepared and caspase activities were determined using the caspase-3/-7 substrate Ac-DEVD-MCA or the caspase-1 substrate Ac-WEHD-MCA. AMC released due to caspase activity was measured fluorometrically. One unit is defined as the amount of enzyme capable of generating 1 nmol AMC in 1 h. Data represent means ± SD from three separate experiments.

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cell death indicates that Shigella-induced cell death is only partly caspase-dependent.

**Shigella infection of macrophage-like cells stimulates DNA fragmentation without nuclear condensation**

To further assess whether the rapid cell death caused by wild-type Shigella infection is apoptosis or necrosis, we stained infected cells by TUNEL labelling. U937RA and J774 cells infected with wild-type bacteria were fixed and incubated with the TUNEL reaction mixtures, and observed by confocal laser microscopy. As shown in Fig. 5(a, b), the condensed nuclear morphology typical of apoptosis was detected in both macrophage-like cell lines when they had been treated with stsp as a positive control for apoptosis. With regard to the Shigella-infected cells, Shigella invasion into both host cell lines could be observed even 1 h after infection but DNA fragmentation was not detected at this point. After 3 h incubation, however, DNA fragmentation was observed, but unlike the condensed pattern seen in the stsp-treated cells, the nicked chromatin in the infected cells stained diffusely. This is in agreement with a previous report on macrophage cell death induced by Salmonella infection (Brennan & Cookson, 2000). Moreover, the frequency of TUNEL-positive infected J774 cells was not reduced at all in the presence of either the caspase-1 or caspase-3/-7 inhibitors (Ac-YVKD-CHO and Ac-DEVD-CHO, respectively) (data not shown). Thus, Shigella infection of macrophage-like cells induces DNA fragmentation that is distinguishable from that observed in typical apoptotic cells.

**Shigella infection of macrophage-like cell lines induces rapid membrane damage**

In order to characterize the cell death induced by Shigella, we used a flow cytometric assay that discriminates between apoptosis and necrosis. Here a fluorescence-labelled phospholipid-binding protein, annexin V, is used to specifically detect phosphatidylserine (PS), a membrane lipid that normally localizes to the inner leaflet of the plasma membrane. Early apoptotic cells display PS on their outer leaflet while maintaining membrane integrity. Necrotic cells also expose PS but this is due to membrane damage that can be detected by their uptake of membrane-impermeable dyes such as propidium iodide (PI). Apoptotic and necrotic cells can thus be separated by flow cytometry,

**Fig. 3.** PARP is not cleaved in Shigella-infected macrophage-like cells. U937UD, U937IFN, U937RA and J774 cells were infected at an m.o.i. of 50 with either the wild-type YSH6000 Shigella strain (a) or the avirulent mutant N1411 that lacks the ipaBCDA genes (b), or they were incubated with the apoptosis-inducing agent stsp (c) for the indicated times. Approximately 5 x 10^4 cells per lane were separated by SDS-PAGE and the gel was immunoblotted with a polyclonal antibody specific for the amino-terminal region of the 85 kDa fragment of human PARP that is generated by caspase-3/-7 cleavage of PARP. Results are representative of at least three experiments.
with apoptotic cells falling in the annexin V-FITC-positive and PI-negative region and necrotic cells appearing in the annexin V-FITC- and PI-double-positive region (Fig. 6). Stsp-treated U937 and J774 cells were used as a positive control for apoptosis and almost 30–40% of these cells were detected in the annexin V-FITC-positive and PI-negative region (Fig. 6). Of the cell lines infected with wild-type YSH6000, U937IFN cells prominently exposed PS without PI uptake in a time-dependent manner as shown in Table 1 (see further discussion below). The infected U937UD cells reacted similarly but to a lesser degree. In contrast, less than 10% of the Shigella-infected U937RA and J774 cells were found in the annexin V-FITC-positive and PI-negative region. Instead, increasing numbers of cells were detected in the annexin V-FITC- and PI-double-positive necrotic region, indicating that the infected U937RA and J774 cells are undergoing necrosis with rapid membrane damage (Table 1).

**Macrophage-like cell death caused by internalized Shigella is due to osmotic lysis with the insertion of pores into the membrane**

We first examined whether the phagocytosis of YSH6000 was an important step in the cytolytic pathway in infected macrophage-like host cells. Experiments were thus performed in the presence of cytochalasin D, which inhibits actin polymerization and consequently blocks phagocytosis. We confirmed that cytochalasin D has no cytoxicity on cells used here (data not shown). U937RA and J774 cells were pre-incubated with cytochalasin D and then infected with *Shigella*. In the presence of cytochalasin D, the phagocytosis of the wild-type bacteria was dramatically suppressed (data not shown) and the release of LDH remained at the level of uninfected control cells (Fig. 4). Necrotic cell death could be inhibited by cytochalasin D, suggesting that bacterial internalization could be critical in the induction of the necrotic response.

*Shigella* is known to lyse red blood cells (RBCs) when they are brought into close contact with the cells by centrifugation. This is known as contact haemolysis and it is apparently mediated by the *Shigella*-mediated insertion of a 2–5 nm pore into the red blood cell membrane (Blocker et al., 1999). A similar mechanism is also observed with *Yersinia* species. Here, the formation of the 1–2–3·2 nm pore in RBCs requires the *yopB* gene product (Hakansson et al., 1996). It is thus possible that the *Shigella*-mediated rapid cell death of infected host cells may also be due to osmotic lysis caused by the formation of a pore in the host cell membrane. Polyethylene glycols (PEGs) with various molecular masses have been used to measure the size of pores induced by several pathogens (Moran et al., 1992; Kirby et al., 1998; Dacheux et al., 2001). We tested the ability of various osmoprotectants to protect infected U937RA cells from death by pre-incubating host cells with osmoprotectants and then monitoring LDH release 2 h after infection. Although sucrose (0·9 nm diameter) did not prevent the lysis of infected cells, PEGs with a molecular mass of 600 (1·6 nm diameter) and 1000 (2 nm diameter) reproducibly suppressed LDH release, and PEG 2000 (2·8 nm diameter) was the most effective osmoprotectant, as shown in Fig. 7. From a plot of the relative cell death in the presence of osmoprotectants versus their diameter (not shown), we estimated that the functional diameter of the pore is 2·87 nm (± 0·4 nm, standard deviation, n = 4). In analyses of *Shigella*-infected J774 cells, the similar size of pores was also determined (data not shown).

**Shigella infection of U937 cells differentiated with IFN induces apoptosis that is not dependent on Shigella pathogenicity**

Our previous report (Nonaka et al., 1999) showed that *S. flexneri YSH6000* induces apoptosis in U937 cells that
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(a) Transmission  TUNEL  Anti-LPS
+ WT for 1 h
+ WT for 3 h
+ step for 5 h

(b) + WT for 1 h
+ WT for 3 h
+ step for 5 h
have been differentiated with IFN but not in U937 cells differentiated with RA. Shigella-infected U937IFN showed the morphological features of typical apoptosis, namely cytoplasmic shrinkage and nuclear fragmentation (Nonaka et al., 1999). To explore the caspases involved in infected U937IFN cell death, we prepared a cytosolic fraction of infected U937IFN cells and assayed its caspase activities using several peptide-MCA substrates. Although the cytotoxicity of U937IFN by wild-type Shigella is not as high as that of infected U937RA and J774 cells (Fig. 1a), caspase-3/-7 activity was observed in infected U937IFN lysates, much higher than observed for infected U937RA and J774 cells (Fig. 2a). The cleavage of PARP was also clearly detected only in infected U937IFN cells (Fig. 3a). In contrast, caspase-1 activity was not detected at all (Fig. 2a). U937IFN cells infected with Shigella also showed the most

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**Fig. 6.** *Shigella*-infected macrophage-like cells expose PS as a result of rapid membrane damage. Undifferentiated and differentiated U937 (a) and J774 (b) cells were infected with wild-type bacteria at an m.o.i. of 50 or treated with 0.5 μM stsp. Infected cells were then stained with annexin V-FITC and the vital dye PI and immediately analysed by FACSscan (Becton Dickinson). Apoptotic cells are annexin V-FITC-positive and PI-negative (lower right region in the FACS panels), while necrotic infected cells are annexin V-FITC- and PI-double positive (upper right region in the FACS panels). Results are representative of at least three experiments.
Table 1. Flow cytometric analyses of infected and stsp-treated cells treated with annexin V/PI

The percentages of annexin V- and PI-double-positive (necrosis) or annexin V-positive and PI-negative (apoptosis) cells in infected cells were calculated with the software CELLQuest (Becton Dickinson). A minimum of 20,000 cells were evaluated per sample. Each value represents the mean ± SD from triplicate measurements.

<table>
<thead>
<tr>
<th>Time post-infection (h)</th>
<th>Necrosis (%)</th>
<th>Apoptosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U937UD</td>
<td>U937IFN</td>
</tr>
<tr>
<td>0</td>
<td>2.1 ± 0.3</td>
<td>3.5 ± 0.8</td>
</tr>
<tr>
<td>1</td>
<td>3.1 ± 0.5</td>
<td>5.0 ± 1.1</td>
</tr>
<tr>
<td>3</td>
<td>6.4 ± 0.7</td>
<td>11.3 ± 1.3</td>
</tr>
<tr>
<td>5</td>
<td>8.7 ± 0.8</td>
<td>19.2 ± 0.8</td>
</tr>
<tr>
<td>Stsp for 3 h</td>
<td>1.8 ± 0.7</td>
<td>3.2 ± 0.9</td>
</tr>
</tbody>
</table>

noticeable exposure of PS in the absence of PI uptake compared to the other cell lines tested (Table 1). Thus, *Shigella*-induced U937IFN apoptosis is accompanied by activation of caspase-3/-7, but not caspase-1, and exposes PS while its membrane is still intact.

To determine whether the ability of *Shigella* to generate apoptosis is dependent on its virulence, we performed infection experiments using the avirulent *S. flexneri* mutant N1411, which lacks *ipaBCDA*. As shown in Fig. 1(b), mutant N1411 did not induce rapid necrotic cell death in any cell line used. However, the cleavage of PARP by caspase-3/-7 was seen in infected U937IFN and U937UD cells irrespective of pathogenicity (Fig. 3b). Furthermore, U937IFN cells were incubated with N1411 at an m.o.i. of 50 for 5 h and caspase-3/-7 and caspase-1 activity in the lysate was measured. Caspase-3/-7 but not caspase-1 activity was detected as summarized in Table 2. Thus, avirulent strains can also induce apoptosis in U937IFN cells. We then examined whether *S. flexneri* killed by pre-treatment with antibiotics or the *E. coli* strain JM109 could also induce apoptosis in U937IFN cells. U937IFN cells were mixed with the bacterial suspensions at the indicated m.o.i., incubated for 5 h, and cytosolic fractions were prepared. Caspase-3/-7 activity was detected in the U937IFN line when it had been exposed to killed wild-type *Shigella* and even *E. coli* JM109, as summarized in Table 2. Caspase-1 activity was not detected at all (data not shown).

**DISCUSSION**

While it has been reported that *S. flexneri* induces macrophage apoptosis (Zychlinsky et al., 1992; Chen et al., 1996;
Table 2. Activation of caspase-3/-7 by interaction with several bacteria

U937UD, U937IFN, U937RA and J774 cells were infected with wild-type YSH6000, avirulent N1411, YSH6000 killed by pre-treatment with 100 μg Gm ml⁻¹, or E. coli strain JM109 at an m.o.i. of 50, or treated with 0.5 μM stp. After 5 h incubation, caspase-3/-7 activities were determined by using the peptide substrate Ac-DEVD-MCA as described in Methods. Each value represents the mean ± SD from triplicate measurements.

<table>
<thead>
<tr>
<th>Caspase-3/-7 activity [nmol AMC h⁻¹ (mg protein)⁻¹]</th>
<th>U937UD</th>
<th>U937IFN</th>
<th>U937RA</th>
<th>J774</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>0.8 ± 0.1</td>
<td>1.2 ± 0.6</td>
<td>0.6 ± 0.4</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Wild-type</td>
<td>2.3 ± 0.6</td>
<td>11.0 ± 0.4</td>
<td>1.5 ± 0.9</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>ΔipaBCDA</td>
<td>2.9 ± 0.7</td>
<td>8.5 ± 1.1</td>
<td>0.6 ± 0.4</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Gm-treated wild-type</td>
<td>2.3 ± 0.3</td>
<td>10.6 ± 0.9</td>
<td>1.8 ± 0.6</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>E. coli JM109</td>
<td>1.8 ± 0.6</td>
<td>6.5 ± 0.4</td>
<td>1.0 ± 0.6</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>0.5 μM stp</td>
<td>37.5 ± 2.1</td>
<td>34.3 ± 1.3</td>
<td>31.3 ± 2.4</td>
<td>32.9 ± 1.9</td>
</tr>
</tbody>
</table>

Hilbi et al., 1998), this pathogen-induced cell death has unusual features that distinguish it from typical apoptosis in that activation of caspase-1 but not caspase-3/-7 occurs. In general, during apoptosis, the executioner caspases, which include caspase-3 and -7, are activated and these are predominantly responsible for the limited proteolysis that characterizes the apoptotic dismantling of the cell. Caspase-1 is now thought not to be essential for the induction of apoptosis (Kuida et al., 1995; Li et al., 1995, 1997). Rather, it appears to be associated with the production of cytokines during inflammation.

Zychlinsky and co-workers previously showed that Shigella induces IpaB-mediated apoptosis in macrophages (Zychlinsky et al., 1992; Chen et al., 1996; Hilbi et al., 1998), so we tried to reproduce their results using similar methods. However, we detected only virulence-dependent cell death of infected macrophage-like cells (Fig. 1), but not the effective suppression of cell death by caspase-1 inhibitor (Fig. 4), the binding of caspase-1 and IpaB, or the activation of caspase-1 (i.e. processing of pro-caspase-1) in infected cells (data not shown). Recently, it has been reported that the release of mature IL-1β appears to be linked to the processing of precursor forms by caspase-1, although functional caspase-1 has not been detected in monocytes and macrophages (Ayala et al., 1994; Wewers et al., 1997; Mehta et al., 2001). The researchers also suggested that caspase-1 may have a role in release of mature IL-1β that is separate from its function as a protease. Furthermore, it has been also reported that another protease(s) could be involved in the processing of the pro-form of IL-1β (Schonbeck et al., 1998). These findings raised the possibility that caspase-1 may not be associated with induction of cell death by infection of Shigella. Therefore, we tried to re-analyse cell death of infected macrophages by different methods, including direct assay of caspase activity using synthetic peptide substrates, flow cytometric analysis using annexin V/PI staining, and confocal laser microscopic analysis with TUNEL staining. To examine if the activity of caspase-1 could be required for induction of cell death by Shigella infection, we directly assayed the activity of caspase-1 with a specific synthetic peptide substrate, Ac-WEHD-MCA. However, no activity was detected in lysates from U937RA and J774 cells infected with wild-type Shigella. Similarly, the activity of caspase-3 was not detected at all. We also observed that caspase-1 inhibitor, Ac-YVKD-CHO, had only partial effects on cell death by Shigella early during...
infection, correlating with the results of the caspase-1 activity assay. We conclude that caspase-1 may have only partial effects on Shigella-induced cell death early during infection under our experimental conditions, but caspase-3/-7 do not contribute to this cell death. Also, we can not rule out the possibility that caspase(s) other than caspase-1,
-3 and -7 are associated with induction of Shigella-induced cell death, because Z-VAD-fmk significantly suppressed cell death by Shigella at 2 h post-infection. Furthermore, to distinguish clearly necrosis from apoptosis in infected cells, we examined infected cells with annexin V/PI staining or the TUNEL method. It has been reported that the cleavage of chromatin DNA is caused not only in apoptotic cells but also in necrotic cells (Dong et al., 1997; de Torres et al., 1997). In apoptotic cells, the cleavage of chromatin DNA is accompanied by nuclear condensation. On the other hand, necrotic cells showed DNA fragmentation without nuclear condensation. Our results clearly revealed that macrophage-like cell death by infection of Shigella is necrosis rather than classical apoptosis.

A possible mechanism by which Shigella kills macrophage-like cells is that it inserts a pore into the plasma membrane that causes osmotic lysis. This is suggested by the work of Blocker et al. (1999), who showed that Shigella can lyse RBCs by inserting a 2-5 nm pore into the cell membrane. However, it was initially not clear if the macrophage-like cell death induced by wild-type Shigella occurs through a similar mechanism, because Blocker et al. (1999) examined the causes of Shigella-induced cytotoxicity only with RBCs, not with living macrophage cells. Furthermore, the Shigella-induced haemolysis requires centrifugation (Blocker et al., 1999), indicating that Shigella can lyse the plasma membrane by contact from the outside of the RBCs. In contrast, we have shown here that the Shigella-induced macrophage-like cell death can be completely inhibited by the pre-treatment of the host cells with cytochalasin D. Thus, phagocytosis is a prerequisite for Shigella-induced macrophage-like cell death, which indicates that only internalized bacteria can lyse the plasma membrane of macrophage-like cells. Nevertheless, we have shown that Shigella kills macrophage-like cells in a manner that resembles haemolysis, at least superficially, in that it forms a pore that could result in osmotic lysis. Although the pore size we measured (2.87 ± 0.4 nm) on macrophage-like cells was very similar to that (2.5 nm) on RBCs (Blocker et al., 1999), it is not yet clear if the same pore is involved in both phenomena. Supporting the possibility that the same pore is involved is that Blocker et al. (1999) have found that pore formation in haemolysis is dependent on both IpaB and IpaC. Similarly, macrophage-induced cell death is dependent on the ipaBCDA genes, because Shigella-induced killing does not occur upon infection with the avirulent mutant of the YSH6000 strain, N1411, which lacks ipaBCDA.

In intestinal mucosa, phagocytic cells such as macrophages have a variety of phenotypes. It is known that CD11b is one of the representative surface markers of macrophages, and the expression level is not constant between cell types (i.e. source organs or differentiation stages) (Rogler et al., 1998). Therefore, it has been suggested that phagocytic cells with different levels of CD11b could be a relevant target for Shigella. The U937 cell line can be induced to differentiate into macrophage-like cell lines by treatment with RA or IFN, generating the cell lines we have denoted as U937RA and U937IFN. These two lines differ in that U937RA appears to be more macrophage-like since the cells generate more superoxide and express higher levels of CD11b (Kikuchi et al., 1994, 1996). In this study, we used U937IFN and U937RA as reproducibly available cell lines representing the different cell types in the intestinal mucosa, not the partially differentiated myeloid cells. We have shown that Shigella induces necrosis in macrophage-like cells with higher expression of CD11b and apoptosis in cells with lower expression levels. Surprisingly, the inhibition of phagocytosis by cytochalasin D did not suppress Shigella-induced U937IFN apoptosis; rather, it appeared to enhance it. In addition, not only the wild-type YSH6000 strain but also the avirulent N1411 mutant, Gm-killed YSH6000, and the E. coli strain JM109 could induce apoptosis in U937IFN. These observations suggest that extracellular bacteria can transmit apoptotic signals in a manner that is independent of their pathogenicity. Susceptibility to such apoptotic signals was not observed for U937RA and J774 cells. One possible molecule involved in this process could be the Toll-like receptor (TLR)-2 (Kirschning et al., 1998). Aliprantis et al. (1999) have reported that bacterial lipoproteins, which are expressed by all bacteria, are potent activators of TLR-2 and that TLR-2 transmits a pro-apoptotic signal. Recently, Y. Niikura and co-workers (personal communication) showed that Fas is up-regulated in U937IFN cells, while the receptor is down-regulated in U937RA cells. U937IFN is more sensitive to apoptotic stimuli through Fas than U937RA. Similar regulation may be seen in the case of other receptor(s) e.g. TLR-2 and porimin (Ma et al., 2001), which is thought to be a receptor associated with induction of oncosis. These findings suggest that the expression level of such receptors involved in induction of cell death could be associated with Shigella’s ability to induce necrosis or apoptosis.

In summary, we have found that infection of macrophage-like cell lines with S. flexneri strain YSH6000 results in death that is due mainly to the rapid induction of necrosis. The mechanism may involve the formation of a pore in the plasma membrane of infected cells, which is dependent on the ipaBCDA virulence genes. Shigella can also induce death in other cells, without even being taken up, through a mechanism that does not involve the ipaBCDA genes and results in typical apoptosis. These observations point to the ability of Shigella to control the way it kills host cells, as it clearly commands mechanisms that lead to either apoptosis or necrosis. This ability most likely plays a crucial role in its initiation of infection, survival, and escape from host immune responses.

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REFERENCES


