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BARD

FINAL REPORT

PROJECT NO. US-3159-99

**Streptococcus Iniae Infections in Trout and Tilapia:
Host-Pathogen Interactions, the Immune Response
Towards the Pathogen and Vaccine Formulation**

D.L. Evans, A. Eldar, L. Jaso-Friedmann, H. Bercovier

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Investigators

Institutions

Principal Investigator (PI):

Donald L. Evans

University of Georgia
Research Foundation

Co-Principal Investigator (Co-PI):

Avi Eldar

Kimron Veterinary Institute

Collaborating Investigators:

Liliana Jaso-Friedmann

University of Georgia
Research Foundation

Herve Bercovier

Hebrew University

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Nonspecific cytotoxic cells, antibody responses, apoptosis, differential
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Abbreviations commonly used in the report, in alphabetical order:

CPS/capsular polysaccharide; NCC/nonspecific cytotoxic cell; PCD/programmed cell death

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BARD Final Scientific Report

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Mustapha Oumouna, PhD 669-07-5951 (USA)

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings				0
Longer Visits (Sabbaticals)				0

Description Cooperation:

Cooperation involved sharing of bacterial isolates from diseased
fish and many email "conversations"

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted		1		1
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ABSTRACT

The objectives of the BARD proposal were to determine the mechanisms of nonspecific cytotoxic cells (NCC) that are necessary to provide heightened innate resistance to infection and to identify the antigenic determinants in *Streptococcus iniae* that are best suited for vaccine development. Our **central hypothesis** was that **anti-bacterial immunity in trout and tilapia can only be acquired by combining "innate" NCC responses with antibody responses to polysaccharide antigens**. These Objectives were accomplished by experiments delineated by the following Specific Aims: Specific aim (SA) #1 (USA) "Clone and Identify the Apoptosis Regulatory Genes in NCC"; Specific aim #2 (USA) "Identify Regulatory Factors that Control NCC Responses to *S. iniae*"; Specific aim #3 (Israel) "Characterize the Biological Properties of the *S. iniae* Capsular Polysaccharide"; and Specific aim #4 (Israel) "Development of an Acellular Vaccine". Our model of *S. iniae* pathogenesis encompassed two approaches, identify apoptosis regulatory genes and proteins in tilapia that affected NCC activities (USA group) and determine the participation of *S. iniae* capsular polysaccharides as potential immunogens for the development of an acellular vaccine (Israel group). We previously established that it was possible to immunize tilapia and trout against experimental *S. difficile/iniae* infections. However these studies indicated that antibody responses in protected fish were short lived (3-4 months). Thus available vaccines were useful for short-term protection only. To address the issues of regulation of pathogenesis and immunogens of *S. iniae*, we have emphasized the role of the innate immune response regarding activation of NCC and mechanisms of invasiveness.

Considerable progress was made toward accomplishing SA #1. We have cloned the cDNA of the following tilapia genes: cellular apoptosis susceptibility (CAS/AF547173); tumor necrosis factor alpha (TNF/AY428948); and nascent polypeptide-associated complex alpha polypeptide (NACA/AY168640). Similar attempts were made to sequence the tilapia FasL gene/cDNA, however these experiments were not successful. Aim #2 was to "Identify Regulatory Factors that Control NCC Responses to *S. iniae*." To accomplish this, a new membrane receptor has been identified that may control innate responses (including apoptosis) of NCC to *S. iniae*. The receptor is a membrane protein on teleost NCC. This protein (NCC cationic antimicrobial protein-1/ncamp-1/AAQ99138) has been sequenced and the cDNA cloned (AY324398). In recombinant form, ncamp-1 kills *S. iniae* *in vitro*. Specific aim 3 ("Characterize the Biological Properties of the *S. iniae* Capsular Polysaccharide") utilized an *in-vitro* model using rainbow trout primary skin epithelial cell monolayers. These experiments demonstrated colonization into epithelial cells followed by a rapid decline of viable intracellular bacteria and translocation out of the cell. This pathogenesis model suggested that the bacterium escapes the endosome and translocates through the rainbow trout skin barrier to further invade and infect the host. Specific aim #4 ("Development of an Acellular Vaccine") was not specifically addressed.

These studies demonstrated that several different apoptotic regulatory genes/proteins are expressed by tilapia NCC. These are the first studies demonstrating that such factors exist in tilapia. Because tilapia NCC bind to and are activated by *S. iniae* bacterial DNA, we predict that the apoptotic regulatory activity of *S. iniae* previously demonstrated by our group may be associated with innate antibacterial responses in tilapia.

ACHIEVEMENTS

We have identified a new class of DNA binding proteins (ncamp-1) that kill *Streptococcus iniae* (see Appendix manuscript #1). In addition, several apoptosis regulatory genes/proteins from tilapia nonspecific cytotoxic cells (NCC) have been sequenced and cloned. The bacterial DNA binding protein and the apoptosis regulatory genes (may) function in both up- and down regulation of apoptosis in tilapia during innate anti-bacterial immune responses. Previous studies by the Israel scientists have indicated that the teleost's ability to resist acute bacterial infections may depend less on primary and secondary antibody responses than on cellular innate immunity. Their studies on the pathogenesis of cellular invasion provided the stimulus for the collaborative experiments of the USA and Israel scientists. The research objectives of the combined studies were to clarify the mechanisms of anti-bacterial innate cellular (NCC) resistance that may be related to the pathogenesis and pathology of cellular invasion by *S. iniae*. These combined approaches provided the long term goals of the BARD proposal.

1. Accomplishments of the USA team. We have published evidence demonstrating that (*in vivo*) *S. iniae* activate tilapia NCC cytotoxicity. One mechanism of this activation may be inhibition of apoptosis via binding of bacterial DNA to membrane anti-apoptotic proteins. We identified and cloned three apoptosis regulatory cDNAs from tilapia NCC: cellular apoptosis susceptibility protein (CAS/Appendix manuscript #2, Figures 1-2), TNF-alpha (12) and nascent polypeptide-associated complex alpha polypeptide (NACA) from activated tilapia NCC. CAS is stress up-regulated (Appendix manuscript #2, Figures 4-5) in tilapia. This finding is significant because by preventing apoptosis, CAS may also up-regulate innate immune responses in tilapia thus giving NCC the ability to recycle. This could have widespread therapeutic applications for tilapia and other related teleosts. Our studies have also provided new information about potential roles for TNF in the immune response of tilapia (and of course other cyprinid fish). As a direct result of this work, the biological activities of recombinant tilapia TNF have been investigated as well as its expression in fish following infection (Appendix manuscript #3). This is the first report where a cell other than a macrophage has been identified as the source as well as the target of TNF alpha. In addition, the apoptosis protective effect of recombinant tilapia TNF are similar to the actions of activation cytokines. Since cytokine genes in teleosts have not been sequenced, the TNF report carries added significance for the future developments of immune activators.

Studies from the USA team have also identified a unique new class of pattern recognition receptors (PRR) that bind *S. iniae* DNA. We named this protein NCC cationic antimicrobial protein-1 (ncamp-1) and suggest that it plays an important role in mediating resistance to *S. iniae*. The data provided in Appendix manuscript #1 demonstrate that ncamp-1 is related to histone 1X. Polyclonal anti-camp-1 antibodies were generated against the soluble recombinant form of ncamp-1 and this antiserum bound tilapia NCC from PBL, anterior kidney and spleen. This recombinant ncamp-1 also had high specific bacterial killing activity (Table 1) against *S. iniae* (Table 2), *E. coli* (Appendix manuscript #1) and *Micrococcus luteus* (unpublished data). The truncated ncamp-1 recombinants shown in Table 1 (d1 and d2) are explained in Appendix manuscript #1.

Table 1		
Species	Recombinant	^a MIC ₅₀
<i>E. coli</i>	ncamp-1	0.43 uM
	ncamp-d1	0.63 uM
	ncamp-d2	1.0 uM
<i>S. iniae</i>	ncamp-1	0.86 uM

^a Minimal inhibitory concentration required to produce 50% killing.

Table 2. Susceptibility of <i>S. iniae</i> to ncamp-1 killing.		
TYPE I:	++	DAN 12, KFP 164
	+	DAN 7, KFP 166, KFP 169
	+/-	DAN 1, DAN 14, DAN 15, DAN 16
TYPE II:	+/-	KFP 173

++ completely susceptible, + partial susceptibility, +/- no or low susceptibility.

How will our projected studies of NCC help improve animal health? While many sequencing projects have provided molecular information about some of the effectors of immunity in fish, there is still a lack of information about the biological roles of some of these molecules. Our studies have provided new information about potential roles for CAS and TNF in the immune response of tilapia, and this may also affect other cyprinid fish. As a direct result of this work, the biological activities of recombinant tilapia TNF have been investigated as well as its expression in fish following infection. In addition to TNF, studies of other apoptosis regulatory proteins (e.g. FasL and ncamp-1) are significant in that by directly or indirectly regulating apoptosis they up-regulate innate immune responses in fish. This could have important therapeutic applications for tilapia and other related teleosts.

Accomplishments of the Israel team. The objectives specified in SA#3 were to characterize the binding and intracellular pathogenesis of *S. iniae* using an *in vitro* cell culture model. A novel mechanism of translocation through trout skin by *S. iniae* was proposed (data

not shown). Additional pathogenesis and epidemiological studies (4, 11) identified type II *S. iniae* from diseased fish.

Details of cooperation. All *S. iniae* isolates used by the USA team were furnished by Dr. Eldar. Additional interactions included the sharing (by email) of information and new ideas between Drs. Evans and Eldar. There were no visits between the scientists in either country.

PUBLICATIONS (with BARD acknowledgements) and REFERENCES of the ACHIEVEMENTS

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APPENDIX

PUBLISHED ABSTRACTS

1. Kaur, H., Oumouna, M., Jaso-Friedmann, L., and Evans, D. L. Role of bacterial DNA and oligonucleotide motifs in innate immune defense mechanisms in fish. Vet. Res. Day Proc., Coll. Vet. Med., UGA, 12-13, Feb., 2001.
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MANUSCRIPTS SUBMITTED (appendix manuscript #1).

1. **Evans, D. L.**, Kaur, H., Leary III, J. H., Praveen, K. and Jaso-Friedmann, L. 2005. Molecular characterization of a novel pattern recognition protein from nonspecific cytotoxic cells: sequence analysis, phylogenetic comparisons and antimicrobial activity of a recombinant homologue. *Developmental and Comparative Immunology* (in revision).

MANUSCRIPTS IN PREPARATION (appendix manuscript #2).

1. Kesavannair Praveen, K., Leary III, J. H., **Evans, D. L.**, and Jaso-Friedmann, L. Molecular cloning of cellular apoptosis susceptibility (CAS) gene in *Oreochromis niloticus* and its role in regulation of nonspecific cytotoxic cell (NCC) functions. To be submitted to *Fish and Shellfish Immunology*.