Trojan Horse Effect: Phagocyte-Mediated *Streptococcus iniae* Infection of Fish

Amir Zlotkin,¹ Stefan Chilmonczyk,² Marina Eyngor,³ Avshalom Hurvitz,⁴
Claudio Ghittino,⁵ and Avi Eldar^{3*}

Department of Clinical Microbiology, The Hebrew University—Hadassah Medical School, Jerusalem 91120,¹ Department of Poultry and Fish Diseases, The Kimron Veterinary Institute, Bet Dagan 50250,³ and Dan Fish Farms, Kibbutz Dan, Upper Galilee 12245,⁴ Israel; Unité de Virologie et Immunologie Moléculaires, Institut National de la Recherche Agronomique, Jouy en Josas 78352, France²; and Department of Fish Pathology, IZS—State Veterinary Institute, 06126 Perugia, Italy⁵

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The salmonid macrophage-like cell line RTS-11 and purified trout pronephros phagocytes were used to analyze in vitro entry and survival of two *Streptococcus iniae* serotypes. Efficient invasion by *S. iniae* occurred in both cells, but only the type II strain persisted in pronephros phagocytes for at least 48 h. Ex vivo models of opsonin-dependent phagocytosis by pronephros phagocytes demonstrated increased phagocytosis efficacy. Analysis of phagocytes collected from diseased fish demonstrated that ~70% of the bacteria contained in the blood during the septic phase of the disease were located within phagocytes, suggesting an in vivo intracellular lifestyle. In addition to the augmented levels of bacteremia and enhanced survival within phagocytes, *S. iniae* type II induces considerable apoptosis of phagocytes. These variabilities in intramacrophage lifestyle might explain differences in the outcomes of infections caused by different serotypes. The generalized septic disease associated with serotype II strains is linked not only to the ability to enter and multiply within macrophages but also to the ability to cause considerable death of macrophages via apoptotic processes, leading to a highly virulent infection. We assume that the phenomenon of survival within phagocytes coupled to their apoptosis plays a crucial role in *S. iniae* infection. In addition, it may provide the pathogen an efficient mechanism of translocation into the central nervous system.

In the United States and in Israel, *Streptococcus iniae* is an endemic fish pathogen associated with bacterial meningitis of salmonids and other fish species (12, 29). However, accidental injuries following handling of fish can also lead to human infections (44). Restriction fragment length polymorphism ribotyping of fish isolates has shown that North American and Israeli isolates cluster in two distinct epidemiological clones, demonstrating the independence of the evolution of this pathogen in each of the countries. Pulsed-field gel electrophoresis of North American strains has indicated that isolates recovered either from infected fish or from diseased humans cluster in two virtually identical clones, while isolates from nondiseased fish are genetically different (44).

More recently, it has been shown that following a 5-year routine vaccination program, a novel serotype, capable of producing a generalized bacterial meningitis, has emerged (4). In this case, *S. iniae* probably gains access to the bloodstream and maintains a high level of bacteremia, leading to the onset of a generalized disease and meningitis, as described for other diseases (14, 27). Similarly to *Streptococcus pyogenes* (group A streptococcus) infection in humans, where serotype replacement in a population (24) is most likely the result of the immune status of the individuals along with the introduction of a highly virulent organism (8), the propensity of *S. iniae* to cause an invasive disease in fish is likely related not only to the

immune status of the fish but also to the pathogenetic mechanism(s) of virulent strains. One of the features that allow *S. iniae* to establish an infection is related to its ability to overcome the immune response of macrophages, which play a role in initial phagocytosis and eventual killing of streptococci and other pathogens. Invasion and intracellular survival of *S. iniae* in host cells might thus represent an important pathogenicity mechanism in invasive infections.

To gain more insight as to the ability of *S. iniae* to initiate infection, we studied the various interactions between noninvasive (type I) and invasive (type II) strains of the pathogen and salmonid macrophages.

By using salmonid-specific cellular models, we demonstrate that *S. iniae* is capable of (i) invading and surviving in fish phagocytes and (ii) specifically inducing their apoptosis. The role of this coupled phenomenon in the infectivity of two *S. iniae* serotypes and in their induction of meningitis is discussed.

MATERIALS AND METHODS

Bacterial strains and tissue culture cells. Bacterial strains used in this study were collected from diseased rainbow trout. *S. iniae* Dan-15 (serotype I) was isolated in 1992 from a fish farm sited in the Upper Galilee, while *S. iniae* KFP 404 (serotype II) was collected from the same location in 2000. All strains were stored at –70°C in brain heart infusion broth (Difco) with 15% glycerol. Cultures were initially grown on Columbia blood agar at 18°C; for infection assays, bacteria were grown for 8 h in brain heart infusion. Optical density at 640 nm was measured with a spectrophotometer (Shimadzu Corporation, Kyoto, Japan), and viable counts were determined. Mid-log-phase cultures (10⁸ CFU) were found to correspond to an optical density of 0.30 to 0.35 for both strains.

The established salmonid macrophage cell line RTS-11 (17), the salmonid embryonic cell line CHSE-214, and the rainbow trout gonad cell line RTG-2, used for adhesion and invasion assays, were cultured at 18°C in Dulbecco's

* Corresponding author. Mailing address: Department of Poultry and Fish Diseases, The Kimron Veterinary Institute, P. O. Box 12, Bet Dagan 50250, Israel. Phone: 972-3-9681760. Fax: 972-3-9681739. E-mail: eldar@agri.huji.ac.il.

modified Eagle medium (DMEM) (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum (GIBCO), HEPES (1%), penicillin (100 µg/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml). Cell lines were subcultured every 3 weeks.

PN and blood leukocyte collection. Blood was collected in heparinized tubes and diluted (1:20) with DMEM. Pronephros phagocytes (PN) from hind-kidneys were obtained by forcing the tissues over a TG 100 separating gauze (Schleicher and Schuell, Dassel, Germany). PN and blood leukocytes were isolated through a Histopaque ($d = 1.077$) gradient (Sigma, St. Louis, Mo.). Cell suspensions were centrifuged for 20 min at $400 \times g$ to remove erythrocytes and debris. The leukocyte-enriched interphase was collected, washed in phosphate-buffered saline (PBS) (15 mM Na_2HPO_4 –145 mM NaCl [pH 7.20]), counted (by trypan blue exclusion), and resuspended in DMEM supplemented with 10% fetal calf serum.

Infection assays. For preliminary assays, RTS-11 cells were infected (multiplicity of infection [MOI], 100) for 0, 20, 40, 60, 120, or 180 min before cells were washed three times in PBS or washed and reincubated for 2 h in complete medium supplemented with ampicillin (100 µg/ml). The results showed that at time zero, the level of *S. iniae* adhesion to RTS cells was 0.01 to 0.03 CFU/cell. After 60 min of infection, total cell-associated bacteria were 3.8 to 6.8 CFU/cell, with only 0.4 CFU of intracellular bacteria; after 180 min, each cell harbored 2.5 to 5 CFU/cell. Therefore, in the following experiments, the RTS-11, CHSE-214, and RTG-2 cell lines were infected (MOI, 100) for 60 min for the adhesion assay and for 180 min for the invasion assay. All experiments were performed (in triplicate) at least three times.

Adhesion assay. Adhesion of *S. iniae* to cell lines was performed as previously described (11, 34). Mid-log-phase *S. iniae* bacterial suspensions were added to 2×10^5 RTS-11 macrophages and to prewashed confluent CHSE-214 and RTG-2 cells in 12-well tissue culture plates (Costar Co., Cambridge, Mass.) at an MOI of 100 bacteria per eukaryotic cell. After 15, 30, and 60 min of incubation, nonadherent bacteria were removed by washing the cells three times with PBS. For viable count determinations, infected cells were treated for 5 min with 0.05 ml of 0.25% trypsin and of 0.1% EDTA (Sigma) in Hanks balanced salt solution (GIBCO), and streptococci were harvested by adding 0.15 ml of 0.025% Triton X-100 (U.S. Biochemicals, Cleveland, Ohio) in sterile distilled water to each well. After 3 min, cell lysates were collected and serially diluted in PBS, and aliquots (in triplicates) were plated onto blood agar for assessment of bacterial CFU.

Total counts of cell-associated (invading plus surface-adherent) bacteria were determined. Adherent bacteria were quantified by subtracting the number of invasive bacteria (determined as described under "Invasion and survival assay" below) from the total cell-associated bacteria.

Invasion and survival assay. Invasion was assessed as described by Rubens et al. (33) with some minor modifications. Briefly, RTS-11, CHSE-214, and RTG-2 cells and PN were infected as described above. After 3 h, extracellular bacteria were removed by three washes with PBS, and the original volume was reconstituted with DMEM supplemented with 10% fetal calf serum and ampicillin (100 µg/ml). After the addition of ampicillin, incubation was allowed to proceed for an additional 3 h. For both *S. iniae* strains, the MIC (Etest; AB Biodisk, Solna, Sweden) of ampicillin is >0.016 mg/ml; incubation of *S. iniae* in the presence of ampicillin (100 µg/ml) for 3 h resulted in 100% killing of bacteria with no toxic effects to fish PN or cell lines.

For assays of survival of bacteria in phagocytes, PN were isolated from 100-g naïve trout as described above. Cells were collected, washed, and counted by dye exclusion. PN were infected for 3 h (as previously described), washed, and resuspended in DMEM supplemented with 10% fetal calf serum and ampicillin (100 µg/ml). Viable counts of intracellular bacteria were determined at time zero (3 h post-antibiotic addition) and after 24 and 48 h.

Opsonin-dependent invasion of trout PN. PN were isolated from 100-g naïve trout (nonvaccinated controls) and from trout specifically vaccinated against KFP 404 (with 10^9 CFU of formalin-killed bacteria emulsified in incomplete Freund's adjuvant [Sigma]/fish; immunization was performed as described previously [13]). Assays for assessment of PN invasion and opsonin-dependent phagocytosis were based on a previously described protocol (26), which we modified as follows. Briefly, 75 µl of KFP 404 bacterial solutions plus 25 µl of serum samples were distributed (in triplicate) in a 96-well microtiter plate and incubated for 45 min at 18°C. To obtain the maximal assay sensitivity, undiluted serum was applied to the first wells of the serial dilution (1:1, 1:5, and 1:20) and no sera were added to the last wells. Trout PN were suspended in DMEM supplemented with 0.1% gelatin and 10% fetal calf serum; 100 µl containing 2×10^5 cells (MOI, 100) was then added to each well. Cells from a given fish were combined with the serum samples obtained from the same fish. The mixture was incubated for 60 min at 18°C with shaking, and invasion was stopped by an additional 3-h incubation with ampicillin (100 µg/ml). Cells were then lysed with

0.025% Triton X-100, and intracellular bacteria were quantified by plate counting.

Survival in whole blood. Resistance to phagocytosis in whole blood was determined as described by Fuller et al. (16), with minor modifications which were found necessary due to the limited amount of blood that can be drawn from each fish. Bacterial suspensions (10 µl, containing 10^2 or 10^3 CFU) were added to 120 µl of fresh, heparinized trout or human blood in sterile glass tubes and incubated on an orbital shaker for 1.5 to 72 h at 18°C (trout blood) or 37°C (human blood). For bacterial enumeration, 20 µl of blood sample was added to 40 µl of 0.025% Triton X-100, vortexed, serially diluted, and plate counted.

Partition of bacteria in blood of diseased fish. Naïve fish were infected with *S. iniae* by cohabitation. Three clinically diseased fish (previously infected by intraperitoneal [i.p.] injection with 100 50% lethal doses [LD_{50}] of *S. iniae* Dan-15 or *S. iniae* KFP 404) were placed with 100 naïve fish (two groups of 50 fish in separated tanks); the course of the infection by cohabitation was monitored daily. Fish were considered to be diseased when they simultaneously exhibited three out of the four following clinical symptoms: lethargy, black discoloration, loss of orientation, and ocular pathologies.

To assess the number of free and ingested bacteria in the blood of diseased fish, a three-step protocol consisting of (i) fractionation between the various blood components, (ii) separation of leukocytes by Percoll purification, and (iii) enumeration of bacteria by plate counting was used. Blood was drawn from the caudal veins of diseased trout, collected in sterile heparinized tubes, and divided into three aliquots. For determination of the total number of bacteria, 20 µl of blood was lysed by addition of 40 µl of 0.025% Triton X-100 in sterile distilled water, serially diluted, and plate counted. Bacteria in serum were enumerated by plate counting of serum (obtained after full decantation of leukocytes and erythrocytes). For quantification of intracellular organisms, peripheral blood leukocytes (PBL) were purified over a 1077 Histopaque gradient and lysed with 0.025% Triton X-100, and bacteria were plate counted.

Transmission electron microscopy. Infected and control cell cultures were directly fixed in culture flasks, in cold (0 to 4°C) 1.25% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), for 45 min. Cells were then rinsed in buffer and postfixed for 45 min in 1% osmium tetroxide. After ethanol dehydration, samples were embedded in Epon resin. Ultrathin sections were stained with uranyl acetate and lead citrate according to the Reynolds method (31) and observed under an EM12 Philips transmission electron microscope at 80 kV.

Assessment of apoptosis. Apoptosis of PBL and PN was monitored by analysis of DNA fragmentation and quantified with a flow cytometer.

DNA fragmentation, visualized in gel electrophoresis as "DNA laddering," was analyzed as described previously (20), with modifications. Briefly, PBL and PN (5×10^5 per well) were infected with *S. iniae* as previously described. After 2 h, extracellular bacteria were removed by three washes with PBS, and the original volume was reconstituted with DMEM supplemented with 10% fetal calf serum, penicillin (100 µg/ml), and gentamicin (50 µg/ml). Incubation was allowed to proceed for an additional 22 h. Macrophages incubated with 1 µg of actinomycin D (Act D; Sigma)/ml for 24 h were used as a positive control for apoptosis (15). Cells were harvested by addition of 2 ml of 0.25% trypsin and of 0.1% EDTA (Sigma) in Hanks balanced salt solution (GIBCO) and were lysed with lysis buffer (0.2% Triton X-100, 20 mM Tris [pH 7.4], 10 mM EDTA [pH 8.0]) at room temperature for 10 min. After centrifugation, the supernatant was treated with proteinase K (100 mg/ml) for 1 h at 50°C, and then RNase (0.5 mg/ml) was added for 1 h at 50°C. Lysates were extracted twice with an equal volume of phenol-chloroform (1:1, vol/vol) and once with an equal volume of chloroform-isoamyl alcohol (24:1, vol/vol) before precipitation with ethanol. Precipitates were dried and solubilized in $1 \times \text{TE}$ (10 mM Tris [pH 8.0]–1 mM EDTA). Electrophoresis was performed with a 2% agarose gel, and DNA was stained with ethidium bromide. As a negative control, noninfected cells were treated by the same procedure as the infected cells.

For flow cytometric (FCM) assessment of apoptosis, assays were performed ex vivo on PN and PBL collected from naïve trout. Leukocytes suspended in DMEM were infected (MOI, 100) as described above. FCM assays were carried out 24 h after in vitro infection on cells permeabilized and stained with propidium iodide (a fluorescent probe for DNA) in nuclear isolation medium (NIM) for 20 min in the dark at 4°C (6). Acquisitions were carried out by using a standard fluorescence-activated cell analyzer (FACScan; Becton Dickinson, Mountain View, Calif.) on cells kept in NIM. For each sample, 10,000 individual leukocytes were recorded according to the FCM procedures used for the cell cycle analysis. Debris and cell aggregates were gated out of the acquisition. To estimate the percentage of apoptotic cells, we used an FCM method based on detection of the extensive cleavage of nuclear DNA (propidium iodide red fluorescence) occurring in apoptotic cells. Apoptotic nuclei display a DNA con-

FIG. 1. Adhesion to and invasion of salmonid cell lines by *S. iniae*. CHSE-211, RTG-2, and RTS-11 cells were infected with *S. iniae* type I (open bars) or type II (solid bars) at an MOI of 100. After 60 min of incubation, nonadherent bacteria were washed, cells were lysed, and total cell-associated bacteria were plate counted. For the invasion assay, the same procedure was allowed to continue for 3 h before extracellular bacteria were washed, the original volume was reconstituted, and ampicillin (100 μ g/ml) was added for 2 h of additional incubation. Data are means $\pm$ SD from six experiments performed in triplicate. $P < 0.01$ (*S. iniae* Dan-15-infected cells versus *S. iniae* KFP 404-infected cells) according to Student's *t* test.

tent lower than that contained in the diploid (G_0/G_1) state. Data were collected and analyzed using Cellquest software.

Cells were incubated in NIM (6) for 20 min in the dark at 4°C prior to FCM analysis. We use a two-parameter dot plot of a fluorescence peak width versus fluorescence peak area signals to gate out cell aggregates from cell suspensions.

Infection of fish by free and macrophage-associated bacteria. RTS-11 macrophage monolayers were infected with *S. iniae* Dan-15 or *S. iniae* KFP 404, harvested as described above (under "Invasion and survival assay"), and suspended (10^3 to 10^7 cells/ml) in DMEM; 0.2-ml was injected i.p. into recipient fish (10 rainbow trout, 100 g each, obtained from a specific disease-free site). A second group of naive fish was infected by i.p. injection (0.2 ml) of PBL (10^3 , 10^4 , 10^5 , 10^6 , and 10^7 cells/ml) purified from diseased fish naturally infected by a serotype II strain. Bacterial CFU associated with RTS-11 macrophages and PBL were quantified by plating on agar. A third group was infected with (0.2-ml) mid-log-phase bacterial cultures diluted to the required inocula (10^2 to 10^6 CFU). Morbidity and mortality were monitored daily, and dead fish were subjected to bacterial examination.

Statistical analysis. Data are presented as means $\pm$ standard deviations (SD) from at least four independent experiments performed in triplicate. Results were analyzed by Student's *t* test. The results of partitioning of bacteria in the blood of diseased fish were analyzed by a one-way analysis of variance (SAS software, version 5). Results of infection of fish by free and macrophage-associated bacteria were analyzed by linear regression analysis (SAS software, version 5). The

electron microscopy analysis and analysis of DNA fragmentation by flow cytometry and agarose gel electrophoresis were repeated six times, and data from a typical experiment are reported.

RESULTS

Both strains readily bound to salmonid RTS-11 macrophages. After 20 min, each RTS-11 macrophage harbored 1.6 CFU of Dan-15 or KFP 404 (data not shown). However, 60 min postinfection, the degree of binding was strain dependent: Dan-15 (serotype I) bound to macrophages at a ratio of 3.8:1 (bacterial CFU/cell), whereas the ability of strain KFP 404 (serotype II) to adhere to macrophages was roughly 50% higher (6.8 CFU/cell). In contrast, bacterial adhesion to non-professional phagocytic cells was considerably lower: Dan-15 and KFP 404 bound to CHSE cells at rates of 1.2 and 1.5 CFU/cell, respectively. For both serotypes, insignificant binding of bacteria to RTG cells was observed (0.05 to 0.1 CFU/cell) (Fig. 1).

FIG. 2. Invasion and survival of *S. iniae* in PN macrophages. PN were infected for 3 h (as described above), washed, and resuspended in DMEM supplemented with 10% fetal calf serum and ampicillin (100 μ g/ml). Viable counts of intracellular bacteria were determined at time zero (3 h post-antibiotic addition) and after 24 and 48 h. Data are means $\pm$ SD from three experiments performed in triplicate. $P < 0.01$ (*S. iniae* Dan-15-infected macrophages versus *S. iniae* KFP 404-infected macrophages) by Student's *t* test.

FIG. 3. Electron micrograph of PN macrophages infected with *S. iniae* KFP 404 for 2 h, showing streptococci internalized within the cells 22 h postinfection.

Internalization and intracellular survival. A recent study (16) demonstrated that *S. iniae* is capable of invading the mammalian HEP-2 and brain microvascular endothelial cells (BMEC) cell lines when they are cultured at 37°C. In our study, we analyzed internalization of *S. iniae* in a specific immunocompetent salmonid macrophage cell line, RTS-11, and in PN grown at the temperature at which the natural disease occurs. Monolayers were infected with *S. iniae* (as described above) for 3 h before ampicillin was added to eliminate extracellular bacteria. After two additional hours, cells were washed and lysed. Intracellular bacteria (protected from antibiotics) were plate counted. The embryonic salmonid cell line CHSE-214 and the trout gonadic cell line RTG-2 were also included. As shown in Fig. 1, *S. iniae* is able to invade RTS-11 macrophages, but the extent of invasion is serotype dependent. The serotype II strain reproducibly yielded ~50% higher CFU counts (4.8 CFU/cell) than the serotype I strain (2.5 CFU/cell), demonstrating that the efficient adhesion of the type II strain is followed by a successful intracellular invasion. PN are invaded by *S. iniae* (see Fig. 3) to a greater extent than RTS-11 macrophages, and as seen in RTS-11 invasion, the extent of PN invasion is also serotype dependent (11 CFU/cell for KFP 404 and 4 CFU/cell for Dan-15). The chronological events that

succeeded PN invasion were monitored for 48 h. While PN invasion by the type I strain is a transitory event after which the bacterium is almost eradicated, invasion by the type II strain is accompanied by a series of events that (i) allow its survival (2.2 CFU/cell 24 h postinfection) (Fig. 2 and 3) and (ii) are followed by its multiplication (3.6 CFU/cell 48 h postinfection) (Fig. 2).

Opsonin-dependent phagocytosis. Sera obtained from fish specifically vaccinated against *S. iniae* KFP 404 and sera from naïve fish were analyzed for antibody levels by microagglutination (13). Specific antibody titers, in serum samples drawn from vaccinated fish 6 weeks postvaccination, varied from 1:560 to 1:1,120, while no specific antibodies were detected among nonvaccinated fish. The opsonic activities of these antibodies in serum were determined by phagocytosis and killing assays, in which (2×10^5) PN (purified from vaccinated and naïve fish) were infected by *S. iniae* KFP 404 (preincubated with specific antisera). Infection was allowed to proceed for 60 min before ampicillin (100 µg/ml) was added to the mixture for an additional 3 h. As shown in Fig. 4, increased bacterial uptake was observed in PN macrophages isolated from the vaccinated fish (5.0 CFU/cell in vaccinated fish versus 0.5 CFU/cell in nonvaccinated fish), indicating that effective opsonization had occurred. The results obtained with PN purified from nonvaccinated fish resemble those obtained with RTS-11 macrophages (~0.4 CFU/cell after 60 min). In contrast, PN obtained from vaccinated fish and incubated for 60 min with opsonized bacteria harbored a number of bacteria per cell similar to that in RTS-11 macrophages incubated for 3 h. This finding suggests that specific antibodies increase the efficacy of phagocytosis.

In vitro viability. Interestingly, in vitro viability and partitioning of *S. iniae* within whole blood were also shown to be host dependent (Fig. 5). Unlike bacterial killing in human whole blood, *S. iniae* cells of both serotypes were able to survive and multiply in trout whole blood, as determined by an increase in viable cell numbers. Survival was monitored up to 72 h postinfection, and CFU counts steadily augmented (Fig. 5).

Partition and serotype infectivity. All parameters indicated that bacterial loads in the bloodstream were significantly lower

FIG. 4. Opsonin-dependent invasion of trout PN. PN and serum samples were obtained from naïve trout and from trout specifically vaccinated against KFP 404. PN were infected (by incubation for 60 min at 18°C with shaking) with KFP 404 preincubated (45 min at 18°C) with serum obtained from the same fish in serial dilution (1:1, 1:5, and 1:20) or with PBS (no sera) as a control. After 60 min of infection (MOI, 100), ampicillin (100 µg/ml) was added for 2 h of additional incubation. For invasion assays, cells were immediately lysed with 0.025% Triton X-100 and intracellular bacteria were plate counted. Data are means $\pm$ SD from three experiments performed in triplicate. $P < 0.01$ (macrophages infected with *S. iniae* KFP 404 preincubated with sera from vaccinated fish versus macrophages infected with *S. iniae* KFP 404 preincubated with sera from nonvaccinated fish) by Student's *t* test.

FIG. 5. Resistance of *S. iniae* in whole blood. Dan-15 and KFP 404 bacterial suspensions were added to fresh heparinized trout or human blood and incubated 1.5 to 72 h at 18°C (trout blood) or 37°C (human blood). For bacterial enumeration, 20 μ l of blood samples was added to 40 μ l of 0.025% Triton X-100, vortexed, serially diluted, and plate counted.

in *S. iniae* type I-infected fish [$(4.27 \pm 1.47) \times 10^4$ CFU/ml (mean $\pm$ 3 standard errors of the mean)] than in *S. iniae* type II-infected fish [$(4.50 \pm 0.76) \times 10^6$ CFU/ml (mean $\pm$ 3 standard errors of the mean)]. The breakdown of the data indicates that while for the type I strain 54.5% $\pm$ 9.7% of the total bacterial count is free and only 45.5% $\pm$ 5.6% is cell associated ($P > 0.05$), the type II strain exhibits a tendency toward cell association, with 69.1% $\pm$ 6.4% of the bacteria present in the pellet and 30.9% $\pm$ 6.4% free in serum ($P < 0.02$) (for both strains, data are means $\pm$ SD from three experiments performed in triplicate).

Serotype and apoptosis induction. As shown in Fig. 6, DNA fragmentation, visualized in gel electrophoresis as “DNA laddering,” is a clear outcome of PN infection by *S. iniae* type II. The type I strain causes a less apparent apoptotic effect. Identification and quantitation of cells undergoing apoptosis was based on evaluation of the subdiploid percentage of the cell

FIG. 6. DNA fragmentation of PN leukocytes incubated for 2 h with *S. iniae*. Total cellular DNA was isolated from noninfected (lane C), serotype I-infected (lane 1), serotype II-infected (lane 2), and Act D-treated (1 μ g/ml for 24 h) (lane 3) macrophages at 22 h postinfection. Lane M, molecular weight marker; 100-bp DNA ladder. DNA samples were analyzed by electrophoresis in a 2% agarose gel with ethidium bromide.

population (Fig. 7). Our data showed that *S. iniae*-treated leukocytes had higher percentages of nuclei with subdiploid DNA content. Percentages of cells with decreased DNA content in leukocyte cultures exposed to bacteria or left unexposed were recorded, and an apoptotic index was calculated (percentage of apoptotic cells in culture with bacteria/percentage of apoptotic cells in control). As shown in Fig. 8, the apoptosis induced in vitro with the serotype II strain (KFP 404) was stronger than that induced with the serotype I strain (Dan-15).

Increased infectivity of bacteria in phagocytes. Approximately 1 log unit of difference was noticed between the LD₅₀s of Dan-15 and KFP 404 suspended in PBS (free bacteria) (2×10^6 CFU for Dan-15 and 4×10^5 CFU for KFP 404). Attainment of similar mortality rates required infection of fish by only 1×10^3 RTS-11 macrophages harboring Dan-15 or KFP 404, equivalent to 2.5×10^3 or 5.0×10^3 CFU (of KFP 404 or Dan-15, respectively). The association between the number of bacteria and the percentage of mortality was examined by linear regression analysis; it was significant at a P value of <0.10 ($r = 0.91$) for Dan-15 and was not significant for KFP 404, strengthening the results given under “Partition and serotype infectivity” above and emphasizing the tendency of KFP 404 to be loaded in macrophages after injection of free bacteria. When unloaded phagocytic cells and viable bacteria were injected simultaneously, the LD₅₀s were identical to those of free bacteria. When fish were infected with macrophages loaded with type II bacteria, 2×10^4 PN, containing 2.5×10^4 to 1×10^5 CFU, sufficed to induce mortality in 100% of the fish.

DISCUSSION

Most studies on interactions of streptococci (including group B streptococci [21, 35] and pneumococci [2, 9, 22, 23, 36, 42]) with macrophages have focused on the role of antibodies and complement in phagocytic killing. Although immunity to pneumococcal disease is a complex phenomenon which involves elicitation of T-cell-dependent responses and priming for increased responses to capsular polysaccharide (1, 30), immunoglobulin G antibody concentrations are known to correlate

FIG. 7. FCM DNA fluorescence profiles of PN leukocytes incubated for 24 h with *S. iniae*. Detection of apoptotic cells is based on DNA content analysis of propidium iodide-stained PN leukocytes. Apoptotic nuclei appear as a broad hypodiploid DNA peak, which is easily distinguishable from the narrow peak of nonapoptotic cells with normal diploid DNA content (G0/G1 peak). DNA fluorescence histogram of noninfected cells displays a large number of diploid nuclei (dotted curve). An increased amount of hypodiploid cells is observed in PN leukocytes incubated for 24 h with *S. iniae* (MOI, 100). Results shown here demonstrate that serotype I (thin curve) induces less apoptosis than serotype II (thick curve).

with opsonophagocytic (functional) activity and protection (3, 23, 32, 36, 40, 41). In this study we provided evidence that phagocytosis of *S. iniae* by macrophages is most vigorous in the presence of opsonizing serum, and most importantly, we report that the ingested type II strain survives in macrophages. Therefore, immunity to *S. iniae* infection cannot be assessed through in vitro opsonophagocytic killing assays, and the presence of specific antibodies is only the evidence of previous elicitation of an immune system response.

FIG. 8. Induction of apoptosis by *S. iniae*. The apoptotic index (percentage of apoptotic cells in culture with bacteria/percentage of apoptotic cells in control) was calculated from data recorded for leukocyte cultures exposed to *S. iniae* or left unexposed. FCM acquisitions were carried out 24 h after in vitro infection. Data are means $\pm$ SD from six experiments performed in triplicate. $P < 0.01$ (*S. iniae* Dan-15-infected macrophages versus *S. iniae* KFP 404-infected macrophages) by Student's *t* test.

The pathogenesis of the meningitis and septic meningitis caused by *S. iniae* is unclear and is possibly the result of a multistep process. It is likely that disease starts with colonization of external tissue, followed by local spread and subsequent invasion of the bloodstream. The outcome of the infection is also related to the host's reaction: failure of initial phagocytosis and killing of the pathogen will allow the establishment of the disease. The salmonid-specific models that we have constructed allow the determination of certain events during type I and type II *S. iniae* infections. Since the incidence of central nervous system (CNS) infection is directly correlated to the concentration of the pathogen in blood and the length of time bacteremia is maintained (5, 25, 28), the abilities of the type II strain to survive within phagocytes and to induce high levels of bacteremia are advantageous for the onset of generalized bacterial meningitis. Increased levels of bacteremia with invasion and intracellular survival of *S. iniae* type II strains in host circulating macrophages thus represents an important evolution of the pathogenicity mechanisms in invasive *S. iniae* infections.

The ability of extracellular bacteria to invade the CNS from the bloodstream has been made clear by means of in vitro and in vivo models of infection (49). The mechanisms which enable *S. iniae* to disseminate in the body, and more particularly in the CNS, are unclear. At one point, free bacteria would have to pass the brain-blood barrier or by translocation through or between endothelial cells and underlying tissues before entering the CNS (45). Most meningeal pathogens, such as *Streptococcus pneumoniae*, *Escherichia coli* K1, and group B streptococci, are known to interact directly with cells of the brain-blood barrier as free bacteria (39). The possibility that a similar

event takes place also in *S. iniae* infection is strengthened by a recent report (16) showing that *S. iniae* causes damage to (human) BMEC monolayers, suggesting that CNS involvement may be attributed in part to the ability of *S. iniae* to promote cell injury and disruption of the blood-brain barrier. Another possibility, as occurs in *Streptococcus suis* type II (46) or *Listeria monocytogenes* infections of the CNS (11), is that bacteria could be carried into the CNS in association with monocytes (or phagocytes) migrating into the CNS compartment to maintain populations of resident macrophages. CNS infection can be initiated by cell-to-cell spread from infected leukocytes to the endothelium or by migration of infected leukocytes to the CNS. Our finding that the quantity of bacteria required for the establishment of experimental infection (with CNS involvement) is considerably diminished if bacteria are loaded within macrophages substantiates the idea that *S. iniae* can enter the CNS compartment in association with migrating monocytes, and it highlights the importance of macrophages as "Trojan horses."

Infected macrophages not only act as transporters, which assist in disseminating the bacteria throughout the organism, but since they undergo apoptotic death, they also fail in priming an immune response. Apoptosis regulates multiple physiological processes, including immune response, and reduces inflammatory tissue injury by removing damaged cells (19). Although apoptosis of *Salmonella*-infected macrophages does not alter antigen presentation ability (48), and apoptosis of *S. pneumoniae*-infected macrophages has been associated with successful clearance of bacteria (10), the ability of pathogens to promote apoptosis is considered an important mechanism for counteracting host immune defenses by avoidance of immune system-mediated killing and for initiation of infection (20, 37, 50). Apoptosis of immune cells has been associated with several streptococcal infections. Group B streptococci induce neuronal and monocyte apoptosis (15, 26), *S. pneumoniae* induces neuronal and neutrophil apoptosis (51), and *S. pyogenes* avoids host responses through apoptosis of lymphocytes and epithelial cells (38, 43).

Our results suggest that *S. iniae* also developed an antihost strategy based on apoptotic killing of blood leukocytes and PN. In fact, because apoptosis occurs without the release of cellular components, it reduces or suppresses inflammation (7, 18, 47). Therefore, apoptosis may be advantageous for the pathogen, as it might avoid the triggering and recruitment of nonspecific host defense mechanisms. Furthermore, macrophage death could also contribute to delaying or hindering the development of a specific immune response.

In conclusion, this study emphasizes the complexity of the strategy used by *S. iniae* to overcome host immune defenses. These mechanisms are shared by both serotypes, but they differ in extent: the profiles of bacteremia, intracellular survival, and induction of apoptosis by type II strains are far more efficient than those of type I strains. We suggest that the generalized meningitis induced by *S. iniae* type II is a consequence of its capacity to (i) survive in phagocytes and (ii) induce their apoptosis. According to this theory, the apoptotic phagocyte serves as a vector that is loaded in the blood circulation and is unloaded, after blood-brain barrier transcytosis, in the CNS.

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