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Streptococcus iniae inhibition of apoptosis of nonspecific cytotoxic cells: a mechanism of activation of innate immunity in teleosts

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ABSTRACT: Nonspecific cytotoxic cells (NCC) may provide innate anti-bacterial resistance against *Streptococcus iniae* infections in tilapia. The mechanism of immunity would be elaboration and release of various cytokines, augmentation of inflammation and amplification of increased antigen processing. To investigate bacterial regulation of NCC function, 2 different processes of cellular pathology were examined: apoptosis and necrosis. Different isolates of *S. iniae* from diseased teleosts, a dolphin and a human were tested. All isolates were examined for their ability to produce apoptosis and/or necrosis on freshly purified tilapia NCC and on a tilapia continuous cell line (i.e. TMB-8 cells). Two different isolates (9033 and 173) inhibited the outer membrane expression of phosphatidylserine (PS) by NCC, an early sign of apoptosis. This occurred at 4 h post-treatment and lasted throughout the 24 h treatment period. All other isolates either did not differ from control levels or produced a small increase in PS expression by NCC. The early reduction in PS expression occurred concomitantly with increased necrosis associated with nonspecific DNA fragmentation. Two-color flow cytometry (Annexin-V vs propidium iodide staining) demonstrated the specificity of Annexin-V binding. Experiments were also done to determine the effects of *S. iniae* on TMB-8 cells. Treated TMB-8 cells did not produce appreciable Annexin-V binding. Compared to the ATCC strain, 9033 produced high levels of necrosis-associated DNA fragmentation of TMB-8 cells at 4 and 8 h post-treatment. These data indicated that different isolates of *S. iniae* may regulate NCC anti-bacterial resistance by causing reduced levels of programmed cell death (PCD), increased necrosis and associated enhancement of inflammatory responses. Understanding the relevance of these bacterial effects on NCC may be an important consideration in the evaluation of isolates used in vaccine/bacterin production.

KEY WORDS: Nonspecific cytotoxic cells · Tilapia · *Streptococcus iniae* · Apoptosis · Necrosis · Annexin-V · Innate immunity · DNA · Programmed cell death · Teleosts

INTRODUCTION

Nonspecific cytotoxic cells (NCC) may provide essential innate cytokine and cytotoxic responses during bacterial and protozoan infections in teleosts (see reviews: Evans & Cooper 1990, Evans & Jaso-Friedmann 1992, 1993). We have previously shown that the fish pathogen *Edwardsiella ictalurus* stimulates *in vivo* NCC cytotoxicity in catfish (Evans et al. 1998). Studies of the *in vivo* activation of innate resistance with *Streptococcus iniae*, an important fish (tilapia, trout) and human pathogen have shown similar results (Kitao et al. 1981, Eldar et al. 1994, *S. iniae* Study Group 1996, Weinstein et al. 1997). These data indicated that NCC may be important participants in generating innate resistance to bacterial infections.

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Various species of Gram-positive cocci have been isolated from freshwater (Bragg & Broere 1986, Perera et al. 1994, Stoffregen et al. 1996) (tilapia, koi carp, trout, hybrid stiped bass) and marine fish species (eels, yellowtail, flounder, etc.) (Minami et al. 1979). In diseased animals, mechanisms of innate and acquired immunity have not been defined. In trout, *Streptococcus iniae* vaccine studies (Eldar et al. 1995) showed no correlation between antibody titers and protection against homologous challenge. The conclusion was that 'protection might be explained by the involvement of the cellular immune system of the non-specific defense system' (Eldar et al. 1995). Immunization of trout by immersion in *Streptococcus* sp. (Horovitcz et al. 1997) was successful in generating immunity that lasted for only 1 mo post-injection (PI). Immunized fish however, were negative at 2 to 3 mo PI. A similar result (Iida et al. 1981) was reported for immunization of cultured yellowtail with Streptococci. These studies suggested that for certain bacterial infections in tilapia, antibodies may not be principally responsible for protection.

In vivo experiments where apoptosis has been described as an invasive characteristic of bacteria capable of destroying antigen processing and immune cells have not been reported for any aquatic species. However, *in vitro* studies of mammalian cells suggested that apoptosis may be a pathogenesis factor for many different bacterial species (see reviews: Chen & Zychlinsky 1994, Williams et al. 1994, Lilies 1997, Zychlinsky & Sansonette 1997). Bacterial toxins (Lilies 1997), *Actinomycete* leukotoxin, *Staphlococcus aureus* alpha toxin, *Escherichia coli* hemolysins, Staphlococcal enterotoxin B (SEB), etc. as well as inhibitors of protein synthesis (i.e. diphtheria toxin, shigella toxin, etc.) promoted apoptosis and/or necrosis of mammalian cells.

In the present study we investigated the effects of different fish and human isolates of *Streptococcus iniae* on apoptosis and necrosis of NCC. To develop this model *in vitro* system, studies were carried out to determine the effects of *S. iniae* on the expression of phosphotidylserine residues on NCC as detected by Annexin-V binding. Certain isolates increased the necrotic pathology of NCC while simultaneously reducing Annexin-V binding. These data suggested a new mechanism of activation of inflammation by bacteria and participation of NCC in mediation of innate immunity in aquatic species.

MATERIALS AND METHODS

Fish. Outbred tilapia (*Oreochromis niloticus*/*Tilapia nilotica*) of both sexes weighing 60 to 100 g were obtained from Americulture, Inc., Animas, New Mexico, USA. Fish were maintained in flow-through 500 gallon (1900 l) aquaria at a temperature of 24 to 26°C and were fed a commercial diet of pelleted fish food (Goldkist fingerling catfish food). All experimental fish were kept in quarantine for at least 30 d prior to experimentation. To obtain NCC, fish were net captured and anesthetized in 25°C water containing 5 ppm 3-aminobenzoic acid ethyl ester (Sigma Chemical Co., St. Louis, MO).

Media. Cells were cultured in RPMI-1640 (Cellgro, Media Tech, Washington, DC) supplemented with 10% fetal bovine serum (Atlanta Biologics, Atlanta, GA). Bacteria were cultured on sterile 5% defibrinated bovine blood agar (Metro Medical, Elon, NC), Brain-Heart Infusion (BHI) agar and BHI Broth (Difco Lab, Detroit, MI).

Bacteria. Isolates of *Streptococcus iniae* were obtained from Emmett Shotts Jr (University of Georgia), D. E. Low (University of Toronto) and Avi Eldar (Kimron Veterinary Institute, Israel). Only 1 bacterial isolate was from ATCC (i.e. 29178; Amazon dolphin isolate) (Pier & Madin 1976). The remaining isolates were from diseased fish and consisted of: Dan-1, 173 and 169 (all isolated from the brain of diseased rainbow trout) provided by A. Eldar; 164 (isolated from the trunk kidney of a diseased European sea bream) (A. Eldar); and 9033 (isolated from the brain of a diseased tilapia) and 2378-91 (human isolate) (provided by Dr D. E. Low). Bacteria were grown on 5% defibrinated bovine blood agar, transferred to BHI. A single colony was inoculated into BHI broth cultures. Bacteria were grown to log phase, harvested by washing in PBS (pH 7.4) and colony counts were done. Bacteria were then fixed in 3.7% formalin for 1.5 to 2 h. Fixed bacteria were washed 3× in PBS and stored at 4°C. Fixed bacteria preparations from each isolate were sterility checked for contamination. All were negative.

Cell line. The tilapia continuous cell line TMB-8 was provided by Dr R. Hedrick (Universtiy of California-Davis). These cells were originally established from *Oreochromis mossambicus* cardiac tissue (Dr D. Lewis pers. comm.). Cells were maintained at 25°C/5% CO_2, and serially passaged in RPMI-1640 containing 10% fetal bovine serum (FBS).

Purification of NCC. Tilapia were sacrificed by anesthetic euthanasia and anterior kidney and spleens were asceptically removed. Single cell suspensions were made by mechanical disruption of the tissue and NCC were enriched by fractionation over 45.5% Percoll® density gradients as previously described (Jaso-Friedmann et al. 1997). Purity of NCC was determined by staining with the monoclonal antibody (mab) 5C6 as previously reported (Evans et al. 1988). Briefly, cells were stained for 1.5 h at 4°C, washed and anti-mouse IgM-FITC conjugate (SIGMA Chemicals, St. Louis, MO) was added for 45 min at 4°C. Stained cells were analyzed by flow cytometry.

***In vitro* treatment of NCC and TMB-8 cells with *Streptococcus iniae*.** Experiments were carried out in 96-well round bottom microtiter plates. 100 µl of NCC (10^6 cells ml^{-1}) in RPMI-1640 were added to each well. Formalin killed *S. iniae* of each isolate was added to the wells in various dilutions. Following incubation (28°C/5% CO_2) for 0 to 24 h, cells were harvested, counted, washed in PBS and divided into aliquots for testing.

TMB-8 cells were briefly trypsinized, washed and 100 µl (at 10^6 cells ml^{-1}) resuspended into microtiter plates. Different concentrations of the formalin killed bacterial isolates were added in 100 µl and cultures were incubated for the indicated times (28°C/5% CO_2). Following treatment, cells were harvested and analyzed by flow cytometry for Annexin-V binding and propidium iodide uptake.

Annexin-V analysis. Purified (greater than 80% mab 5C6+) and treated NCC were analyzed for Annexin-V binding. Following each respective treatment, NCC were washed and 5 µl of Annexin-V-FITC (Pharmingen; 65874H) was added to 10^5 cells. Cells were incubated in the dark for 15 min and 500 µl of PBS (containing 2.5 mM $CaCl_2$) was added. Cells were then analyzed by flow cytometry. Nonspecific Annexin-V uptake by NCC was determined by addition of propidium iodide (50 µg ml^{-1} in RPMI-1640 without FBS) and 2-color analysis.

Flow cytometry. Flow cytometry analysis was done using an EPICS XL-MCL 4-color SYSTEM II automated cell analysis system (Coulter Electronics Corp, Hileah, FL). Cells were washed with cold (4°C) PAB (PBS/0.1% azide/1.0% bovine serum albumin). Gating was based on forward scatter (FS) versus side scatter (SSc) signals.

RESULTS

Expression of phosphatidylserine (PS) residues on purified NCC

NCC were first examined for the outer membrane expression of PS residues following treatment with the ATCC strain of *Streptococcus iniae* or isolate 9033. NCC were purified from tilapia anterior kidney (AK) tissue and treated for 4 h in culture with different isolates of *S. iniae*. In Fig. 1, PS expression was detected on NCC by Annexin-V binding. Although these cells were treated with the ATCC isolate of *S. iniae*, the level of Annexin-V binding was only slightly higher than media controls. Apoptosis in these cases was dependent on the mechanism of cell death associated with *in vitro* cell culture. NCC treated with 9033 (Fig. 1B) were negative for Annexin-V binding. The insert shows a histogram of mab 5C6 binding to NCC representing the enrichment of the cells used in all experiments.

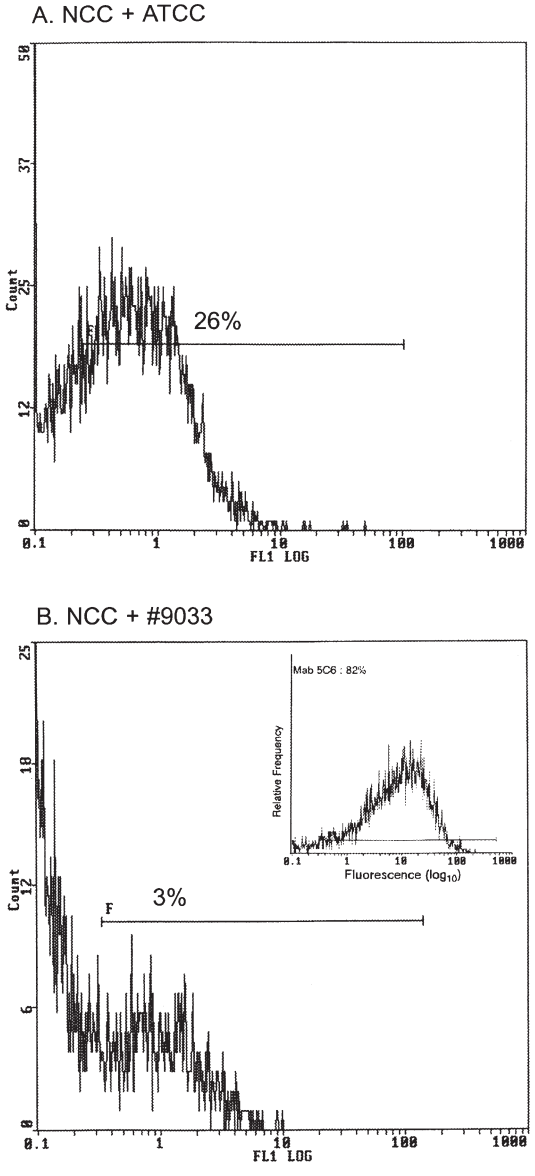

Fig. 1. Effects of treatment of NCC with *Streptococcus iniae* on expression of phosphatidylserine residues determined by Annexin-V binding. Purified NCC were treated with killed *S. iniae* [(A) ATCC strain and (B) isolate 9033] and analyzed by flow cytometry for Annexin-V binding. Isolate 9033 produced an almost 100% reduction in Annexin-V binding. Enrichment of NCC was determined by staining with monoclonal antibody (mab) 5C6 (see insert). NCC were purified over Percoll (45.5%) density gradients and stained with mab 5C6 to determine purity of the NCC population (see 'Materials and methods' for details). The population was greater than 80% mab 5C6+. Data representative of 9 experiments

Effects of *Streptococcus iniae* on expression of PS

NCC were purified from tilapia AK tissue and treated in culture with different isolates of *Streptococcus iniae*. Cells were treated for 0, 4, 12 and 24 h and

Table 1. Effects of *Streptococcus iniae* on protection of NCC from apoptosis. Different isolates of *S. iniae* were compared for the induction of apoptosis in NCC. Purified NCC (10^6) from the anterior kidney were treated with 3×10^5 bacteria. Treated NCC were harvested at 0, 4, 12 and 24 h post-treatment, washed and stained with Annexin-V-FITC (as previously described). Cells were next resuspended in PBS containing 2.5 mM $CaCl_2$. Percent Annexin-V binding was determined by flow cytometry. Results shown (given as mean ± SD) are representative of 3 different treatment groups tested in 27 different experiments consisting of: control (media only); ATCC (negative control representing isolates DAN-1, 169, 2378-91 and 164); and isolate 9033, which produced the same apoptosis modulating effects as isolate 173 (data not shown)

Treatment	Annexin-V binding (h post-treatment)			
	0	4	12	24
Media control	28.2 ± 8.1	39.5 ± 10.3	38.9 ± 12.4	43.0 ± 11.0
ATCC	–	36.8 ± 10.1	43.2 ± 10.3	48.0 ± 14.8
9033	–	13.0 ± 2.8	12.5 ± 2.1	11.0 ± 1.4

examined by flow cytometry for Annexin-V binding (Table 1). The data shown represent 3 different groups: media control, isolates producing negative to moderate increased Annexin-V binding (e.g. ATCC, Dan-1, 169, 2378-91 and 164) and isolate 9033. NCC treated with 9033 or with 173 had a rapid and large decrease in PS expression (Table 1) compared to control levels. PS expression remained low for the duration of the 24 h treatment period. The decrease in binding by Annexin-V was not caused by a reduced number of NCC in the cultures. *S. iniae* was not lytic and numbers of cells in treated cultures did not differ at 4 h post-treatment from controls (data not shown). Greater than 80% of the treated cells were mab 5C6+ determined by flow cytometry.

The reduction in outer leaflet expression of PS by 9033 was dependent on the concentration of 9033

In Fig. 2, purified NCC were treated with different concentrations (undiluted suspension contained 30×10^6 bacteria) of 9033 for 24 h and NCC were then stained with Annexin-V. The reduction in PS expression by treated NCC was dilution dependent. Treatment with the same numbers of the ATCC strain had no effects on the outer membrane expression of PS. Other *Streptococcus iniae* isolates either produced no changes in Annexin-V binding by NCC, or only moderate increases in the percentages of Annexin-V binding were observed (data not shown). These late changes were caused by selective depletion of Annexin-V-negative cells rather than increased late expression.

Treated NCC were next examined by flow cytometry to determine the specificity and optimum time for expression of PS (i.e. Annexin-V binding). This was accomplished by flow cytometric differentiation of intact NCC (no membrane lesions) versus necrotic NCC. NCC were treated for 4 (Fig. 3A) and 24 h (Fig. 3B) and stained with propidium iodide and Annexin-V. NCC were then subjected to 2-color flow analysis. NCC that were stained with both fluorochromes (i.e. double positive for Annexin-V-FITC and PI) were considered nonspecific positives for Annexin-V. Most cells in all treatment cultures were single positive with little nonspecific uptake of Annexin-V-FITC. Single positive Annexin-V NCC remained relatively constant from 4 to 24 h. Single PI cells occurred at very high percentages in 9033 and 173 treated cultures. These cells were also very low in single positive Annexin-V staining.

Effects of *Streptococcus iniae* on expression of PS by TMB-8 cells

TMB-8 cells were treated for 0, 4 and 8 h with different concentrations of *Streptococcus iniae*. Treatments had no appreciable effects on PS expression by these cultured tilapia cells (data not shown).

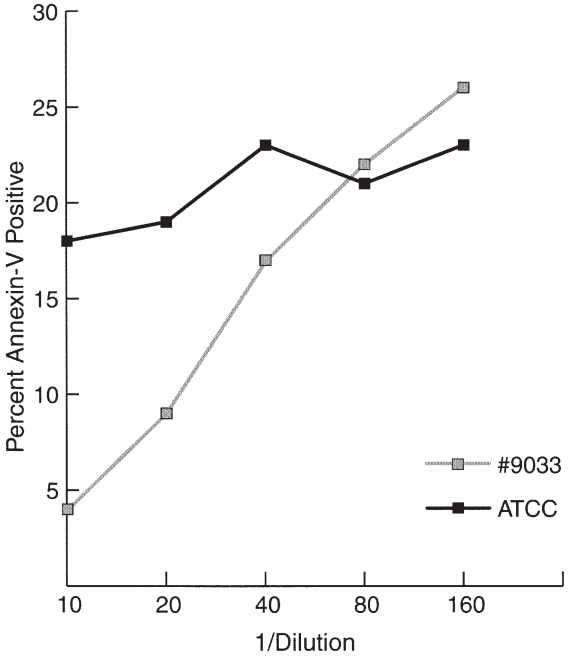

Fig. 2. Effects of bacterial numbers on induction of apoptosis in NCC. Purified NCC (1×10^6) were treated with varying dilutions of *Streptococcus iniae* 9033 from a stock which contained 30×10^6 organisms. They were incubated 18 to 24 h at 28°C, washed, stained with Annexin-V-FITC for 15 min in the dark at 4°C and resuspended in PBS containing 2.5 mM $CaCl_2$ for flow cytometric analysis. Log_{10} fluorescence is shown. Data representative of 3 experiments

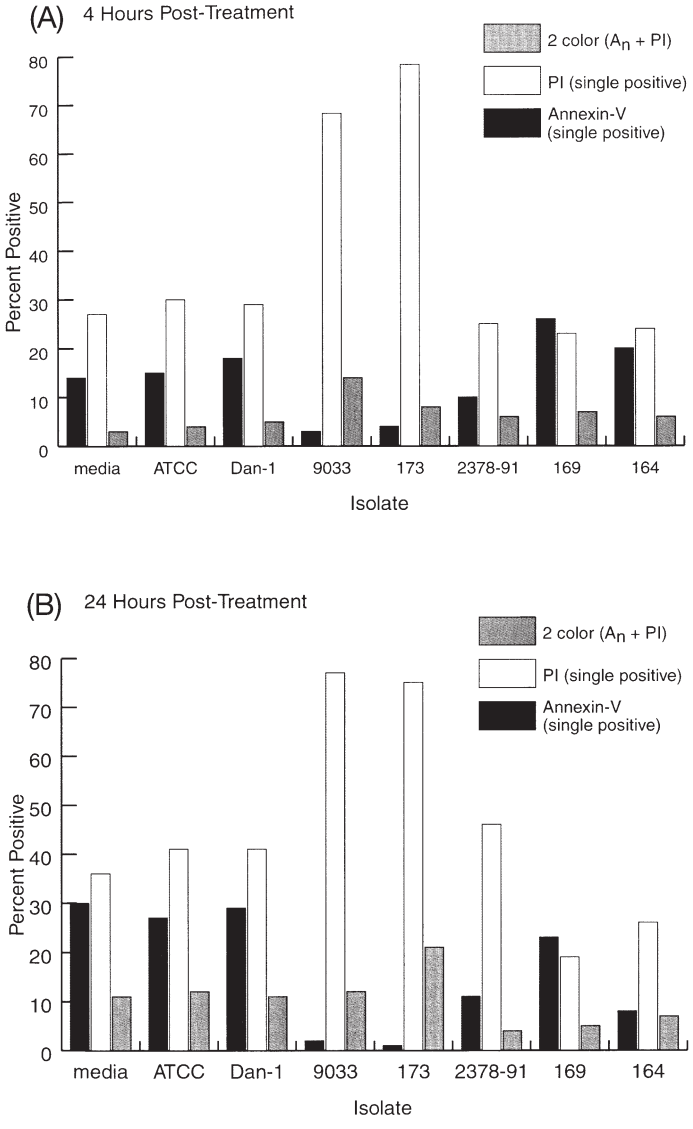

Fig. 3. Comparisons of (A) early and (B) late apoptosis with necrosis in *Streptococcus iniae*-treated NCC. The time-dependent induction of apoptosis and necrosis was measured by 2-color flow cytometry. Purified anterior kidney cells (1×10^6) were stained with both Annexin-V-FITC and propidium iodide following treatment with the different isolates of *S. iniae* (3×10^6). Cells were treated for either (A) 4 or (B) 24 h. Cells were harvested, washed and stained with Annexin-V-FITC for 15 min. NCC were resuspended in PBS containing 2.5 mM $CaCl_2$ and stained with propidium iodide (50 µg ml^{-1}). Two-color analysis was done by comparing green (FITC/*x*-axis) with red (*y*-axis) fluorescence. Data representative of 27 different experiments

Effect of *Streptococcus iniae* on DNA fragmentation by TMB-8 cells

The isolates 9033 and ATCC produced DNA fragmentation of TMB-8 cells characteristic of necrosis. Table 2 shows increased DNA fragmentation (PI uptake) at different concentrations of bacterial treatment. This occurred at both 4 and 8 h post-treatment. Lower levels of DNA fragmentation and a titration effect was not observed following treatment with the same concentrations of ATCC (Table 2).

DISCUSSION

Previous studies on any species have not investigated the bifunctional immunoregulatory effects of bacteria on the simultaneous production of reduced apoptosis and increased necrosis. Indeed, differentiation of a pathogen from a nonpathogen may be strongly influenced by this relationship. An invasive microorganism would greatly benefit from increased apoptosis of host cells and decreased necrosis of an antigen processing cell or memory B- or T-cell. The only exception to this would be intracellular bacteria (*Listeria*, *Shigella*, *Mycobacteria*) and parasites (*L. donovani*; Moore & Matlashewski 1994). For these intracellular pathogens, decreased apoptosis and decreased necrosis would provide the ideal microenvironment for replication and protection from scavengers and immune responses.

Apoptotic cells are phagocytosed and do not initiate an inflammatory response. Phagocytosis is probably initiated by recognition by macrophages of outer leaflet expressed PS. Cells undergoing necrosis are eventually lysed and may produce an active inflammatory response. Using NCC as the tilapia sentinel target cell, we demonstrated that certain isolates of *Streptococcus iniae* may immunoregulate tilapia immunity by reducing normal levels of apoptosis while increasing

Table 2. Production of DNA fragmentation in TMB-8 cells. TMB-8 cells were treated *in vitro* for 0 to 8 h with ATCC or 9033 isolates of *Streptococcus iniae*. Cells were harvested and examined by flow cytometry for propidium iodide uptake. Net DNA fragmentation = Treatment – Media control. Data shown are representative of 3 experiments

Treatment time (h)	Concentration of bacteria	Percent DNA fragmentation 9033	ATCC
0	–	6	3
4	–	14	16
4	0.156250×10^6	26	5
4	0.312500×10^6	35	7
4	0.625000×10^6	44	10
4	1.250000×10^6	58	13
8	–	16	18
8	0.156250×10^6	18	11
8	0.312500×10^6	32	17
8	0.625000×10^6	45	20
8	1.250000×10^6	60	17

necrosis. This might represent an advantage for the immunologically competent host and aid in identification of an isolate to be used in vaccine development. Cells undergoing apoptosis lack immunocompetence. Initiation of apoptosis of otherwise competent NK cells, lymphocytes, macrophages, etc. might represent an advantage for the survival of an invasive pathogen. Thus, differentiation of these 2 pathways of cellular pathology for a given microorganism may be more relevant in studies of innate immune resistance than determination of bacterial immunogens which initiate acquired antibody responses. We have found *S. iniae* isolates that simultaneously reduce apoptosis while increasing necrosis of NCC. This is the optimum combination for initiation of antigen processing leading to increased cellular recruitment and heightened primary and secondary antibody and cell mediated immune responses. This hypothesis is supported by the observations that all isolates in the present study were obtained from diseased fish and 173 has been used as a vaccine strain. We suggest that at least 1 mechanism for enhanced immunogenicity of this isolate is associated with the ability to inhibit apoptosis of NCC.

We predict that in teleosts, NCC are the principal cells involved in orchestrating innate responses to bacterial infections. NCC become biochemically activated following first encounter with a stressor agent. Activation responses represent augmented NCC cytotoxicity in the absence of recruitment of increased numbers of NCC. This response occurred in tilapia peripheral blood following a stress response (Jaso-Friedmann et al. 1999). In the peripheral blood, NCC become activated to almost 100% killing efficiency within 5 min following encounter with a stressor agent.

These responses are in sharp contrast to those that occur during normal homeostatic regulation of NCC. Immunosurveillance by NCC consists of a random encounter with a target cell (parasite or tumor cell) and following delivery of a lethal hit, the NCC quickly proceed into pathways of programmed cell death (PCD). Under these conditions, NCC do not recycle and their apoptosis induced turnover rate is rapid. *In vivo* and in the presence of *Streptococcus iniae* (or their products), this equilibrium would be disrupted producing a reduction in the rate of normal PCD of NCC. Rather than committing suicide in the face of bacterial insult, NCC would become activated, kill targets, secrete cytokines and eventually undergo necrosis. NCC undergoing necrosis would provide a critical 'secondary' innate response. This stimulation would produce cytokine amplification, increased phagocytosis by macrophages and activation of antigen processing cells.

Our *in vitro* model clearly supports this hypothesis. NCC treated with 9033 and 173 isolates of *Streptococcus iniae* had reduced levels of Annexin-V binding (i.e. reduced apoptosis). These same isolates produced increased necrosis of NCC. The *in vitro* responses of nonhematopoetic TMB-8 cells to *S. iniae* treatment differed from those of purified tilapia NCC. Unlike NCC, the TMB-8 cells did not express increased Annexin-V binding following treatment with 9033. Responses of the TMB-8 cells to treatment with the ATCC strain were likewise different (i.e. no Annexin-V binding) from the NCC data. These responses may have indicated that tissue cultured cells may not be the most appropriate targets for *in vitro* modeling studies of apoptosis because trypsinization and mechanical handling may also produce altered PCD. It is also possible that *S. iniae* produced different pathology on tilapia tissue depending on cell type and that lymphocytes or NCC cells may be the most appropriate targets of these microorganisms.

In tilapia, a focus of infection by *Streptococcus iniae* may produce a microenvironment where apoptosis down regulates immune function, reduces necrosis and eventually suppresses the local inflammatory response. These are the characteristic properties of infection by a pathogen in any species. However, a small percentage of isolates that we have examined produce almost a 90% reduction in apoptosis while simultaneously producing increased necrotic cell death of NCC. We predict that these isolates do not produce disease in fish, not because they are not highly immunogenic but because they elicit aggravated inflammatory responses while reducing apoptotic lesions of NCC. This combined action produces high levels of innate immunity. Our central hypothesis for mechanisms of teleost immunity is that NCC regulate innate responses either by direct lysis of targets or by releasing soluble apoptosis inhibitory factors that exacerbate inflammatory responses.

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