CHAPTER 4

Protection of Hybrid Striped Bass Against *Mycobacterium marinum* After DNA Vaccination

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Abstract

The protective responses induced by a DNA vaccine encoding the *Mycobacterium marinum* Ag85A gene were investigated. Juvenile hybrid striped bass (*Morone saxatilis* x *M. chrysops*) were injected with 25 µg or 50 µg DNA plasmid i.m. and developed specific protective responses to live bacterial challenge 120 days post-vaccination. Both vaccine groups demonstrated increased survival, reduced splenic bacterial counts, and reduced granuloma formation compared to the control groups. The vaccine groups also developed more rapidly and significantly increased antibody and lymphoproliferative responses post-challenge over those of the control groups. However, no significant differences in immune responses were recognized between the 25 µg and 50 µg groups, and these groups eventually experienced increased mortalities, splenic bacterial counts, and granuloma formation.
Keywords: Immunity; DNA; Vaccine; *Mycobacterium*; Fish; Striped bass; *Morone*

Rationale

This DNA vaccine study endeavored to confirm the protective effects of the prior DNA study and to characterize the specific immune responses generated by vaccinated fish after live *M. marinum* challenge. The research was also designed to evaluate the protective responses of the vaccinated fish over a longer time frame (up to 2 months).

Introduction

Mycobacterium marinum is well-recognized as a primary agent of piscine mycobacteriosis. This bacteria was first isolated from marine fish at the Philadelphia Aquarium in 1926 and has been associated with mortalities among numerous species of fish (Aronson, 1926; Austin and Austin, 1993; Chinabut, 1999). After *M. marinum* enters the body via the gastrointestinal tract, external lesions, or by transovarian passage, mycobacterial organisms can spread throughout the body through the lymphatic or circulatory system (Chinabut et al., 1994; Smith, 1997; Chinabut, 1999). These bacteria

can then cause development of internal granulomas which may subsequently lead to organ malfunction or death (Austin and Austin, 1993). Mechanisms of immunity against *M. marinum* have not been completely elucidated, but prior studies indicate that protective effects in fish are based on both specific and non-specific defenses (Bartos and Sommer, 1981; Chen et al., 1996).

Protection by DNA vaccines has been largely correlated with specific immune responses to the encoded antigen. In fish, DNA vaccines have been shown to provide protection against various piscine pathogens, such as viral hemorrhagic septicemia, infectious hematopoietic necrosis virus, and channel catfish virus (Boudinot et al., 1998; Lorenzen et al., 1998; Nusbaum et al., 2002); protection among vaccinated fish was conferred by immunity developed to the encoded viral glycoproteins. The synthesis of antigen by DNA vaccination imitates natural infection and leads to the subsequent specific humoral and cellular responses and ultimately the generation of memory lymphocyte responses (Donnelly et al., 1997; Heppell and Davis, 2000). In mammals, DNA vaccines encoding *M. tuberculosis* and *M. bovis* antigen 85A (Ag85A) have been shown to provide protection against live bacterial challenge (Denis et al., 1998; Tanghe et al., 2001). The induced protective immune responses included Ag85A-specific T-cell proliferation and cytotoxic T-cell activity. Because DNA vaccines have been shown to provide protection through specific responses, a DNA vaccine encoding the *M. marinum* Ag85A will putatively provide protection though induction through similar specific immune mechanisms. The research presented here characterized the specific protective immunity created by DNA vaccinated fish following live *M. marinum* challenge 120 days post-vaccination.

Materials and methods

Fish

Fingerling hybrid striped bass (*Morone saxatilis* x *M. chrysops*) were obtained from a commercial supplier and housed at the Aquatic Medicine Laboratory of the VMRCVM in Blacksburg, Virginia. Fish were maintained in multiple 2272 liter recirculation aquaculture systems with appropriate biological filtration and aeration.

Water temperature was maintained at approximately 24-26°C, and fish were fed daily at 3-5% body weight (41% protein; AquaMax; Purina, St. Louis, MO). Water quality parameters (ammonia, nitrite, nitrate, and pH) were monitored daily using a water analysis kit (HACH Co., Loveland, CO). Care of animals was in compliance with the guidelines of the Institutional Animal Care and Use Committee of the VPI&SU.

DNA construct preparation

Mycobacterium marinum (ATCC no. 927) was grown in Middlebrook 7H9 broth (Difco, Detroit, MI) for 9 days at 28°C (Wolf and Smith, 1999). Genomic DNA was isolated from *M. marinum* based on the methods of Whipple *et al*. (1987) and the gene for the Ag85A (*fbpA*) was then amplified by PCR. Ready-To-Go PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ) were used for the PCR, and amplification was performed using an Omni Gene thermocycler (Hybaid Limited, Franklin, MA) at 95°C for 5 minutes, followed by 30 cycles of 1 minute of denaturation at 95°C, 1 minute of annealing at 60°C, and 1 minute of extension at 72°C. Amplification products were analyzed on 1.5% agarose gels (Vemulapalli et al., 2002), and reaction products of the predicted size were subcloned into an expression vector, pcDNA 3.1 (Invitrogen, San Diego, CA) to create the pCMV-85A construct.

The resulting recombinant pCMV-85A plasmid was then transformed in *E. coli* TOP10 chemically-competent cells (Invitrogen). Samples from this transformation were spread on Luria-Bertani (LB) agar plates with ampicillin (100 µg/ml) and X-gal (1.6 mg) and grown overnight at 37°C. White colonies were chosen and grown overnight at 37°C in LB broth with ampicillin (100 µg/ml). Plasmids were isolated using Plasmid Maxi and Mega Kits according to the manufacturers instructions (Qiagen, Valencia, CA). The DNA was then washed with 70% ethanol, dried, and resuspended in 30 µl of 10 mM Tris hydrochloride-1 mM EDTA. Clones were screened for the *M. marinum* Ag85A gene by DNA sequencing (GenBank accession no. AY225215), and final DNA concentrations in solution were determined by absorption spectrophotometry (UV-1201 Spectrophotometer; Shimadzu, Santa Clara, CA).

DNA sequencing

Nucleotide sequencing of the *M. marinum* Ag85A gene was determined at the Core Laboratory Facility, Virginia Bioinformatics Institute, VPI&SU, using an Applied Biosystems Inc. model 377 automated DNA sequencer (ABI, Foster City, CA). Sequencing reactions were performed using the pCMV-85A plasmid template, T7 sequencing primer (Invitrogen), and ABI Big Dye Terminator ready reaction kit. Reactions were purified using Millipore Multiscreen HV plates (Millipore, Bedford, MA), dried, and resuspended as per manufacturer's protocols. LaserGene sequence analysis software (DNASTAR Inc., Madison, WI) was used for nucleotide sequence analysis.

Fish vaccination

Hybrid striped bass were separated into groups and given i.m. injections in the hypaxial muscle. The vaccination groups consisted of fish injected with different doses of the pCMV-85A construct (25 µg or 50 µg groups) on Day 0 and 14. As the control groups, other fish were vaccinated with 25 µg empty pcDNA 3.1 plasmid (pCMV-0 group) or sham-vaccinated with sterile PBS (Saline group). Vaccine doses were contained in 0.20 ml of sterile PBS and injected with a 1 ml syringe and a 27 gauge needle.

Live bacterial challenge

On Day 120 post-vaccination, fish from each control group (Saline and pCMV-0) and vaccination group (25 µg and 50 µg pCMV-85A) were challenged with live *M. marinum* (ATCC no. 927). Fish were identified and arbitrarily distributed into three 150 gallon tanks inside an isolation facility. Challenge was performed by i.m. injection with approximately 8 x 10^5 cfu *M. marinum*/g body weight of fish, a dose designed to cause onset of mortality at approximately 15 days. The isolate was grown in Middlebrook 7H9 broth at 28°C for 15 days. The resulting bacteria sample was briefly sonicated to break up bacterial aggregates, washed three times in PBS, resuspended in PBS, and the concentration of bacteria estimated by absorption spectrophotometry and confimed by

plate counts. Fish were injected with the processed bacterial solution in the dorsal musculature just ventral to the dorsal fin.

During the study, morbid or dead fish were removed immediately and recorded. Vaccine efficiency during the study was defined as:

(1- % mortality vaccinated fish/ % mortality Saline control fish) X 100

This number compared the relative ability of different vaccine doses (25 µg pCMV-0, 25 µg pCMV-85A, and 50 µg pCMV-85A) to reduce mortality after live bacterial challenge.

Immune responses of vaccinated fish after live bacterial challenge

On Day 42 post-vaccination and on Day 0, 7, 14, 21, and 35 post-challenge, a subsample of fish from each available group were anesthetized with tricaine methanesulfonate (MS-222; Sigma Chemical Co., St. Louis, MO) and blood samples taken for ELISA and lymphoproliferative assays.

Production of recombinant Mycobacterium marinum Ag85A fusion protein

Recombinant Ag85A was generated for the ELISA and lymphoproliferative assay using the methods of Vemulapalli et al. (2002). Briefly, PCR was utilized to amplify the open reading frame of the *M. marinum fbp*A. The resulting fragment was cloned into pCR2.1 vector (Invitrogen) and then subcloned into the *Eco*RI and *Xba*I sites of the pMalC2 vector (New England Biolabs, Beverly, MA). Ag85A was expressed in *E. coli* (GIBCO-BRL, Carlsbad, CA) as a fusion with maltose-binding protein. The fusion protein was purified by affinity chromatography (New England Biolabs), lipopolysaccharide was removed using polymyxin B beads (Affi-Prep Polymyxin support; BioRad Laboratories, Hercules, CA) and the protein was dialyzed with PBS. Aliquots of the purified protein were stored at –70°C until use.

ELISA

The antibody responses of fish from each group were evaluated for the presence of specific immunoglobulin against the *M. marinum* Ag85A using an indirect ELISA (Vemulapalli et al., 2002; Pasnik et al., 2003a). The *M. marinum* Ag85A was diluted to a 1 µg/50 µl concentration in bicarbonate coating buffer (pH 9.6) and the solution used to

coat polystyrene plates (Corning, Corning, NY) with 50 µl/well. The plates were incubated at 4°C overnight, washed four times with wash buffer (Tris-buffered saline [TBS] at pH 7.4, 0.05% Tween 20), and blocked with 2% BSA in TBS for 2 hours at room temperature (22°C). The blocking solution was then removed and diluted fish serum samples (1:100 dilution in blocking solution) were added to individual triplicate wells at 100 µl /well. A positive control serum sample and a diluent only sample were tested in the same manner. The plates were incubated for 4 hours at room temperature and then washed four times with wash buffer.

The secondary antibody solution, a protein A-peroxidase conjugate (Sigma), was added at 100 µl/well at a 1:500 dilution. After 1 hour incubation at room temperature, the plates were washed four times and 100 µl of substrate solution (TMB Microwell peroxidase substrate; Kirkegaard & Perry Laboratories, Gaithersburg, MD) added to each well. After 20 minutes incubation at room temperature, 100 µl of stop solution (0.185 M sulfuric acid) was added. The absorbance at 450 nm was then recorded with a microplate reader (Molecular Devices, Menlo Park, CA) and each sample serum mean absorbance compared against the control well mean absorbance.

Lymphoproliferative assay

Peripheral blood lymphocytes of fish from each group were evaluated for the presence of specific immunoglobulin against the *M. marinum* Ag85A and ConA using a lymphoproliferative assay (Marsden et al., 1996; Pasnik et al., 2003a). Blood from fish in each control and vaccination group was collected and diluted with sterile RPMI-1640 medium (Mediatech, Cellgro, Herndon, VA). The resulting cell suspension was washed twice in RPMI-1640 medium and resuspended in 3 ml RPMI-1640 medium. Using aseptic technique, phagocytes were placed over the Lymphoprep separation medium (1.077; Nycomed, Norway) and centrifuged at 400 x *g*. The buffy coat layer was collected and washed twice in cold RPMI-1640 medium, resuspended in 3 ml medium, and enumerated and size-analyzed with a CASY 1 model TTC cell counter and analyzer system (Scharfe System GmbH, Germany). The cells were then adjusted to 1 x 10^6 cells/ml with RPMI-1640 medium supplemented with 2 mM L-glutamine, 10% heat-inactivated FBS (Sigma), 50 IU/ml penicillin, and 50 mg/ml streptomycin (Cellgro).

Aliquots of cells were cultured in triplicate in 96-well plates (Corning) with supplemented 1640-RPMI medium containing 1.0 µg *M. marinum* Ag85A, 1.25 µg ConA (positive control; Sigma), or no additives (negative control) (Ahmed et al., 1994; Gogal et al., 1999; Vemulapalli et al., 2002). The cells were cultured at 28°C in 5% CO_2 for 24 hours. After 24 hours, 20 µl Alamar Blue (Trek Diagnostic, Westlake, OH) was added to the wells, and the cells were incubated for an additional 48 hours. Absorbance was then measured at 570 nm and 600 nm with a kinetic microplate reader (Molecular Devices), and specific absorbance of the unstimulated cells (negative control) was subtracted from the specific absorbance of the cells to yield a delta-specific absorbance.

Splenic bacterial counts

On Day 14 and 28 post-challenge (134 and 148 days post-vaccination), samples of unchallenged and challenged fish were killed by MS-222 overdose and the spleen aseptically removed. A 50 mg sample of each spleen was homogenized in 10 ml of sterile PBS, serially diluted 1:1000, and plated on Middlebrook 7H10 agar. Colonies of bacteria were counted after incubation for 10 days at 28°C and confirmed as *M. marinum* by PCR.

Histopathologic examination of granuloma formation

Histopathologic lesions of selected internal organs were studied after challenge based on the methods of Talaat et al. (1998) and Wolf and Smith (1999). Tissues of five fish from each available control and vaccination group were sampled on Day 14 and 28 post-challenge and immersed in 10% neutral buffered formalin for a period of at least 72 hours. Samples of the spleen, posterior kidney, liver, and heart were routinely processed for paraffin embedding. Tissue sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy.

Visceral granulomas in each tissue were counted and compared using 5 random 20x fields. A granuloma was defined as an aggregate of approximately 20 or more activated macrophages, with various amounts of central necrosis, peripheral fibrous connective tissue, or a base of mature lymphocytes (Wolf and Smith 1999). A granuloma count was determined for each sampled tissue from each sampled fish; this count was

then utilized to establish a mean granuloma count for each tissue from each control and vaccination group.

Statistical analysis

Statistical analysis was performed with SAS software (SAS Institute, Cary, NC). Splenic bacterial count data was subjected to pairwise comparisons using the Log-Rank test, and the results were Bonferroni corrected for the number of comparisons. The ELISA data was subjected to a mixed model repeated measures analysis of variance, while the lymphocyte proliferation was subjected to one-way analysis of variance; Bonferroni correction was performed to compare the various experimental dose groups within each sampling day. The data for each test was reported as means with S.E.M. determined from pooled means. An overall significance level of $p < 0.05$ was accepted.

Results

Protective effects against live bacterial challenge

Vaccinated fish were exposed to live *M. marinum* at a dose designed to cause the onset of mortality at approximately 15 days post-challenge. When fish were challenged 120 days post-vaccination, the Saline control and pCMV-0 group fish experienced onset of mortalities early in the experiment, reaching 100% mortality within 21 days (Fig. 1). In contrast, the 25 and 50 μg groups showed a considerably delayed onset of mortality, with the first mortalities on Day 23 and 25, respectively. The vaccination groups also exhibited a significantly increased mean survival times (Table 1), though all of the fish in the vaccination groups eventually died within 48 days. After the first mortalities in each group, all of the groups experienced a fairly rapid progression of disease to 100% mortality. However, while all of the fish in the control groups died within approximately 15 days of the onset of mortalities, the fish in the vaccinated groups died within approximately 25 days of the first mortalities.

Splenic bacterial cultures were obtained on Day 14 and 28 post-challenge and incubated for 10 days. On Day 14 post-challenge, the control groups exhibited significantly higher mycobacterial growth in the spleen samples than either the 25 μg or

50 μg pCMV-85A groups. The relative percent survival on Day 14 post-challenge for the control groups were 64% each, while the relative percent survival for the vaccination groups were 100% each. Later, the relative percent survival on Day 28 post-challenge showed vaccine efficacies for the 25 μg and 50 μg groups at 61% and 55%, respectively, compared to 0% for the other groups (Table 2). On Day 28 post-challenge, splenic samples from the 25 μg and 50 μg groups also indicated increased growth of bacteria within the fish, though these bacterial counts were still not as high as the bacterial counts from the control groups on Day 14 post-challenge.

The histopathology of the challenged fish indicated rapidly progressing granuloma formation in the control fish. The inflammatory reaction was characterized by formation of discrete non-necrotizing and necrotizing granulomas with associated macrophages, lymphocytes, bacteria, and connective tissue. When fish were examined on Day 14 post-challenge, the control groups exhibited significant numbers of granulomas within the spleen, posterior kidney, liver, and heart (Table 3). The spleens of the control groups were most affected, and mean splenic granuloma counts for the Saline and pCMV-0 groups were 13.6 and 15.2, respectively. Furthermore, the numerous splenic granulomas almost obliterated the normal tissue architecture (Fig. 2A). The splenic tissues of the 25 μg and 50 μg groups exhibited only a low number of granulomas and appeared largely unaffected; mean splenic scores for the 25 μg and 50 μg groups were 1.0 and 0.3, respectively, and were significantly lower than those of the control groups (Fig. 2B). However, on Day 28 post-challenge, the 25 μg and 50 μg groups had developed numerous granulomas with mean granuloma counts of all sampled tissues approaching the granuloma counts of the control groups on Day 14 post-challenge (Fig. 2C).

Ag85A-specific antibody production

The concentration of *M. marinum* Ag85A-specific antibodies were measured by ELISA at an optical density of 450 nm. On Day 42 post-vaccination, the control groups exhibited low concentrations of specific antibodies (Saline – 0.005 +/- 0.011; pCMV-0 – 0.011 +/- 0.011). Meanwhile, the vaccination groups showed significant levels of antibodies (25 μg – 0.469 +/- 0.011; 50 μg – 0.487 +/- 0.011). On Day 0 post-challenge

(Day 120 post-vaccination), negligible levels of specific antibodies were detected in all pCMV-85A vaccinated fish (Fig. 3). However, rapidly increasing concentrations of antibodies were generated by the vaccination groups up to Day 28 post-challenge. Significantly greater responses were seen among the vaccination groups than the control groups, though there were no significant differences between the two vaccination groups. After Day 28 post-challenge, the concentrations of antibodies began to peak, but fish continued to maintain elevated levels of antibodies. Specific antibody responses were detected in the Saline and pCMV-0 and groups after challenge, though these specific responses were minimal and increased slowly.

Lymphoproliferative responses

Anterior kidney lymphocytes were cultured *in vitro* in the presence of ConA (Fig. 4) or Ag85A (Fig. 5). On Day 42 post-vaccination, ConA-stimulated cells from the vaccination groups (25 μg – 0.504 +/- 0.018; 50 μg – 0.538 +/- 0.018) demonstrated significantly increased proliferation above those of the control groups (Saline – 0.372 +/- 0.018; pCMV-0 – 0.332 +/- 0.018). This significant difference was diminished on Day 0 post-challenge (Day 120 post-vaccination). After live bacterial challenge, all of the groups still exhibited proliferative lymphocyte responses after ConA stimulation. However, the 25 μg and 50 μg groups again demonstrated significant responses above those of the control groups, and these responses continued to increase after the controls experienced expected 100% mortalities.

On Day 42 post-vaccination, Ag85A-stimulated cells from the vaccination groups (25 μg – 0.368 +/- 0.012; 50 μg – 0.431 +/- 0.012) showed significantly increased proliferation above those of the control groups (Saline – 0.001 +/- 0.012; pCMV-0 – 0.002 +/- 0.012). This significant difference was diminished on Day 0 post-challenge (Day 120 post-vaccination). Following live bacterial challenge, significantly increased lymphoproliferative responses were once again detected among the vaccination groups on Day 7 post-challenge. Both the 25 μg and 50 μg groups demonstrated significant lymphocyte responses that continued to rapidly increase until Day 21 post-vaccination. Minimal antigen-specific lymphoproliferative responses were detected in the Saline and pCMV-0 control groups after challenge with *M. marinum*.

Discussion

Treatment of diseased fish is difficult, partially because only two antibiotics are currently approved by the Food and Drug Administration (FDA) and available for use in foodfish in the U.S.A. (Center for Veterinary Medicine, 2000). Neither antibiotic is licensed by the FDA for use against piscine mycobacteriosis, and in fact, no chemotherapeutic is licensed for the treatment of *Mycobacterium* spp. in tropical, ornamental or foodfish. Therefore, the prevention of mycobacteriosis is the only current means of controlling the disease, and prevention in fish has historically been limited to husbandry considerations. Despite potentially significant financial losses due to piscine mycobacteriosis, no effective treatments or vaccines have been developed for this disease in fish.

Our laboratory has studied the development of immunity to piscine mycobacteriosis and past experiments have indicated that the *M. marinum* Ag85A can be significantly immunostimulatory and/or protective against *M. marinum* infection (Pasnik et al., 2003a; Pasnik et al., 2003b). The study presented here confirms the protective effects by the pCMV-85A construct and indicates the importance of Ag85A-specific immune responses generated after challenge. Vaccinated fish (25 µg and 50 µg groups) exhibited very low levels of specific responses at Day 0 post-challenge (Day 120 post-vaccination), suggesting that fish were not protected by preexisting elevated levels of immune responses induced by vaccination. Instead, the protective effects were most likely conferred by immunologic memory and the rapidly increasing specific immune responses post-challenge. These responses included rapidly increasing antibody concentrations and lymphocyte responsiveness. The control groups (Saline and pCMV-0 groups) exhibited only minimal, slowly-increasing specific responses, which themselves were putatively generated by the *M. marinum* challenge. Furthermore, only a slight increase in non-specific lymphocyte stimulation was noted after ConA treatment and no protective effects were demonstrated among the control groups.

Mortality patterns post-challenge indicated protection based on delayed onset of mortalities and increased relative percent survival among the 25 µg and 50 µg groups.

By Day 22 post-challenge, both control groups had reached 100% moralities, while mortalities among the vaccine groups were 0%. However, the protective effect was diminished though still significant at Day 35, and the 25 μg and 50 μg groups showed relative percent survival of 61% and 55%, respectively. These findings indicate a significant protective effect of the DNA vaccine.

Previous studies by other researchers have shown similar protection in fish following DNA vaccine administration. DNA vaccines for viral piscine pathogens are well-examined and indicate that plasmid constructs encoding certain antigens are capable of inducing significant numbers of virus neutralizing antibodies and reducing mortalities among vaccinated fish populations after live viral challenge (Boudinot et al., 1998; Lorenzen et al., 1998; Nusbaum et al., 2002). Some of these studies have also shown that the efficacy of a DNA vaccine depends on the utilized encoded antigen. For example, Anderson et al. (1996) determined that a DNA vaccine encoding the infectious hematopoietic necrosis virus (IHNV) nucleoprotein was not immunostimulatory or protective; however, the vaccine construct encoding the IHNV glycoprotein conferred significant protection against live IHNV challenge. DNA vaccines against bacterial piscine pathogens have not been largely examined, though Gomez-Chiarri et al. (1996) were able to generate significant protection with DNA vaccines against *Renibacterium salmoninarum*. While these studies offer promising results, the long-term efficacies of many of these DNA vaccines have not been assessed to date.

Previous study in our laboratory found that a recombinant vaccine expressing a mammalian *Mycobacterium* sp. Ag85A was significantly immunostimulatory in *Morone* sp., but no protective effects were conferred when fish were challenged with a high dose of *M. marinum* (Pasnik et al, 2003a). In the study presented here, significant protection was most likely conferred due to the utilization of a DNA vaccine as the method of delivery for the *M. marinum* Ag85A. However, the data from this study also suggests that the vaccine only delays the pathogenesis of the disease. This conclusion was supported by the splenic bacterial counts and histopathology evaluations. On Day 14 post-challenge, splenic bacterial counts for the vaccination groups were significantly lower than the counts for the control groups. Futhermore, tissue samples from this time point indicated a clear difference in mean granuloma counts between the vaccination and

the control groups. The bacterial counts and degree of granulomatous inflammation roughly corresponded to the percent mortalities among the groups; the Saline and pCMV-0 groups exhibited high splenic bacterial growth, significant lesion development, and high percent mortalities, while the vaccination groups had low bacterial growth, minimal lesion development, and 0% mortalities. However, at Day 28, samples of the vaccination groups exhibited bacterial growth and lesions similar in magnitude and severity to those of the control groups at Day 14. Though the 25 µg and 50 µg groups had only 9% and 12% mortalities, respectively, at Day 28, the increasing bacterial counts and developing granulomas could account for the subsequent increase in mortalities.

The high challenge dose of 8×10^5 cfu *M. marinum*/g body weight fish i.m. was designed to cause acute mortalities among naïve fish (Wolf and Smith, 1999). The vaccine-induced specific immune responses may hinder the dissemination and/or replication of the *M. marinum* in the host, but these responses only appear to delay development of clinical signs and mortalities instead of eliminating the disease. Long-term immunity to the administered *M. marinum* may have been deterred by immunosuppression of the fish and/or by the unnatural high bacterial challenge dose. In the study presented here, the high *M. marinum* dose may have ultimately overwhelmed the induced immune responses or immunosuppressed the fish in the vaccination groups, allowing for unhindered disease development. Immunosuppression is an important factor in the development of mycobacteriosis (Talaat et al., 1998; Gauthier et al., 2003). Cellular components of *Mycobacterium* spp. are known to be immunosuppressive themselves (Geijtenbeek et al., 2003), thereby encumbering immunity. These factors may have prevented long-term vaccine-induced protection against the experimental high dose exposures and allowed the eventual development of 100% mortalities in the vaccination groups. However, fish would most likely be naturally exposed to mycobacterial organisms at lower levels. As such, the vaccine produced as a result of this research may confer better protective effects against a lower dose by inducing immune responses capable of controlling environmentally relevant numbers of mycobacteria.

Objectives Met

The new DNA vaccine (pCMV-85A) was determined to rapidly stimulate specific humoral and cellular immune responses after live *M. marinum* challenge. The protective effects of the vaccine were limited, and splenic bacterial counts, granuloma numbers, and mortality rates eventually reached levels close to those of the control groups.

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Table 1. Comparisons of mean survival, percent survival, and splenic bacterial counts on Day 14 post-challenge following live bacterial challenge of pCVM-85A-vaccinated hybrid striped bass (*Morone saxatilis* x *M. chrysops*).

Vaccination Group	Mean Survival Time in Days	Mean Colony-Forming Units in log (*M. marinum* CFUs)	RPS
Saline	15.3+/-0.68	5.0690+/-0.09732[a]	N/A
pCMV-0	14.6+/-0.65	5.1495+/-0.09732[a]	63
25 µg	37.7+/-1.12	3.8122+/-0.09732[b]	100
50 µg	36.9+/-1.09	3.8215+/-0.09732[b]	100

Significant differences ($p < 0.05$) among groups were determined according to Bonferroni corrected data and are indicated on the table by different alphanumeric letters.

Relative percent survival = (1- % mortality vaccinated fish/ % mortality Saline control fish) X 100

Table 2. Comparisons of mean survival, percent survival, and splenic bacterial counts on Day 28 post-challenge following live bacterial challenge of pCMV-85A-vaccinated hybrid striped bass (*Morone saxatilis* x *M. chrysops*).

Vaccination Group*	Mean Survival Time in Days	Mean Colony-Forming Units in log (*M. marinum* CFUs)	RPS
25 µg	37.7+/-1.12	4.7227+/-0.05385	91
50 µg	36.9+/-1.09	4.6709+/-0.05385	88

*All fish in Saline and pCMV-0 control groups were dead by Day 28 post-challenge and are not included.

No significant differences ($p < 0.05$) among vaccination groups were found according to Bonferroni corrected data.

Relative percent survival = (1- % mortality vaccinated fish/ % mortality Saline control fish) X 100

Table 3. Comparison of mean granuloma counts following live bacterial challenge of pCMV-85A-vaccinated hybrid striped bass (*Morone saxatilis* x *M. chrysops*).

Vaccination Group	Day Post-Challenge	Mean Granuloma Count			
		Spleen	Posterior Kidney	Liver	Heart
Saline	14	13.6	4.2	2.6	4.2
pCMV-0	14	15.2	5.1	2.4	4.5
25 µg	14	1*	0.1*	0*	0*
50 µg	14	0.3*	0*	0*	0*
25 µg	28	12	2.5*	1.7	0.2*
50 µg	28	12.6	4	1.6	2.1

All fish in Saline and pCMV-0 control groups were dead by Day 28 post-challenge.

*Significant differences ($p < 0.05$) between groups and Saline control are indicated for each sampled internal organ. The mean granuloma counts for the vaccination groups on Day 28 were compared to those of the Saline control group on Day 14.

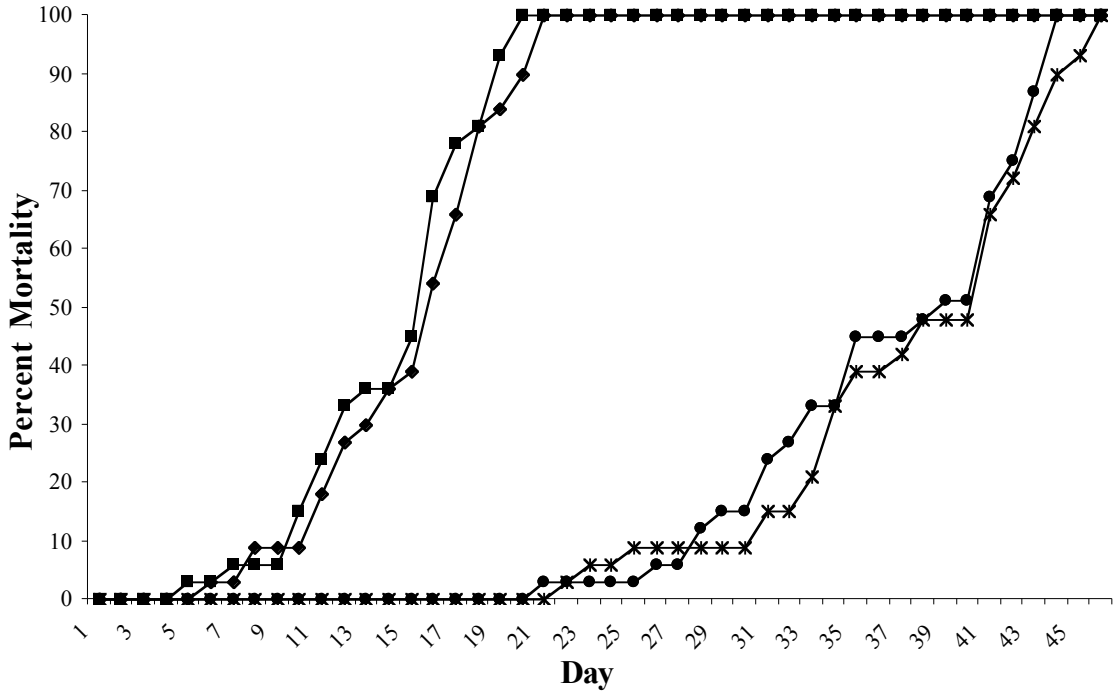

Fig. 1. Cumulative mortality for pCMV-85A-vaccinated hybrid striped bass (*Morone saxatilis* x *M. chrysops*) following challenge with approximately 8 x 10^5 cfu *M. marinum*/g body weight of fish on Day 120 post-vaccination. Groups included were: Saline control (diamond), pCMV-0 (square), 25 µg pCMV-85A (asterisk), and 50 µg pCMV-85A (circle).

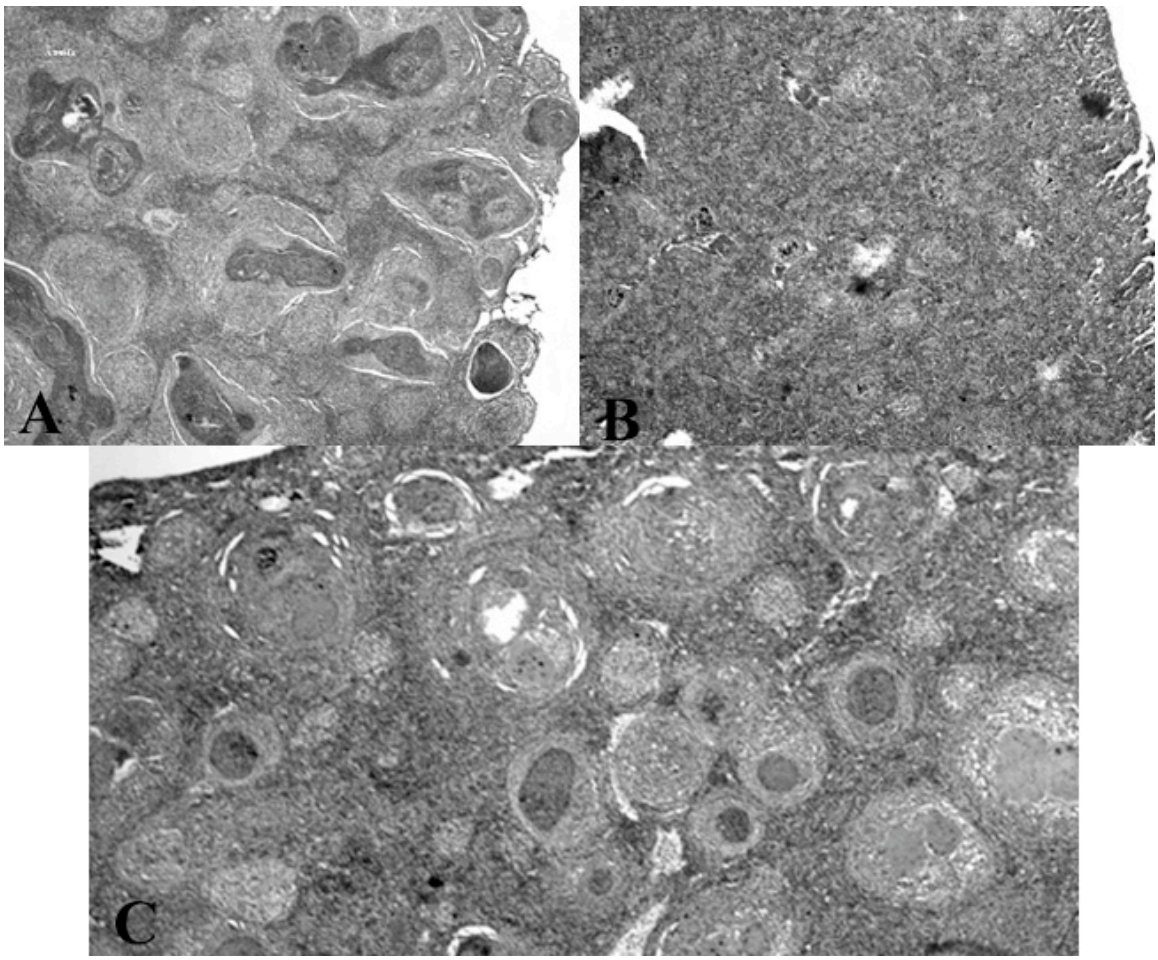

Fig. 2. Histopathology of typical lesions observed in hybrid striped bass (*Morone saxatilis* x *M. chrysops*) challenged with approximately 8 x 10^5 cfu *M. marinum*/g body weight of fish on Day 120 post-vaccination. (A) Splenic tissue from Saline control fish exhibiting characteristic extensive granuloma formation on Day 14 post-challenge. (B) Representative splenic section from pCMV-85A-vaccinated fish showing rare granuloma formation and relatively normal tissue on Day 14 post-challenge. (C) Splenic section from pCMV-85A-vaccinated fish exhibiting extensive granuloma formation on Day 28 post-challenge. H&E stains with 200x magnification.

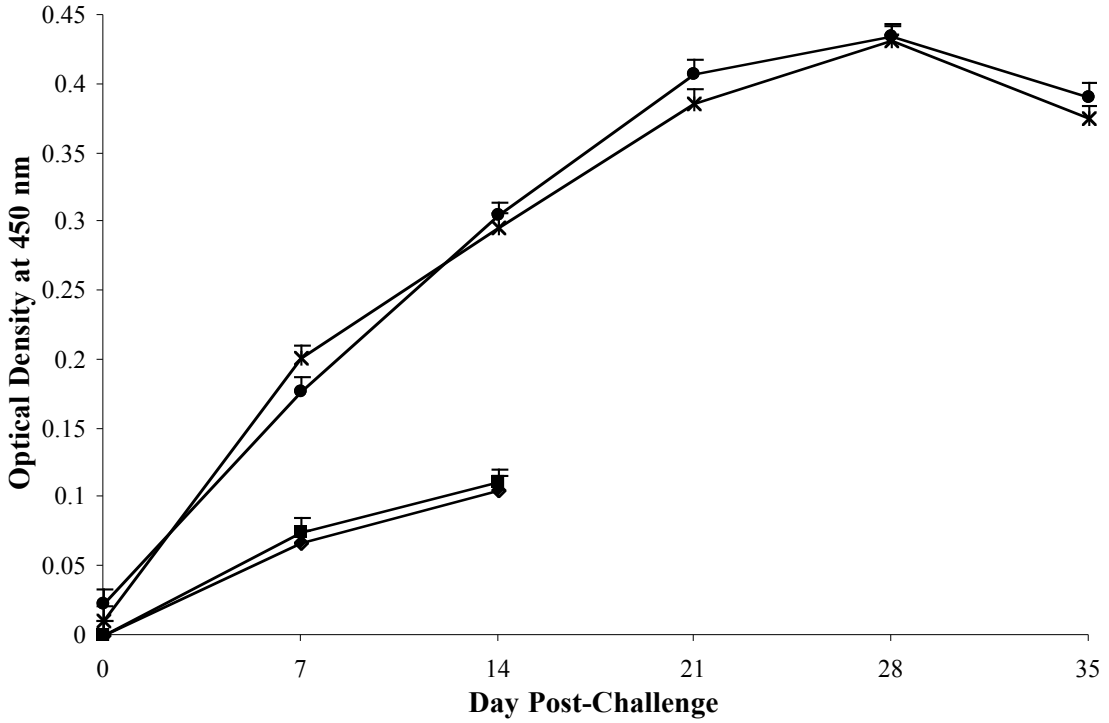

Fig. 3. Detection of Ag85A-specific antibodies by ELISA from serum of pCMV-85A-vaccinated, *M. marinum*-challenged hybrid striped bass (*Morone saxatilis* x *M. chrysops*). Sera were collected from available vaccine groups on Days 0, 7, 14, 21, 28, and 35 post-challenge, diluted 1:100, and assayed for the presence of antibodies to *M. marinum* Ag85A using absorption spectrophotometry. Groups included were: Saline control (diamond), pCMV-0 (square), 25 µg pCMV-85A (asterisk), and 50 µg pCMV-85A (circle). Both control groups experienced expected 100% mortalities during the experiment and thus samples were not available for all time points. Results are shown as the mean + S.E.M. of OD_{450} of the developed color. The pCVM-85A vaccination groups were significantly different ($p < 0.05$) than the control groups at all time points after Day 0 post-challenge, but no significant differences were noted between the 25 µg and 50 µg groups at any time point.

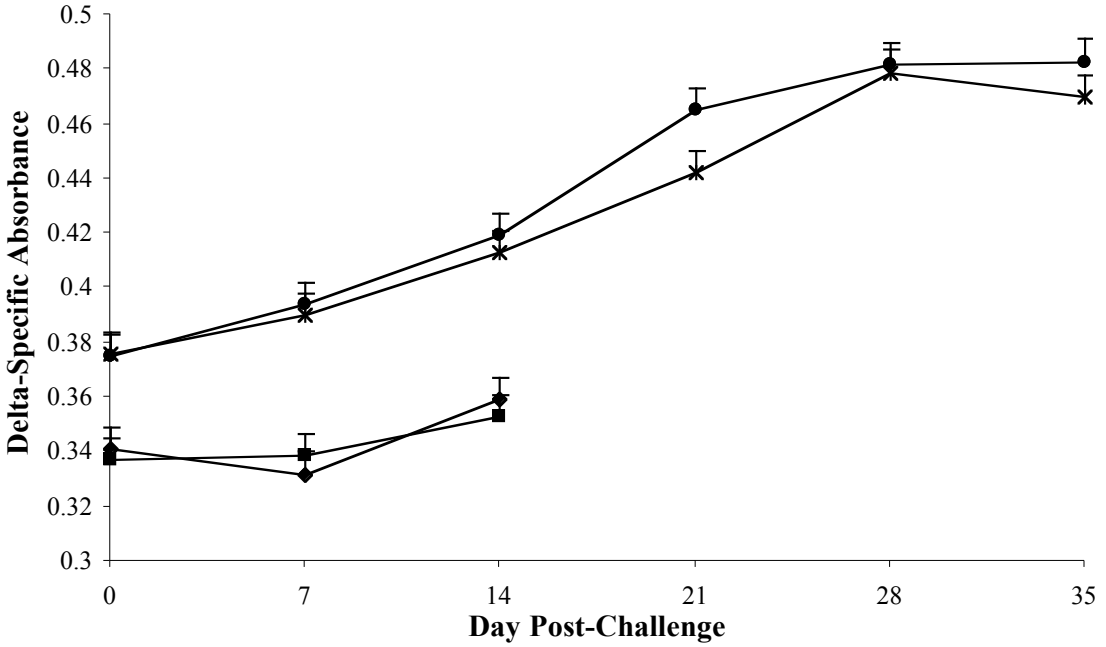

Fig. 4. Proliferation of anterior kidney lymphocytes from pCMV-85A-vaccinated, *M. marinum*-challenged hybrid striped bass (*Morone saxatilis* x *M. chrysops*) after *in vitro* stimulation with ConA. Cells were harvested from available vaccine groups on Day 0, 7, 14, 21, 28, and 35 post-challenge, cultured for 3 days, and lymphocyte proliferation determined by Alamar blue assay. Groups included were: Saline control (diamond), pCMV-0 (square), 25 µg pCMV-85A (asterisk), and 50 µg pCMV-85A (circle). Both control groups experienced expected 100% mortalities during the experiment and thus samples were not available for all time points. Results are shown as the mean + S.E.M. of the delta-specific absorbance of the developed color. The pCVM-85A vaccination groups were significantly different ($p < 0.05$) than the control groups, though no significant differences were noted between the 25 µg and 50 µg groups.

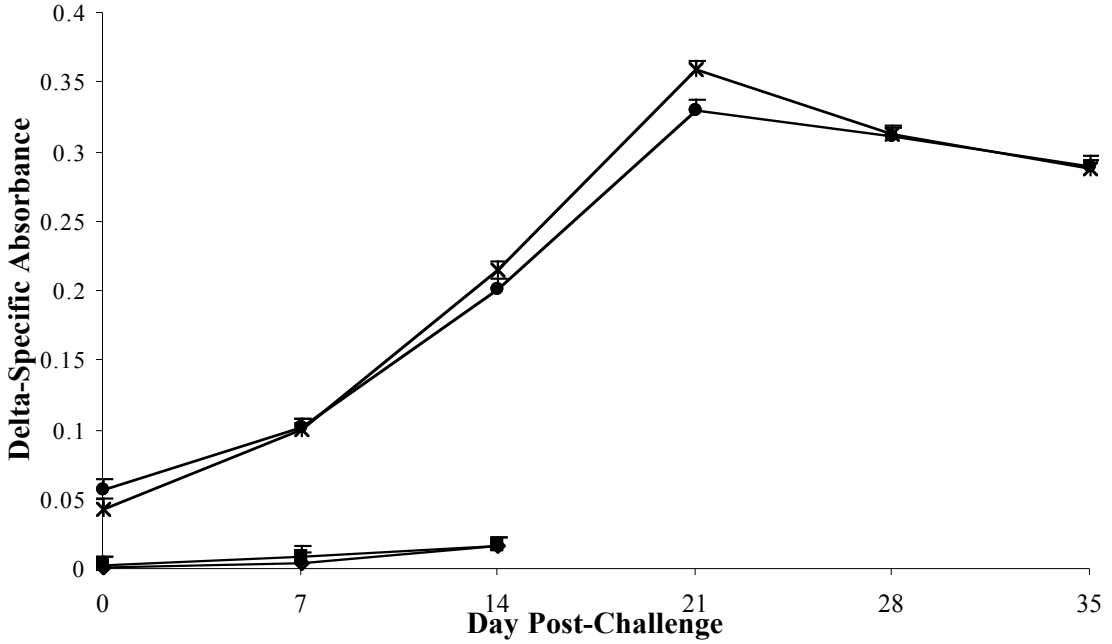

Fig. 5. Proliferation of anterior kidney lymphocytes from pCMV-85A-vaccinated, *M. marinum*-challenged hybrid striped bass (*Morone saxatilis* x *M. chrysops*) after *in vitro* stimulation with Ag85A. Cells were harvested from available vaccine groups on Day 0, 7, 14, 21, 28, and 35 post-challenge, cultured for 3 days, and lymphocyte proliferation determined by Alamar blue assay. Groups included were: Saline control (diamond), pCMV-0 (square), 25 µg pCMV-85A (asterisk), and 50 µg pCMV-85A (circle). Both control groups experienced expected 100% mortalities during the experiment and thus samples were not available for all time points. Results are shown as the mean + S.E.M. of the delta-specific absorbance of the developed color. The pCVM-85A vaccination groups were significantly different ($p < 0.05$) than the control groups, though no significant differences were noted between the 25 µg and 50 µg groups.