

Piscirickettsia salmonis Induces Apoptosis in Macrophages and Monocyte-Like Cells From Rainbow Trout

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ABSTRACT

Piscirickettsia salmonis is the etiologic agent of the salmonid rickettsial septicemia (SRS) which causes significant losses in salmon production in Chile and other and in other regions in the southern hemisphere. As the killing of phagocytes is an important pathogenic mechanism for other bacteria to establish infections in vertebrates, we investigated whether *P. salmonis* kills trout macrophages by apoptosis. Apoptosis in infected macrophages was demonstrated by techniques based on morphological changes and host cell DNA fragmentation. Transmission electron microscopy showed classic apoptotic characteristics and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling showed fragmented DNA. Programmed cell death type I was further confirmed by increased binding of annexin V to externalized phosphatidylserine in infected macrophages. Moreover, significant increases of caspase 3 activation were detected in infected cells and treatment with caspase inhibitor caused a decrease in levels of apoptosis. This is the first evidence that *P. salmonis* induces cell death in trout macrophages. This could lead to bacterial survival and evasion of the host immune response and play an important role in the establishment of infection in the host. *J. Cell. Biochem.* 110: 468–476, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: *P. SALMONIS*; APOPTOSIS; RTS11 CELL LINE; MACROPHAGES; MONOCYTES

Piscirickettsia salmonis is the etiologic agent of the salmonid rickettsial septicemia (SRS), or piscirickettsiosis [Fryer et al., 1990, 1992; Fryer and Hedrick, 2003], a systemic infection which affects all cultured salmon fish species causing significant losses in salmon production all around the world [Mauel and Miller, 2002; Fryer and Hedrick, 2003].

P. salmonis was described as a non-motile, not encapsulated, pleomorphic but generally coccoid bacteria, with a diameter ranging from 0.2 to 1.5 μm [Bravo and Campos, 1989; Rojas et al., 2007]. It is a Gram-negative intracellular bacterium that replicates within membrane-bound cytoplasmic vacuoles in tissues from infected fish and in cell cultures derived from fish [Cvitanich et al., 1991; Fryer and Hedrick, 2003]. However, two recent reports have suggested that the bacterium may be able to grow in an artificial cell-free media [Mauel et al., 2008; Mikalsen et al., 2008]. Nevertheless, mainly due

to experimental limitations imposed by the intracellular nature of the bacterium, little is known about its infective strategy.

In a previous study we found that *P. salmonis* is able to infect, survive, and propagate inside salmonid macrophages and monocyte-like cells without inducing cytopathic effect and maintaining its expression capacity [Rojas et al., 2009]. These properties may be part of a strategy leading to the survival of this bacterium which may include resistance to killing by macrophages. Therefore, a combination of intracellular growth of the bacteria and regulation of macrophage cell death may be considered as the basis for *P. salmonis* pathogenesis.

Numerous experiments with a variety of microbes demonstrate that apoptosis of professional phagocytes is a common event in pathogenesis and plays a pivotal role in the initiation of the infection, survival of the pathogens, and evasion of the first line of defense of the immune system [Hilbi et al., 1997]. Bacteria

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promoting the destruction of phagocytic cells by apoptosis include *Shigella flexneri* [Zychlinsky et al., 1992], *Legionella pneumophila*, and *Legionella longbeachae* [Müller et al., 1996; Arakaki et al., 2002], *Yersinia enterocolitica* [Monack et al., 1997; Ruckdeschel et al., 1997; Zhang and Bliska, 2005], *Listeria monocytogenes* [Rogers et al., 1996], *Salmonella typhimurium* [Lindgren et al., 1996; Hersh et al., 1999; Valle and Guiney, 2005], and *Chlamydia psittaci* [Ojcius et al., 1998; Byrne and Ojcius, 2004; Miyairi and Byrne, 2006]. This act serves to eliminate key defense cells that are necessary to eradicate the pathogens and results in a decrease in the effectiveness of the immune response and in a further spread of the pathogens to other tissues, often epithelial cells. Nevertheless, other pathogens including *Mycobacterium tuberculosis*, *Chlamydia pneumoniae*, and *Listeria monocytogenes* [Cornelsen et al., 2003; Byrne and Ojcius, 2004; Miyairi and Byrne, 2006] have a totally opposite strategy and prevent the programmed cell death in host cells.

Macrophages are professional phagocytes serving as sentinels in the innate immune response against invading microorganisms. The innate ability of these phagocytes to kill bacteria is crucial for host defense because it is immediate, non-specific, and not dependent on previous pathogen exposure. Nevertheless, although phagocytes are highly adapted at destroying bacterial pathogens, modulation of phagocyte cell death has emerged as a mechanism of pathogenesis [Hilbi et al., 1997; Zychlinsky and Sansonetti, 1997a,b; Weinrauch and Zychlinsky, 1999; Zychlinsky and Sansonetti, 1997a,b; Gao and Abu Kwaik, 2000; Navarre and Zychlinsky, 2000; DeLeo, 2004].

Apoptosis is an evolutionarily conserved and genetically controlled multistep process of cell death in response to a wide variety of stimuli that can be signaled from external environment or from within the cell [Arnoult et al., 2002; Koonin and Aravind, 2002]. It occurs in isolated single cells by controlled autodigestion, which is induced by the activation of specific endogenous cysteine proteases, the caspases. Programmed cell death progresses through a series of morphological and biochemical changes including cytoskeleton disruption, cell shrinkage, membrane blebbing, and cell fragmentation into apoptotic bodies. Though apoptotic cells maintain their plasma membrane integrity alterations such as phosphatidylserine (PS) exposition at the cell surface signal neighboring phagocytic cells to engulf them. In addition, apoptosis also involves chromatin condensation and cleavage into oligonucleosomes size fragments [Vaux and Strasser, 1996; Nagata, 1997, 2000; Song and Steller, 1999; Hengartner, 2000; Fan et al., 2005; Yan and Shi, 2005; Elmore, 2007].

In this study we report that *P. salmonis* induces apoptosis in salmonid macrophages in vitro. The macrophages were the rainbow trout monocyte/macrophage cell line RTS11 [Ganassin and Bols, 1998], which in response to the viral mimic poly IC, more readily undergoes apoptosis than fibroblasts and epithelial cells [DeWitte-Orr et al., 2005]. We propose that *P. salmonis* induces cell death as part of a strategy to modulate host immune response and to establish infection in the host.

MATERIALS AND METHODS

PISCIRICKETTSIA SALMONIS

The prototype strain LF-89 (ATCC VR 1361) was propagated in CHSE-214 cells in MEM medium (Gibco BRL) supplemented with

15 mM HEPES, 10 mM sodium bicarbonate, and 10% FBS (Gibco BRL) [Rojas et al., 2007, 2009].

RTS11

RTS11 was cultured at 20°C in Leibovitz's L-15 medium (Sigma) supplemented with 15% FBS (Gibco BRL). Cells were replicated every 15 days by dividing the cells into two 25 cm² flasks (Falcon) along with their spent medium, and adding an equivalent volume of fresh growth medium [Rojas et al., 2009].

In this original condition, RTS11 cell line presents two different cell types: small, round, and non-adherent cells named monocyte-like cells, and large and adherent cells with typical morphology of macrophages [Ganassin and Bols, 1998; Rojas et al., 2009]. In order to increase macrophage population, RTS11 cells were cultivated with L-15 medium supplemented with 7.5% FBS.

INFECTING RTS11 CULTURES

RTS11 cells were seeded onto poly-L-lysine-coated coverslips in six-well plates with L-15 medium supplemented with 7.5% FBS and then cultivated for 7 days to 50–70% confluence.

Monolayers containing adherent macrophages-like cells were infected for 1 h with *P. salmonis* at a multiplicity of infection (MOI) of 50 [Rojas et al., 2009]. Then, adherent macrophages were rinsed with PBS and incubated with fresh medium until 2, 5, or 10 days post-infection. Conditioned medium containing round non-adherent cells detached by effect of infection were centrifuged at 300g for 20 min, cells were suspended in PBS, recovered onto microscope slides using a cytocentrifuge Hettich Mikro 22, and analyzed as macrophages cells.

IMMUNOFLUORESCENCE STAINING

Macrophages adhered onto coverslips and non-adherent monocyte-like cells recovered by cytospin were fixed and permeabilized at 2, 5, and 10 days post-infection with methanol-acetic acid (3:1, v/v) for 10 min at 20°C.

For *P. salmonis* detection cells were incubated in the dark for 1 h at 20°C with a 1:75 (v/v) dilution in BSA 1%, saponin 0.1% in PBS of the oligoclonal antibody anti-*P. salmonis* (SRS Immunotest Bios Chile) conjugated to FITC. Alternatively infected cells were incubated with 1:10 (v/v) of an anti-*P. salmonis* antibody for 1 h at 20°C and then incubated in the same conditions with 1:200 (v/v) of an anti-rabbit secondary antibody conjugated to Alexa Fluor 532 (Molecular Probes). Afterwards, cells were washed three times with PBS, mounted with Vectashield mounting medium (Vector Lab., Inc.) and viewed with a Nikon Eclipse 400 fluorescence microscope. Color photography was performed with a Nikon Coolpix 4500 digital camera.

TRANSMISSION ELECTRON MICROSCOPY

Macrophages RTS11 were grown in 10-mm culture dishes (Orange) and infected as described above. Cells were fixed for 4–8 h at 4°C with 2.5% glutaraldehyde–0.1 M phosphate buffer (pH 7.4) and post-fixed for 1 h with 1% osmium tetroxide. Samples were dehydrated by incubations in a graded ethanol series, embedding with 1.5% agarose and then, infiltrated with Epon 812 (Shell Chemical Co.). Serial sections were analyzed with a Zeiss-EM-109 transmission electron microscope (TEM).

TUNEL ASSAY

DNA double-strand ruptures occurring during apoptosis were detected in situ by TUNEL assay according to manufacturer's protocol (DeadEnd™ Fluorometric TUNEL System, Promega). All growing, infection, and fixation–permeabilization cell procedures were carried out at 2, 5, and 10 days post-infection as described previously. Macrophages incubated for 30 h with 2 µg/ml of actinomycin D were used as a positive control for apoptotic [DeWitte-Orr et al., 2005]. Macrophages and detached monocytes-like cells were incubated with a reaction mix containing dUTP-FITC. Nuclei were counterstained with DAPI (1 µg/ml) for 10 min. *P. salmonis* infection was evaluated by indirect immunofluorescence as described previously. Fluorescence was observed in a Nikon Eclipse E 400 microscopy and color photography was performed with a digital camera Nikon Coolpix 4500. Results were quantified counting about 500 cells in triplicate from two independent experiments. The means and standard deviations (SD) were calculated.

Caspase-3 activation. Activated caspase-3 was detected by immunocytochemistry with an anti-active caspase-3 polyclonal antibody (BD Pharmingen). All growing, infection, and fixation–permeabilization procedures were carried out at 2, 5, and 10 days post-infection as described previously. Infected, non-infected and actinomycin D induced macrophages were blocked for 12 h at 4°C with CASBlock (Zymed). Cells were incubated for 2 h at 37°C with 1:50 (v/v) dilution of the primary antibody in blocking solution. After washing with PBS, cells were subsequently labeled for 1 h at 37°C with 1:400 (v/v) dilution of an anti-rabbit IgG Alexa Fluor 532-conjugated antibody. Afterwards, cells were labeled with the anti-*P. salmonis*-FITC antibody and then incubated with DAPI. Assays were also performed in the presence of the pan-caspase inhibitor Z-VAD-FMK (benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone) (BD Pharmingen). Briefly, macrophages were pre-incubated for 1 h at a final concentration of 50 and 100 µM of the inhibitor and after replacing with fresh medium, cells were infected with *P. salmonis* as previously described. Fluorescence was observed and results were quantified counting about 500 cells in triplicate from three independent experiments.

Flow cytometric analysis. Exposure of PS to the outer leaflet of the plasma membrane was determined by flow cytometry with FITC-conjugated annexin V, a Ca²⁺-dependent protein with high affinity for PS. Viable cells exclude propidium iodide (PI), then dual fluorescent labeling allow specific detection and quantification of

apoptosis by fluorescence-activated cell sorter (FACS) analysis in a FACSort equipment (Becton Dickinson & Co). All growing, infection, and fixation cell procedures were carried out at 5 days post-infection as described previously. Infected, non-infected, and actinomycin D induced cells were trypsinized, harvested by centrifugation, washed once with ice-cold PBS, and resuspended in binding buffer (140 mM NaCl, 5 mM CaCl₂, 10 mM HEPES-Na, pH 7.4) to a concentration of 1 × 10⁶ cells/ml. Next, aliquots of 100,000 cells in 0.1 ml of binding buffer were transferred to a 5-ml FACS tubes and incubated with 5 µl of annexin V-FITC (BD Pharmingen 556420) and 50 µg/ml of PI (Molecular Probes) for 15 min at room temperature in the dark. Finally, additional 400 µl of binding buffer was added, and samples were analyzed within 30 min. All experiments were performed three times in duplicate. Analysis was performed using a WinMDi 2.8 software. The means and the SD were calculated and statistical analysis was performed using the non-parametric Mann–Whitney test for independent means. Results described as different imply a significant difference from control, *P* < 0.05.

RESULTS

MORPHOLOGICAL FEATURES OF APOPTOSIS IN INFECTED MACROPHAGES

Macrophages infected with *P. salmonis* displayed morphological changes characteristic of apoptosis including chromatin condensation at the nuclear boundary, fragmentation of the nucleus as well as cytoplasm vacuolization, and apoptotic body formation (Fig. 1B–E). In contrast, non-infected macrophages present a normal appearance (Fig. 1A).

DNA FRAGMENTATION IN INFECTED MACROPHAGES

A significant number of infected macrophages showed fragmented DNA at different time points after infection, whereas a low level of DNA fragmentation in non-infected RTS11 cells was observed (Fig. 2A). The pattern of DNA cleavage in infected macrophages was similar to that in cells treated with actinomycin D, which is known to induce apoptosis [Jimenez et al., 2008]. By 2, 5, and 10 days post-infection 25–32% of the nuclei were TUNEL-positive (Fig. 2B).

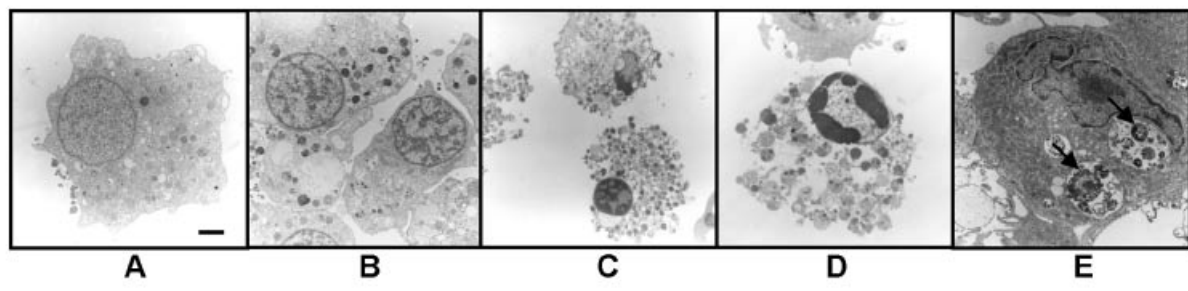


Fig. 1. Transmission electron micrographs of RTS11 macrophages with time after *P. salmonis* infection. A: Non-infected, (B–D) 2, 5, and 10 days post-infection, (E): a higher magnification of an infected cell. Arrows indicate the presence of *P. salmonis*. Scale bar in A–D: 4.5 µm; E: 3 µm.

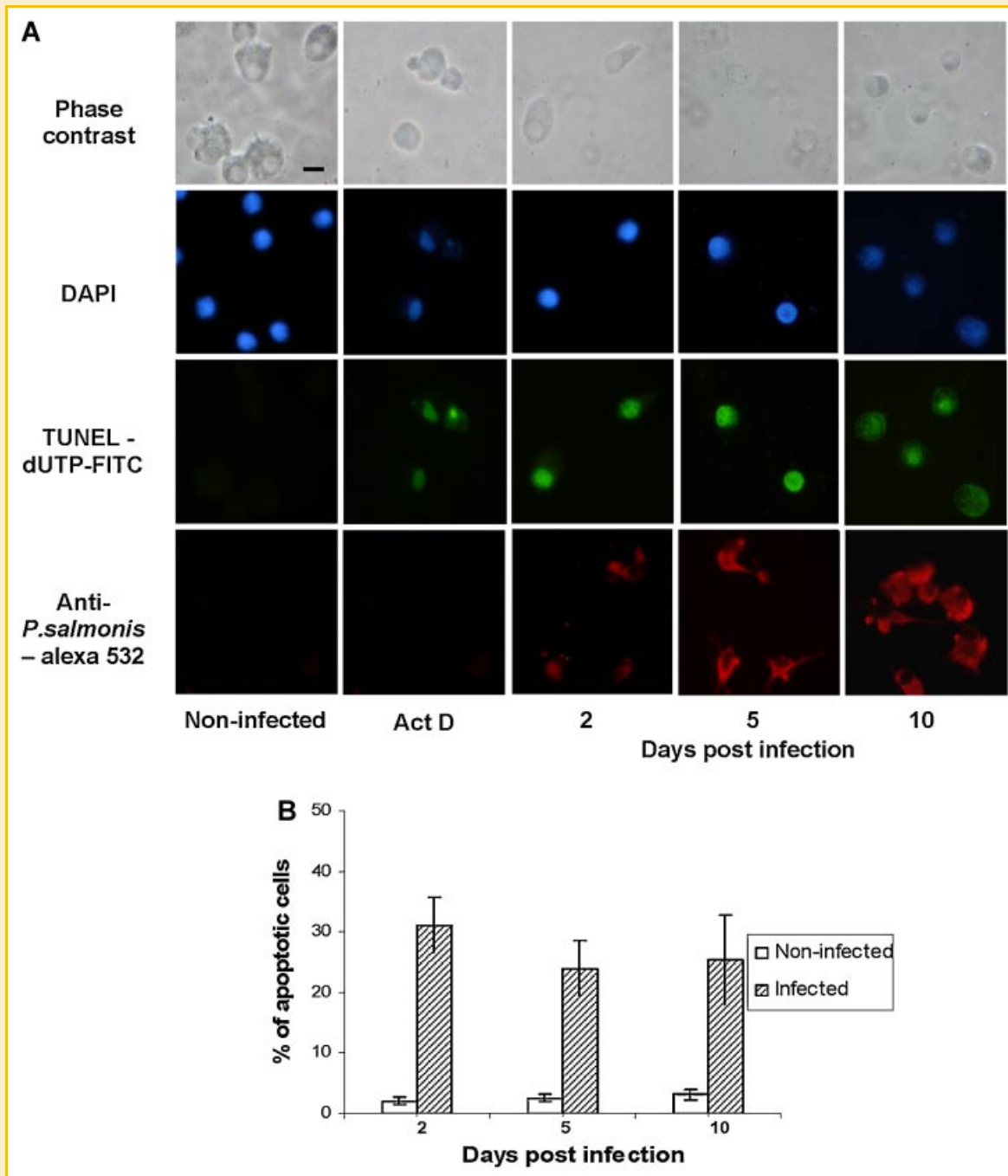


Fig. 2. Nuclear fragmentation in RTS 11 cultures after infection with *P. salmonis* or treatment with actinomycin D. Actinomycin D was used as a positive control for the induction of apoptosis. In panel A RTS 11 are visualized by phase-contrast microscopy (1st row), nuclear DAPI staining (2nd row), and the TUNEL reaction (3rd row). In the 4th row, anti-*P. salmonis* primary antibody was used to visualize bacteria. Scale bar: 20 μ m. In panel B the percentage of apoptotic cells was determined by examination of random fields and the means with standard deviation for 500 infected and non-infected cells are shown.

A fraction of macrophages detaching from macrophage cultures to round non-adherent monocyte-like cells by effect of infection [Rojas et al., 2009] were recovered by centrifugation and analyzed by TUNEL, at 5 days post-infection; 10.5% of monocyte-like cells were found in apoptosis in these infected cells against 1% in non-infected monocytes (not shown).

CASPASE-3 ACTIVATION IN INFECTED MACROPHAGES

Caspase-3 is a central player in apoptosis regulation. To determine whether caspase-3 is essential for apoptosis observed in *P. salmonis*-infected macrophages, the proteolytic activation of this enzyme was measured. As shown in Figure 3A, caspase-3 activity was clearly detected in infected cells but not in non-infected

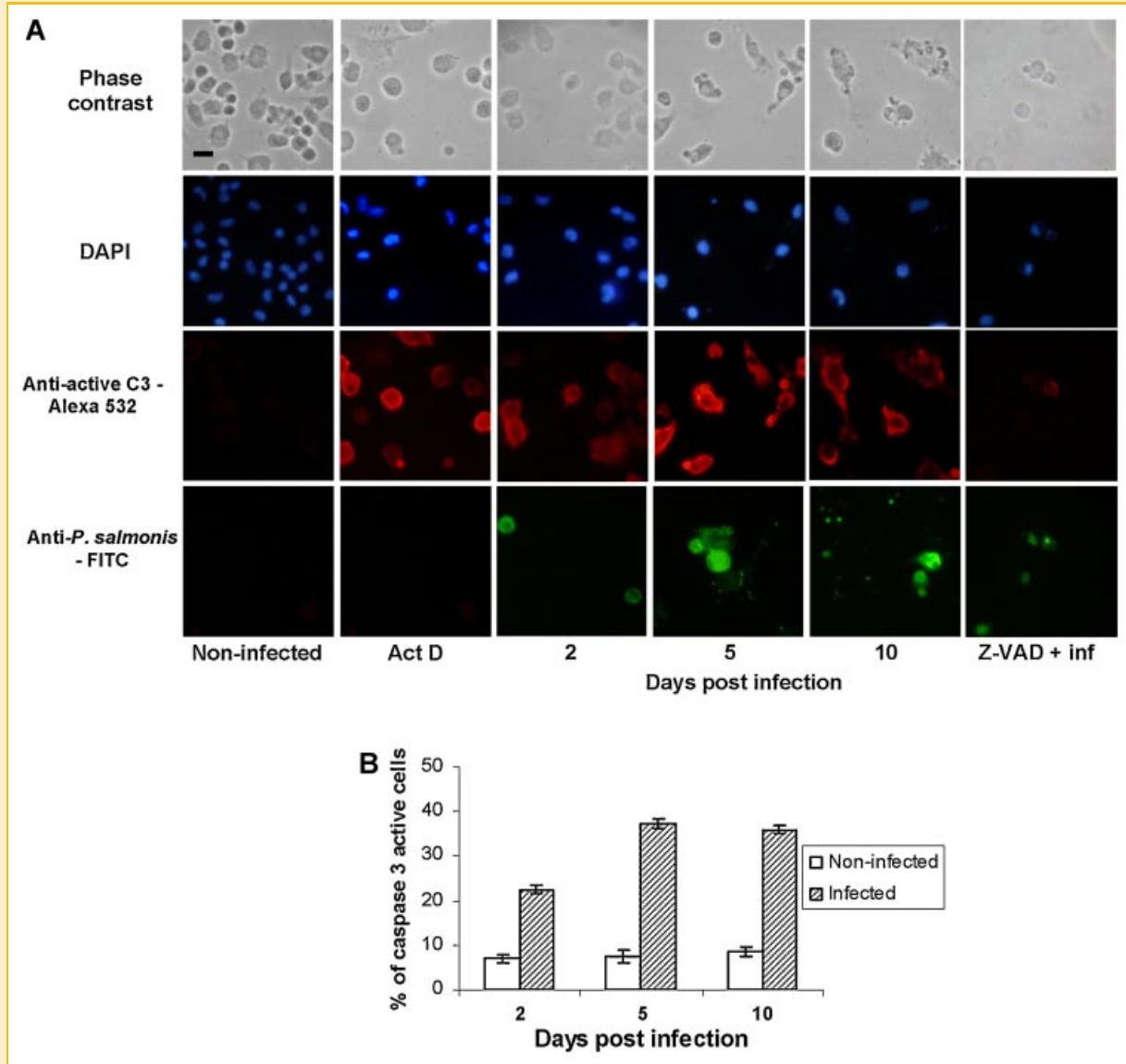


Fig. 3. Caspase-3 activation in RTS 11 cultures after infection with *P. salmonis* or treatment with actinomycin D. RTS11 cultures were infected with *P. salmonis* or treated with actinomycin D in the presence or absence of the caspase inhibitor Z-VAD. In panel A RTS11 are visualized by phase-contrast microscopy (1st row), nuclear DAPI staining (2nd row), and caspase activation (3rd row), while bacteria are visualized in row 4. Scale bar: 20 μ m. B: The percentage of apoptotic cells was determined by examination of random microscope field. The means with standard deviations for 500 cells are shown.

controls cells; moreover, a significant increase in the percentage of macrophages positive to active caspase 3 was observed as the time post-infection increased, from 22% at day 2 to over 35% at 5 and 10 days post-infection (Fig. 3B). Immunoreactivity against caspase-3 suggests that caspase-like proteins could be involved in RTS11 cell death pathways.

In order to confirm that caspase activity was related to apoptosis induced by *P. salmonis*, macrophages were pre-incubated with the pan-caspase inhibitor Z-VAD-FMK. As shown in Figure 3A, pre-treatment of macrophages with Z-VAD led to a reduction in the percentage of macrophages positive for caspase 3 and a decrease in apoptosis induced by *P. salmonis*.

PS EXPOSURE

Apoptosis of *P. salmonis* infected macrophages was further confirmed by exposition of PS at the cell surface. Exposed PS was detected using Annexin V-FITC and analyzed by flow cytometry; moreover, plasma membrane integrity was analyzed by PI. A FACS analysis representative of three separate experiments is depicted in Figure 4. At 5 days post-infection, 76.42% of control non-infected macrophages were viable (Annexin-V⁻ PI⁻), while 23.58 \pm 0.4% of them showed annexin V and PI staining, which correspond to non-viable late apoptotic cells. In contrast, only 55% of *P. salmonis*-infected macrophages were viable, while 44.76 \pm 0.6% were late apoptotic cells. A similar percentage of apoptotic cells (50.48 \pm 0.6%) was

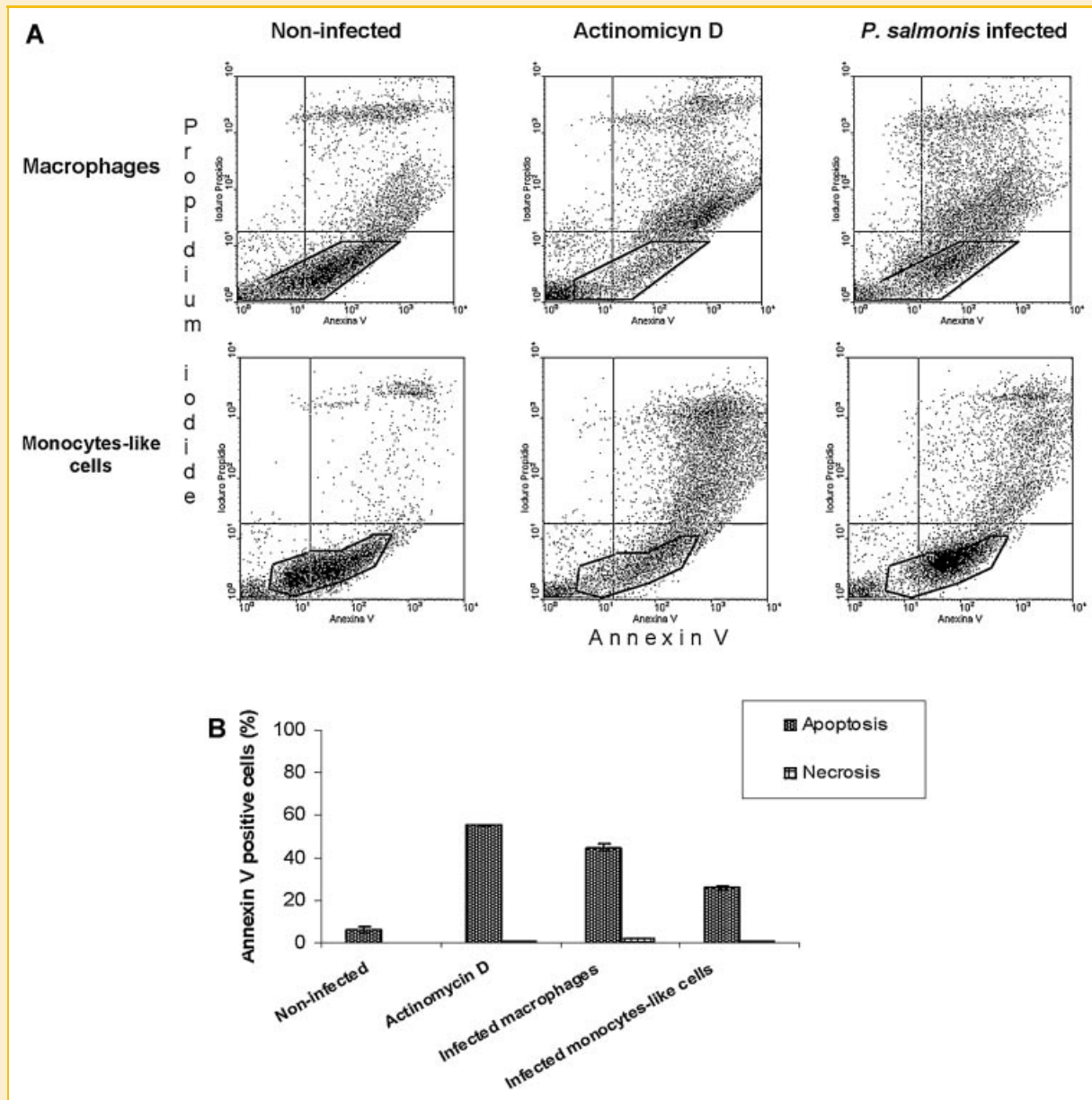


Fig. 4. FACS analysis of RTS 11 cultures after infection with *P. salmonis* or treatment with actinomycin D. Aliquots of 100,000 cells were double labeled with annexin and PI, and analyzed by flow cytometry. In panel A the FACS analysis representative of three separate experiments is shown for uninfected and infected macrophages, and monocytes-like cells detached from infected cultures. B: Quantification of apoptotic cells performed in WinMDi 2.8 software. The means and standard deviations are shown. Statistical analysis was carried out by the Mann-Whitney test ($P < 0.05$).

observed in macrophages treated for 30 h with ActD, a potent inducer of apoptosis. Moreover, $2.41 \pm 0.1\%$ of the cells were stained with PI only; these cells are considered as necrotic.

Detached monocyte-like cells from cultures infected with *P. salmonis* showed $25.73 \pm 1.09\%$ cells annexin positive as compared to only $6.18 \pm 1.05\%$ in detached cells from non-infected cultures. Monocyte-like cells from cultures exposed to actinomycin showed $55.15 \pm 0.58\%$ cells annexin positive while $0.41 \pm 0.06\%$ were IP positive only. The percentage of dead cells measured as PS exposition was significantly higher ($P > 0.05$) in cells infected with *P. salmonis* compared with non-infected cells.

DISCUSSION

Applying several complementary techniques we report that *P. salmonis* induces apoptosis in salmonid macrophages infected in vitro during early, intermediate, and late phases of infection. Under our experimental conditions, apoptosis is observed when live bacteria enter and replicate over a period of time within the host cell.

Macrophage death is evidenced by the characteristic features of apoptosis, such as specific morphological changes, fragmentation of nuclear DNA, caspase-3 activation, and increase in PS

externalization. Apoptosis induction by *P. salmonis* was observed in both, infected macrophages and monocyte-like cells. Surprisingly, in three independent experiments each one in duplicate, non-infected trout macrophages also showed a significant exposition of PS, while a parallel analysis of Vero cells displayed the expected PS distribution of viable mammalian cells. Interestingly, *P. salmonis* infection of the salmonid epithelial cell line CHSE-214 did not cause apoptotic morphological changes or fragmentation of nuclear DNA (data not shown); yet these cells and control CHSE-214 cells also showed an significant exposition of PS, which may be an indicative of a unique feature of healthy fish cells.

Interestingly, only 24–31% of infected macrophages show apoptosis as detected by TUNEL and this agrees with 22–37% of cells showing active caspase-3. These results suggest that the induction of apoptosis by *P. salmonis* is restricted to some monocytes/macrophages. An alternative explanation is that we have measured apoptotic cells at specific days post-infection and dead cells may further increase as a function of time. In agreement with this, monocyte-like cells detached from infected RTS11 cultures also showed only 10–12% of apoptotic cells, both by TUNEL and active caspase-3 (data not shown). Our hypothesis is that the induction of apoptosis by *P. salmonis* in a fraction of the macrophages allows the major part of the macrophage population to be productively infected by the bacteria. These two outcomes are not mutually exclusive and may be influenced by the particular physiological state of both the pathogen and the macrophage.

Several proteins produced by pathogenic bacteria are capable of specifically initiating apoptosis in macrophages [Navarre and Zychlinsky, 2000]. Moreover, there are a variety of reasons to explain the particular susceptibility of macrophages to pathogen-induced apoptosis. As for example, the expression of surface receptors that recognize highly conserved bacterial components, such as lipopolysaccharide (LPS) and bacterial lipoproteins, have been shown to activate pro-apoptotic signaling pathways [Fukui et al., 2008; Navarre and Zychlinsky, 2000].

In fish, apoptosis of macrophages due to bacterial infection has not been reported. Nevertheless, viruses as IPNV induces apoptosis in the zebrafish cell line (ZLE) [Hong et al., 2005; Santi et al., 2005] and in the salmon cell line CHSE-214 [Hong and Wu, 2002]. Moreover, characteristic apoptotic DNA fragmentation was reported in SHK-1 and CHSE 214 cells infected with ISAV [Joseph et al., 2004]. By contrast, another fish virus, chum salmon reovirus (CSV), caused apoptosis in CHSE-214 and RTG-2 but not in RTS11 [DeWitte-Orr and Bols, 2007].

The exact relevance of apoptotic cell death to the pathogenesis of SRS is still undefined. Thus, in addition to other strategies to evade host immune responses, such as enclosing inside vacuoles, induction of macrophage apoptosis may represent a mechanism by which *P. salmonis* escape the host immune response and establish productive infections.

We propose that the ability of *P. salmonis* to promote early apoptosis in a fraction of phagocytic cells may be important for the initiation of infection. Bacteria survive and evade the host immune response, allowing them to spread to other tissues,

which are targeted by the pathogen to establish a productive infection.

Like other pathogens, *P. salmonis* efficiently induce apoptosis in macrophages but not in epithelial cells [Navarre and Zychlinsky, 2000]. For example, the fungal toxin, gliotoxin, induced apoptosis in the monocyte/macrophage cell line RTS11 but not in adherent cells of the salmonid epithelium cell line CHSE-214 [DeWitte-Orr and Bols, 2005]. Therefore, we infected CHSE-214 under the same conditions as RTS11, but no clear evidence of apoptosis was seen as judged by MET, TUNEL, caspase-3 activation, and PS exposition. Probably *P. salmonis* has evolved pathways by which they can selectively kill part of the macrophages, diminishing cellular attack, while residing within a population of epithelial cells that are resistant to apoptosis.

Further studies are necessary to understand the mechanism by which *P. salmonis* triggers apoptosis. It is possible that the bacteria either activates the macrophage intrinsic death program or interferes with factors that inhibit the apoptosis program. As other pathogenic bacteria, *P. salmonis* may produce toxins that can induce macrophage apoptosis. Indeed we also detected cells without positive signal for anti-*P. salmonis* antibody (non-infected cells) that die through apoptosis. Nevertheless, this observation may be explained by a lower loading of bacteria not detectable by immunocytochemistry or that infected cells may elaborate soluble factors that induce apoptosis of neighboring non-infected cells.

The existence in *P. salmonis* of virulence factors analog to IpaB of *Shigella* [Chen et al., 1996] or SipB of *Salmonella* [Hersh et al., 1999], with structural and functional conserved mechanism to induce apoptosis, or Yop(s) proteins of *Yersinia* [Mills et al., 1997] remains to be elucidated. It has been demonstrated that a type III secretion system is used by those and other Gram-negative pathogens to deliver virulence factors to the intracellular compartment of eukaryotic cells, which are required for the induction of apoptosis in macrophages; nevertheless, this secretion system had not yet described in *P. salmonis*.

In summary, we report for the first time that *P. salmonis* induce apoptosis in salmon phagocytic cells but not in epithelial cells. Further analysis in order to understand the molecular mechanisms of apoptosis induced by this pathogen could be important in the development of new potential approaches against piscirickettsiosis.

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