CHAPTER 3

Immunogenic and Protective Effects of a DNA Vaccine
for *Mycobacterium marinum*

Abstract

A DNA vaccine was constructed using the *Mycobacterium marinum* Ag85A gene, which was isolated and subcloned into a commercially-available eukaryotic expression vector. Juvenile hybrid striped bass (*Morone saxatilis* x *M. chrysops*) were immunized by i.m. and i.p. injection with the resulting construct and produced significant specific immune responses towards the Ag85A. Macrophage phagocytic and respiratory burst functions failed to exhibit upregulation after vaccination. Fish receiving the DNA vaccine developed protective responses to live bacterial challenge 90 days post-vaccination, as demonstrated by increased survival over control fish and by reduced splenic bacterial counts. Furthermore, immune responses and protective effects were significantly increased at higher vaccine doses using the i.m. injection route.
Keywords: *Mycobacterium* sp.; DNA vaccine; Fish; Vaccination; Immunity

Rationale

The previous study with RB51SOD/85A indicated that a vaccine expressing the Ag85A from *M. marinum* may be more appropriate for a piscine mycobacteriosis vaccine. This would preclude the argument that the *M. bovis* and *M. marinum* Ag85A did not have sufficient common epitopes to provide cross-protection. This study also utilized a new vaccine technology because of concerns about exposing fish to *Brucella abortus* even though it was killed by irradiation. In addition, the live *M. marinum* challenge dose was decreased, because there was concern that the previously employed dose was too high and may have overwhelmed the fish despite the stimulated immune responses. In the study here, a lower dose was used, though it still caused acute disease.

Introduction

Piscine mycobacteriosis is a common bacterial disease of fish caused by Gram-positive, acid-fast *Mycobacterium* spp., often *M. marinum*. This bacteria can cause a chronic progressive, often fatal disease, causing granulomatous inflammation in virtually

any tissue (Austin and Austin, 1993; Smith, 1997). Given its ubiquitous nature in water and sediment, the organism has been reported worldwide in over 160 species of fresh and saltwater fish (Chinabut, 1999). Presently, no chemotherapeutic agents are approved for treatment of fish mycobacteriosis in the United States. Because of the difficult management of aquatic mycobacteriosis and due to its human zoonotic potential, development of an effective vaccine against fish mycobacteria is vital.

There are numerous commercially-available bacterins for bacterial fish pathogens, yet none exists for aquatic mycobacteriosis (Center for Veterinary Biologics, 2001). However, the potential for a vaccine against piscine mycobacteria has been suggested previously. Bartos and Sommer (1981) elicited immune responses in rainbow trout, *Salmo gairdneri*, from i.p. immunization with *M. salmoniphilum* mixed with adjuvant. Chen et al. (1996) vaccinated rainbow trout with extracellular products from various aquatic *Mycobacterium* spp. and measured enhanced levels of reactive oxygen species, phagocytes, lysozyme, and antibodies produced against the mycobacterial components. Therefore, both nonspecific and specific immune responses could be elicited by immunization with aquatic mycobacteria. However, it was not reported whether these immune responses were sufficient for protection against disease.

The development of DNA vaccines for fish has been increasingly studied in recent years. DNA immunization is based on the introduction of plasmid DNA encoding a protective antigen into animal tissue, which is then able to express the plasmid-encoded protein and induce subsequent immune responses (Heppell et al., 1998). Much effort has been invested into this technology because DNA vaccines have multiple advantages over killed, attenuated, or subunit vaccines (Heppell and Davis, 2000). Indeed, DNA vaccines are known to stimulate both non-specific and specific immune responses without the need for live organisms, replicating vectors, or adjuvants (Tanghe et al., 2001). Antigen synthesis by DNA vaccination imitates natural infection by intracellular pathogens and leads to subsequent cell-mediated responses and ultimately the generation of memory lymphocyte responses (Donnelly et al., 1997). Additionally, DNA vaccines have already been shown to provide protection of fish to various intracellular pathogens, such as viral hemorrhagic septicemia and infectious hematopoietic necrosis virus (Boudinot et al., 1998; Lorenzen et al., 1998). Because these vaccines successfully induce protective

immune responses against intracellular pathogens, they strongly indicate that a DNA vaccine against *M. marinum* infection can be developed.

Multiple experimental vaccines have been constructed against mammalian mycobacteriosis using the Ag85A. This antigen is immunologically important, because the Ag85A is a major secreted fibronectin binding protein of *Mycobacterium* spp., including *M. marinum* (Ohara et al., 1997; Stinear et al., 2000), and the Ag85A induces immune responses and effective protection against several mycobacterial species (Velaz-Faircloth et al., 1999; Naito et al., 2000; Vemulapalli et al., 2002). Furthermore, injection of plasmid DNA encoding mammalian Ag85A results in specific antibody production and protective Th1-type immune responses against mammalian mycobacteriosis (Denis et al., 1998; Tanghe et al., 2001). The research presented here created an Ag85A DNA vaccine that stimulated protective immunity in hybrid striped bass against *M. marinum*.

Materials and methods

Fish

Hybrid striped bass (*Morone saxatilis* x *M. chrysops*), a proven *M. marinum*-susceptible species (Wolf and Smith, 1999), were used as the model to assess vaccine efficacy. Fingerling hybrid striped bass were obtained from a commercial supplier and housed at the Aquatic Medicine Laboratory of the VMRCVM in Blacksburg, Virginia. Ten arbitrarily-selected fish were euthanized by overdose with tricaine methanesulfonate (MS-222; Sigma Chemical Co., St. Louis, MO) and then necropsied, cultured, and submitted for histopathologic examination. This determined that the incoming fish were free of observable parasitic or bacterial disease.

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 the photoperiod consisted of 16 hours light/8 hours dark. Fish were fed daily at 3-5% body weight (41% protein; AquaMax; Purina, St. Louis, MO), and water quality parameters (ammonia, nitrite, nitrate, and pH) were monitored daily using a water analysis kit (HACH Co., Loveland, CO). Fish were acclimated for 5 months before initiation of the study and weighed approximately 40-50 grams at that

time. 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). Bacteria were incubated for 2 hours at 37°C in TEN (50 mM Tris hydrochloride, 100 mM EDTA, 150 mM NaCl) with 8,000 U of lipase (Sigma). Lysozyme (Sigma) was then added to achieve a concentration of 5 mg/ml, and samples were incubated for an additional 2 hours at 37°C. Sodium dodecyl sulfate and proteinase K were added to achieve a final concentration of 1% and 2 mg/ml, respectively, and samples were incubated for 16 hours at 50°C. After the addition of cold 5 M potassium acetate solution, samples were incubated on ice and then centrifuged at 4°C for 10 minutes. The supernatant was then drawn off and placed in microcentrifuge tubes. DNA from this fluid was extracted with the sequential addition of phenol-chloroform-isoamyl alcohol (25:24:1 vol/vol/vol), phenol, and then chloroform-isoamyl alcohol (24:1). Within each sequential step, the sample was inverted 20 times, microcentrifuged for a brief time, and aqueous phase retained. After the last step, 1/10 volume of cold 3 M sodium acetate and 2 volumes of absolute ethanol were added to the aqueous phase. DNA was precipitated with a 2 hour incubation at -20°C and collected by centrifugation. The pellet was washed with 70% ethanol, dried, and resuspended in 50 µl 10 mM Tris hydrochloride-1 mM EDTA. Final sample DNA concentration was estimated by absorption spectrophotometry (UV-1201 Spectrophotometer; Shimadzu, Santa Clara, CA), where 1 absorbance unit equaled 42 µg DNA per ml.

The gene for the Ag85A (*fbpA*) was then amplified by PCR from the *M. marinum* genomic DNA. A primer pair consisting of a forward primer (5' AAC CGG ATG CGC CTA AAT GAA TGA GG 3') and a reverse primer (5' CGC CGC CGT TAA TCG CTG TTC TGT TA 3') were utilized. The primers were selected such that the amplified fragment would contain the ribosomal binding site (RBS), open reading frame, and stop codon of the *fbpA*. Ready-To-Go PCR beads (Amersham Pharmacia Biotech,

Piscataway, NJ) were used for the PCR. 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 in 1XTBE buffer (Vemulapalli et al., 2002). Reaction products of the predicted size were subcloned into an expression vector, pcDNA 3.1 (Invitrogen, San Diego, CA), and the gene expressed under the cytomegalovirus (CMV) promoter to create the pCMV-85A construct (Fig. 1). The cloning reaction contained PCR product, 10 ng pcDNA 3.1 plasmid, 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 0.1 μg BSA, 50 mM NaCl, 2.5 mM $MgCl_2$, 10X ligation buffer, and 1 U DNA ligase (Promega, Madison, WI).

The resulting recombinant pCMV-85A plasmid was then transformed in *E. coli* TOP10 chemically-competent cells (Invitrogen). The plasmid solution was mixed with *E. coli* cells and incubated on ice for 30 minutes. Cells were heat-shocked for 30 seconds at 42°C, immediately transferred to ice, and then incubated on a shaker for 1 hour at 37°C in SOC medium (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). Briefly, the purification involved an alkaline lysis procedure, followed by purification on an anion-exchange resin. The DNA was eluted using a high salt buffer and then desalted and precipitated in isopropanol. 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 *Eco* RI restriction enzyme analysis (with agarose gel analysis) and DNA sequencing (GenBank accession no. AY225215). Final DNA concentrations in solution were determined by absorption spectrophotometry.

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). Cycle 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 the Millipore Multiscreen HV plates (Millipore, Bedford, MA) and dried and resuspended as per manufacturer's protocols for loading on the automated sequencer. 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 with various doses of the pCMV-85A construct (vaccination groups; 5 μg, 25 μg, or 50 μg i.m. groups). The efficacy of i.p. route of administration was also tested with another vaccination group given i.p. injections (25 μg i.p. group). As control groups, other fish were injected with 25 μg empty pcDNA 3.1 plasmid (pCMV-0 group) or sham-vaccinated with sterile PBS (Saline group). Because a pilot study determined two injections were significantly more immunostimulatory than one (data not shown), each group was vaccinated on Day 0 and again on Day 14. Doses were contained in 0.20 ml of sterile PBS and injected with a 1 ml syringe and a 27 gauge needle. Fish were sequestered in different tanks within the same recirculation system, and daily care was continued as previously described. A subsample of fish from each group were anesthetized (for bleeding) or euthanized (for harvesting of lymphocytes and macrophages) with MS-222 on Days 0, 14, 28, 42, 56, and 70.

Production of recombinant Mycobacterium marinum Ag85A fusion protein

Recombinant Ag85A was generated for 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 the 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). 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 the polystyrene plates 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. 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 (21°C) 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 absorbance compared against the control well mean absorbance.

Lymphoproliferative assay

Anterior kidney lymphocytes from fish in each control and vaccination group were aseptically collected by dissection and placed in a Petri dish containing 10 ml sterile RPMI-1640 medium (Mediatech, Cellgro, Herndon, VA). Cells were released by mechanical disruption using curved forceps and a metallic sieve screen (Sigma). 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 for 30 minutes. The buffy coat layer was collected and washed twice in cold RPMI-1640 medium, resuspended, 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 2mM L-glutamine, 10% heat-inactivated FBS (Sigma), and 50 IU/ml penicillin and 50 mg/ml streptomycin (Cellgro), and 1% non-essential amino acids (Cellgro). Aliquots of 100 μl of cells were cultured in triplicate in 96-well plates (Corning, Corning, NY) with 100 μl of 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 at 20 μl/well. The cells were incubated for an additional 48 hours, and then absorbance measured at 570 nm and 600 nm with a kinetic microplate reader (Molecular Devices).

This Alamar Blue assay was developed as a non-radioactive lymphocyte proliferation assay that indirectly measures cell proliferation (Ahmed et al., 1994, Gogal et al., 1999). The dye is added in an oxidized form (blue color) and is reduced (red color) with cell proliferation. The level of proliferation is indicated by the difference between the specific absorbance of the oxidized form (570 nm) and the reduced form (600 nm). The specific absorbance of the unstimulated cells (negative control) is subtracted from the specific absorbance of the cells to yield a delta-specific absorbance.

Cellular phagocytosis

Cellular phagocytosis and H_2O_2 production were assessed from fish in each control and vaccination group based on the techniques of Sakai et al. (1989) and Holladay et al. (1996). The anterior kidney was aseptically collected by dissection and placed in a culture dish containing 10 ml sterile PBS. Cells were released by mechanical disruption using a metallic sieve screen (Sigma). The resulting cell suspension were washed twice in cold, sterile PBS and resuspended. Using aseptic technique, phagocytes

were placed over the Lymphoprep separation medium (Nycomed) and centrifuged. Cells were washed twice in PBS, resuspended, and enumerated and size-analyzed with a CASY 1 model TTC cell counter and analyzer system (Scharfe System GmbH). The cells were then aliquoted in polystyrene round-bottom tubes at 0.5×10^6 cells/ml in 0.5 ml PBS. Fluoresbrite microspheres (1.0 µm; Polysciences, Warrington, PA) were washed twice in 3 ml PBS, resuspended in 200 µl PBS, and then sonicated on ice (Ultrasonic Cell Disrupter; Misonix, Farmingdale, NY) for 30 seconds at 35% to disrupt aggregated microspheres. A 10 µl volume of microspheres were then added to the tubes to give a ratio of 50 beads/cell. Fresh culture media was added to each tube to give a final volume of 5 ml/tube. After 18 hours of incubation at 28ºC at 5% CO_2, cells were analyzed by flow cytometry (See *Flow Cytometry* section). The measure of phagocytosis used for comparison among groups was total fluorescence within the gated population, which was directly proportional to the number of phagocytosed fluorescent beads by phagocytic cells contained within the electronic window.

Phagocyte H_2O_2 production

Cells from the anterior kidney were aseptically collected, prepared as previously described, and aliquoted in 12 x 75 mm polystyrene round-bottom tubes at 0.5×10^6 cells/ml in 0.5 ml sterile PBS. Cells were then incubated for 15 minutes at room temperature with 5 µl of dichlorofluorescin diacetate (DCF-DA; Molecular Probes, Eugene, OR). Following incubation, cells were stimulated with the addition of 10 µl phorbol-12-myristate 13-acetate (PMA; Sigma) with a subsequent 30 minute incubation period. Cells were then placed on ice or immediately analyzed by flow cytometry (See *Flow Cytometry* Section).

Flow cytometry

Flow cytometric analysis of cell phagocytic ability and phagocyte hydrogen peroxide production was performed using a Coulter EPICS V Flow Cytometer interfaced with an MDADS data analysis computer (Coulter Electronics). Standardization of the flow cytometer was performed using fluorescent calibration beads (Coulter, Hialeah, FL) with a 480 nm laser (Coherent, Palo Alto, CA) and excitation set at 300 mW. A

population of phagocytes was isolated on a two-parameter histogram of side angle light scatter (SALS) and forward angle light scatter (FALS) based on the granularity and cell size (respectively) of the cells. The fluorescent distribution was displayed as a 256 channel, single parameter histogram, and green fluorescence was measured through 525 band pass and 550 nm longpass dichromic filters. A 452-515 laser blocker was used to block laser light from other fluorescent measurements. A gated amplifier was used to exclude small particles and cellular debris from evaluation. For each sample, 5,000 events was recorded and analyzed.

Live bacterial challenge

On Day 90 post-vaccination, fish from each control group (Saline and pCMV-0) and vaccination group (5 μg, 25 μg, 50 μg i.m. and 25 μg i.p. pCMV-85A) were challenged with *M. marinum* (ATCC no. 927). Fish were identified and arbitrarily distributed into two 150 gallon tanks inside an isolation facility. Challenge was performed by i.m. injection with approximately 8×10^5 cfu *M. marinum*/g body weight of fish. Previous study by Wolf and Smith (1999) determined that the ATCC no. 927 isolate will cause onset of mortality at approximately 15 days when administered i.m. to *Morone* spp. at this dose. 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, resuspended in phosphate buffered saline (PBS), and the concentration of bacteria estimated by absorption spectrophotometry and confirmed by plate counts. Fish were injected i.m. with the bacterial solution in the dorsal musculature just ventral to the dorsal fin at the dose described previously.

Mortality study

During the study, any moribund or dead fish were removed immediately and recorded. All remaining fish were euthanized 36 days post-challenge at the end point of the experiment; this time point corresponded to twice the number of days from which the control groups reached the expected 100% mortality.

Vaccine efficiency on Day 36 post-challenge was defined as:

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

This number compared the relative ability of different vaccine doses to reduce mortality after live bacterial challenge.

Splenic bacterial counts

On Day 15 post-challenge, representative 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 to 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.

Statistical analysis

The ELISA and macrophage assay data were subjected to a mixed model repeated measures analysis of variance, and a Bonferroni correction was performed to compare the various experimental dose groups within each sampling day. The lymphocyte proliferation and live bacterial challenge data were subjected to one-way analysis of variance, while only the proliferative responses were subjected to Bonferroni correction. The splenic bacterial count data was stratified by vaccine group and subjected to pairwise comparisons using the Log-Rank test; the results were then Bonferroni corrected for the number of comparisons. The data for each test was reported as means with SEM determined from pooled means. An overall significance level of $p < 0.05$ was accepted.

Results

PCR results and cloning

With PCR using the previously described primers, a fragment of approximately 1,100 bp was visualized on agarose gels. This product was isolated, purified, and ligated into the pcDNA 3.1 plasmid (Fig. 1). Subsequent nucleotide sequencing of the gene revealed a 1,014 bp open reading frame containing the transcription region and stop codon (Fig. 2). Forward orientation in the plasmid was also determined by DNA sequencing, and this ensured that Ag85A gene expression would be driven by the CMV promoter.

Ag85A-specific antibody production

Low levels of antibodies specific for Ag85A were detected in all pCMV-85A vaccinated fish after two weeks (Fig. 3). Increasing concentrations of antibodies were generated up to Day 42 post-vaccination, with the greatest increase after booster vaccination on Day 14. Significantly greater responses were seen among the 25 µg and 50 µg i.m. groups than the 25 µg i.p. and 5 µg i.m. groups, though there were no significant differences between the former groups. After Day 42, the concentrations of antibodies detected began to decline, though fish maintained increased levels of antibodies through Day 70. No specific antibody responses were detected in the pCMV-0 or Saline groups.

Lymphoproliferative responses

Anterior kidney lymphocytes were cultured *in vitro* in the presence of ConA (Fig. 4) or Ag85A (Fig. 5). After ConA stimulation, all of the groups exhibited proliferative lymphocyte responses. However, the 25 µg and 50 µg i.m. groups demonstrated significant responses beyond that of the other vaccination and control groups. Following Ag85A stimulation, no significant lymphoproliferative responses were detected until Day 42 post-vaccination. On Days 42, 56, and 70, both the 25 µg and 50 µg i.m. groups demonstrated significant lymphocyte responses beyond that of the Saline control, pCMV-0, 5 µg i.m., and 25 µg i.p. groups. These responses were approximately two- to three-fold higher than that of the next highest group, the 25 µg i.p. group. No antigen-specific lymphoproliferative responses were detected in the pCMV-0 or Saline groups.

Macrophage phagocytosis and hydrogen peroxide production

Both the phagocytic (Fig. 6) and hydrogen peroxide production (Fig. 7) responses were equivocal among the different control and pCMV-85A vaccination groups, irrespective of dose or route of administration.

Protective effects against live bacterial challenge

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 90 days post-vaccination, the Saline control, pCMV-0, and 5 µg i.m. group fish experienced onset of mortalities early in the experiment, rapidly reaching expected 100% mortality (Fig. 8). In contrast, the 25 µg and 50 µg i.m. groups showed considerably delayed onset of mortality and suffered only 20% and 10% mortality, respectively, through 36 days post-challenge. Relative percent survival showed vaccine efficacies for the 25 µg and 50 µg i.m. groups were 80% and 90%, respectively, compared to 0% for the other groups (Table 1). The utilized route of administration again had a significant effect. The 25 µg i.p. group had a slightly delayed onset of mortality compared to the control groups, but reached a total of 80% mortality by 36 days post-challenge. This group experienced a low level of protection, experiencing a 20% relative percent survival rate over 36 days.

Splenic bacterial cultures were obtained on Day 15 post-challenge and incubated for 10 days. The control groups and the 5 µg i.m. group exhibited significantly higher mycobacterial growth in the spleen samples than the other groups. Furthermore, the 25 µg and 50 µg i.m. pCMV-85A groups had significantly lower mycobacterial growth than the 25 µg i.p. group.

Discussion

Because DNA vaccines have been efficacious in the prevention of mycobacterial diseases in mammals, this method of vaccine delivery is promising for use against piscine mycobacteriosis. This study demonstrated that a DNA vaccine encoding the *M. marinum* Ag85A elicited significant levels of protective Ag85A-specific immune responses, the degree of which were largely dose-dependant as well as route-dependant. Furthermore, the degree of immune responses elicited appeared roughly proportional to the amount of protection conferred.

Significant Ag85A-specific immune responses were stimulated by this vaccine, demonstrating that the *fbpA* gene was transcribed and Ag85A successfully expressed on fish cells. Other researchers have similarly shown that fish are able to express foreign

proteins encoded by an eukaryotic expression vector (Boudinot et al., 1998; Heppell and Davis, 2000). Furthermore, the pcDNA 3.1 eukaryotic vector utilized here has been used successfully in other DNA vaccine studies in fish (Heppell et al., 1998; Kanellos et al., 1999). Because the isolated *fbpA* gene contained the entire open reading frame and the stop codon, Ag85A were expressed on fish cells under the influence of the CMV promoter. The hybrid striped bass were then able to generate immune responses against the Ag85A.

Distinct differences were found in vaccine efficiency when comparing various vaccine doses. The Saline negative control, empty plasmid (pCMV-0), and 5 μg pCMV-85A i.m. groups failed to produce detectable specific immune responses beyond that of a negative control. Significant protective immune responses were generated in the 25 μg and 50 μg i.m. groups, with vaccine efficacies of 80% and 90%, respectively, through 36 days. No significant differences were noted between these two groups, and this may indicate that the 25 μg dose was more efficient than the 50 μg dose when considering overall protection and financial cost.

Upon comparison of the different routes of vaccination, it was evident that the i.m. route was significantly more immunostimulatory and protective than the i.p. route. In the 25 μg i.p. group, antibody responses, lymphocyte responses, and survival rates were approximately a third to a quarter that of the 25 μg i.m. group. Nonetheless, administration of 25 μg i.p. was still significantly more efficacious than 5 μg i.m. Yet in order for the i.p. injection route to provide equivalent protective effects as with i.m. injection route, a considerably higher dose or an adjuvant would probably have to be utilized. This mirrors the findings of other studies on fish DNA vaccines (Kanellos et al., 1999). Corbeil et al. (2000) proposed that transfection of somatic cells and antigen presenting cells happens more readily in muscle tissue than in the peritoneal cavity.

Though the specific immune responses varied predictably according to dose and route, a different effect was exhibited by the non-specific macrophage assays. Macrophage activation is known to be vital to proper eradication of *Mycobacterium* spp. in mammals (Govoni and Gros, 1998; Amer and Swanson, 2002). Other researchers have suggested similar importance in fish affected by aquatic mycobacteria. For example, Chen et al. (1996) found that macrophage function was upregulated after exposure to *M.*

marinum extracellular products, suggesting that macrophage function was important in the early, nonspecific immune response to this *Mycobacterium* sp. However, in the study presented here, the macrophage assays did not demonstrate increased phagocytic or respiratory burst functions after vaccination. Thus macrophage function does not appear to play an important role in protection after DNA vaccination. This finding was supported by data from the pCMV-0 group, which indicated that no protection was conferred by the empty vector despite the potential CpG immunostimulatory effects. Other researchers have suggested that the unmethylated CpG motifs found in the plasmid vector backbone are potent macrophage activators (Kanellos et al., 1999; Heppell and Davis, 2000), yet evidence of macrophage upregulation was not exhibited in this study. Based on these findings, it appears that the protection conferred after DNA vaccination can be related to the level of Ag85A-specific immune responses induced.

However, this conclusion is confounded by the fact that macrophages also act as accessory cells in the stimulation of specific immune responses. Macrophages phagocytose microorganisms and degrade them in a phagolysosome, subsequently presenting microorganism-derived peptides to helper T-cells (Secombes, 1996). But given the apparent lack of macrophage stimulation by this DNA vaccine, these findings may reveal that other antigen presenting cells (i.e. dendritic cells and B-cells) may play a more important role in antigen presentation and immunostimulation in DNA vaccinated fish than previously assumed. In mammals vaccinated with DNA vaccines, mature dentritic cells are known mediators of antigen processing and transport to lymphoid tissues for immune response stimulation (Donnelly et al., 2000; Beyer et al., 2001). Thus because dendritic cells are also crucial to Th1 response initiation in mammals challenged with *Mycobacterium* spp. (Demangel and Britton, 2000), these cells may help account for the protective effects of this DNA vaccine.

Because protective immunity against *Mycobacterium* spp. ultimately depends on specific humoral and cellular defenses (Naito et al., 2000; Vemulapalli et al., 2002), results of this study strongly suggest that both humoral and cellular responses were stimulated by the vaccine. We demonstrated that Ag85A-specific humoral responses could be generated with this DNA vaccine. Though antibodies react to extracellular antigens and pathogens, the cellular responses are required to destroy intracellular

pathogens. A significant lymphostimulatory effect was seen in cells from vaccinated fish exposed to Ag85A or a T-cell mitogen. Unfortunately, assays to further characterize cell-mediated immunity in hybrid striped bass are not readily available. However, given that *M. marinum* is a facultative intracellular pathogen, humoral responses alone are most likely not sufficient to confer protection against live bacterial challenge. Thus we believe that significant cellular immune responses must also be generated after vaccination with the pCMV-85A construct. Indeed, both DNA vaccination and Ag85A exposure lend themselves to induction of strong Thl helper T-cell responses, gamma interferon production, and CD8+ T-cell mediated cytotoxicity (Denis et al., 1998; Tanghe et al., 2001).

Mycobacterium spp. Ag85A is a potent immunostimulator, and the selection of extracellular antigens such as Ag85A may be vital for the success of vaccines against intracellular pathogens. In this study, protection was measured by reduced mortality and decreased growth of *M. marinum* within the spleens of immunized fish. Protective immune responses against the high challenge dose administered to these fish would have to be rapidly produced to reduce mortality and bacterial growth. Some of the rapid responses could be attributed to pre-existing immune factors (i.e. antibody titers) produced following vaccination itself. However, a more appropriate explanation is the immunization against a secreted product of *M. marinum*. Because mycobacteria are harbored within cells, they can avoid immune surveillance and destruction (Amer and Swanson, 2002). However, secreted proteins are readily processed by antigen presenting cells and presented for immune system detection. Thus these proteins play a role in early detection of infection, even before the breakdown of the mycobacterial cells themselves (Horwitz et al., 1995). Therefore, secreted proteins like Ag85A are crucial to early immunostimulation and protection against acute *Mycobacterium* spp. infections. Higher DNA vaccine doses appear to have produced sufficient pre-existing and/or rapidly inducible immune responses to protect hybrid striped bass against the acute infection produced in the challenge study. Though this study did not evaluate whether the vaccine confers long-term protection, the initial success of this vaccine offers promise for the development of a protective vaccine for piscine mycobacteriosis.

Objectives Met

A new vaccine (pCMV-85A) was constructed and determined to stimulate humoral and cellular immune responses that were significantly upregulated with higher vaccine doses and i.m. injection. Though the vaccine failed to stimulate detectable macrophage phagocytic and respiratory burst responses, the vaccine provided detectable protection after live *M. marinum* challenge.

Acknowledgements

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Table 1. Comparisons of mean survival time, splenic bacterial counts, and relative percent survival 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)[ψ]	RPS[ω]
Saline	15.2+/-0.6762	5.1326+/-0.07279[a]	N/A
pCMV-0	15.4+/-0.6585	5.0159+/-0.07279[a]	0
5 μg i.m.	16.5+/-0.7964	4.9824+/-0.07279[a]	0
25 μg i.p.	26.7+/-0.7522	4.6404+/-0.07279[b]	20
25 μg i.m.	23.9+/-0.0775	3.5577+/-0.07279[c]	80
50 μg i.m.	27.0+/-0	3.5040+/-0.07279[c]	90

[*] Mean survival times for groups without 100% mortality are underestimated because largest observations were censored and estimation was restricted to largest event time (36 days post-challenge).

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

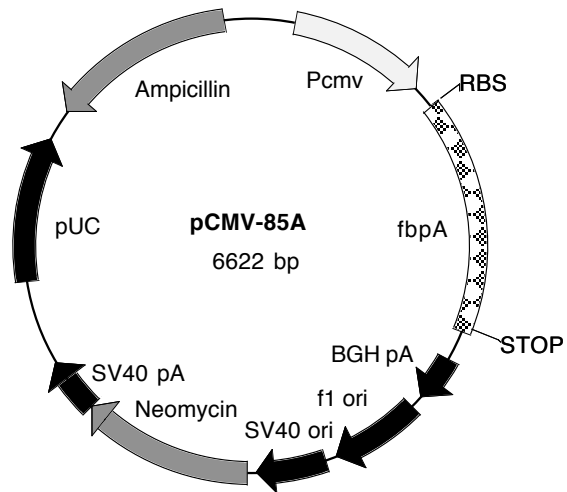[ω]Relative percent survival = (1- % mortality vaccinated fish/ % mortality Saline control fish) X 100

Fig. 1. The 1,100 bp *M. marinum fbpA* PCR product containing the entire open reading
frame, ribosomal binding site, and stop codon was cloned into the pcDNA-3.1 eukaryotic
expression vector to generate the pCMV-85A construct.

ATGAAGCTTGTTGACAGGTTTCGTGGCGCCGTGACGGGTACGCCGCGCCGACTTATGGTGGGAGCCGTTG 70

GCGCGGCCCTGCTATCGGGTCTGGTCGGCTTCGTCGGCGGCTCGGCGACCGCGAGTGCATTTTCGCGGCC 140

GGGCCTGCCGGTGGAATACCTGCAGGTGCCCTCGGCGGCGATGGGCCGCAACATCAAGGTCCAGTTCCAA 210

AGCGGTGGCGCCAACTCTCCGGCGCTGTACCTGCTGGACGGCATGCGTGCGCAGGACGACTTCAGCGGTC 280

GGGACATCAACACCCCCGCGTTCGAGTGGTACTACCGGTCGGGCATCTCGGTCGCCATGCCGGTCGGTGG 350

CCAGTCCAGCTTCTACTCGGACTGGTACAACCCGGCTTGCGGTAAGGCTGGCTGCACCACTTACAAGTGG 420

GAGACCTTCTTGACCAGCGAGCTGCCGCAGTACCTGTCGGCCAACAAGGGTGTCAAGCCCACCGGCAGCG 490

GCGTGGTTGGTCTGTCGATGGCGGGATCGTCGGCCCTGATCCTGGCTGCCTACCACCCCGACCAGTTCGT 560

GTACTCCGGCTCGCTGTCGGCACTGCTGGACCCGTCGCAGGGTATGGGCCCGTCGCTGATCGGCCTGGCC 630

ATGGGTGACGCGGGCGGCTACAAGGCTTCCGACATGTGGGGCCCGAAGGATGACCCGGCCTGGGCGCGTA 700

ACGACCCGATGCTGCAGGTCGGCAAGCTGGTTGCCAACAACACCCGGATCTGGGTGTACTGCGGTAACGG 770

CAAGCCGTCCGACCTGGGTGGCGACAACCTGCCCGCGAAGTTCCTGGAGGGCTTCGTCCGGACCAGCAAC 840

ATGAAGTTCCAGGCCGCGTACAACGCTGCCGGTGGCCACAACGCCGTGTGGAACTTCGATGACAACGGCA 910

CGCACAGCTGGGAGTACTGGGGTGCGCAGCTCAACGCTATGAAGCCTGACCTGCAGCACACGCTGGGTGC 980

GACACCCAACACCGGTGACACCCAGGGCGCCTAG 1014

Fig. 2. Nucleotide sequence of Ag85A from *M. marinum* (ATCC no. 927). Sequence represents the complete open reading frame sequence from the initial codon (ATG) to the stop codon (TAG).
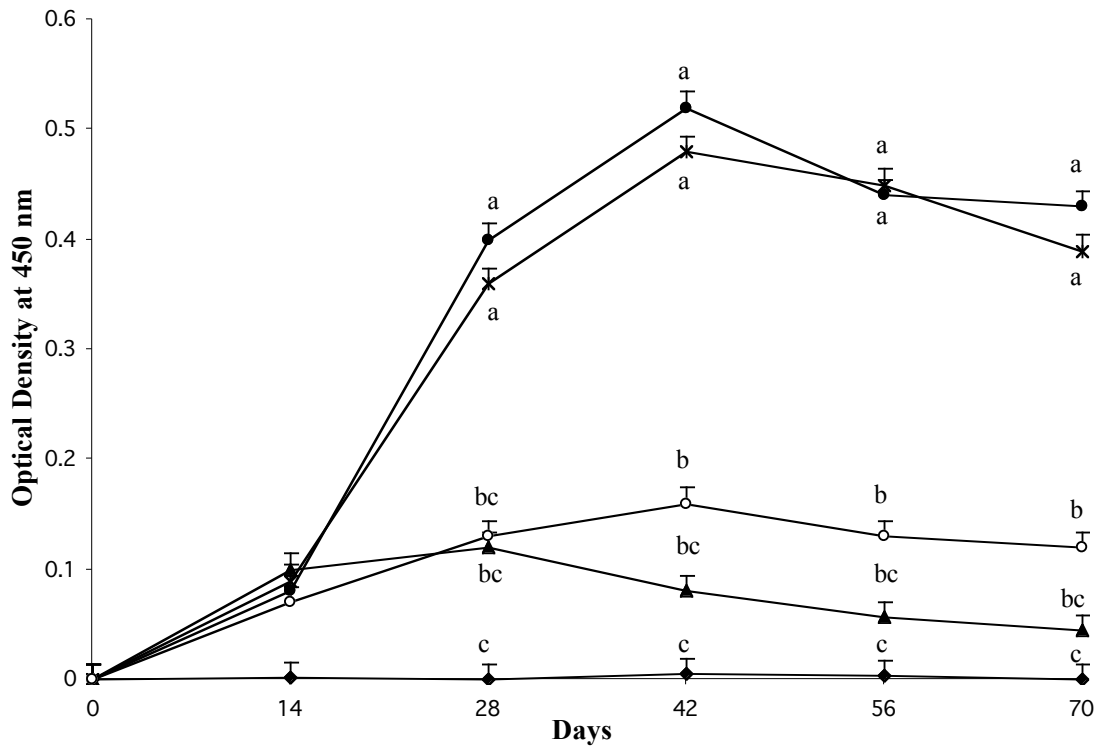
Fig. 3. ELISA detection of Ag85A-specific antibodies from serum of pCMV-85A-vaccinated hybrid striped bass (*Morone saxatilis* x *M. chrysops*). Sera were collected on Days 0, 14, 28, 42, 56, and 70 post-vaccination, diluted 1:100, and assayed for the presence of antibodies to *M. marinum* Ag85A using absorption spectrophotometry. Vaccination groups included were: 50 μg i.m. (closed circle), 25 μg i.m. (asterik), 25 μg i.p. (open circle), and 5 μg i.m. (triangle) pCMV-85A. The Saline control is also included (diamond), and the pCMV-0 control group exhibited responses similar to that of the Saline group. Results are shown as the mean + SEM of OD_{450} of the developed color. Significant differences ($p < 0.05$) among vaccination groups within each sampling day were determined according to Bonferroni corrected data and are indicated on the graph by different alphanumeric letters.
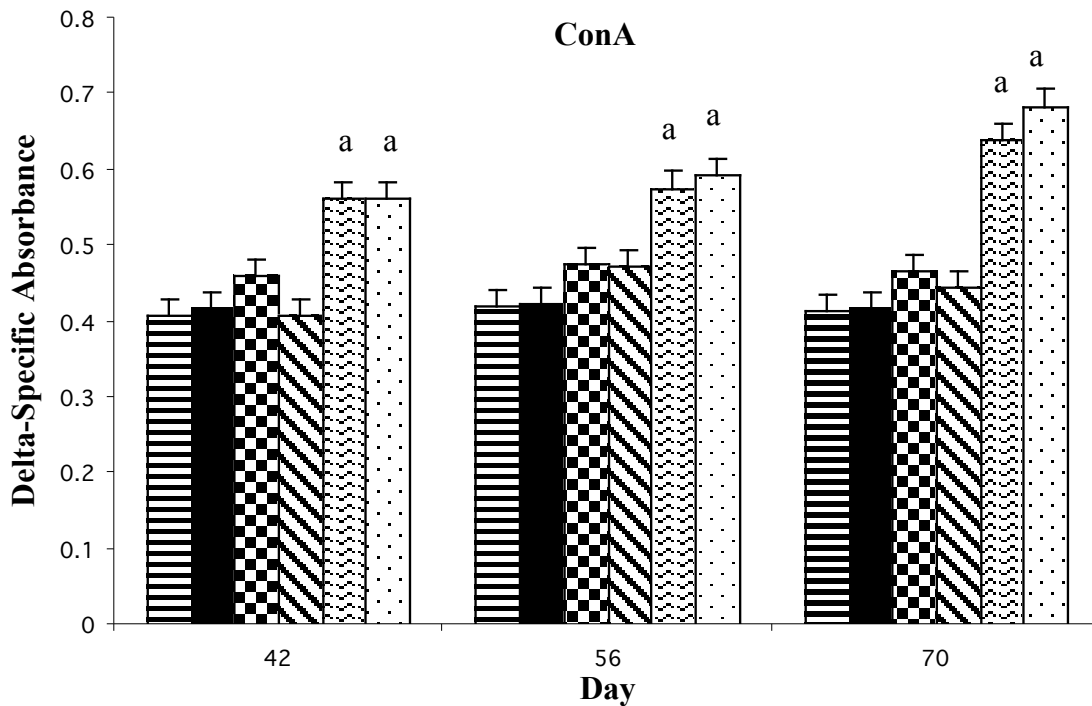
Fig. 4. Proliferation of anterior kidney lymphocytes from pCMV-85A-vaccinated hybrid striped bass (*Morone saxatilis* x *M. chrysops*) after *in vitro* stimulation with ConA. Cells were harvested on Day 0, 14, 28, 42, 56, and 70, cultured for 3 days, and lymphocyte proliferation determined by Alamar blue assay. Vaccination groups included were: 50 μg i.m. (spotted bar), 25 μg i.m. (wavy bar), 25 μg i.p. (diagonal striped bar), and 5 μg i.m. (checkered bar) pCMV-85A. The Saline (horizontal striped bar) and pCMV-0 (solid black bar) control groups are also included. Results are shown as the mean + SEM of the delta-specific absorbance of the developed color. Significant differences ($p < 0.05$) among vaccination groups within each sampling day were determined according to Bonferroni corrected data and are indicated on the graph by alphanumeric letters.
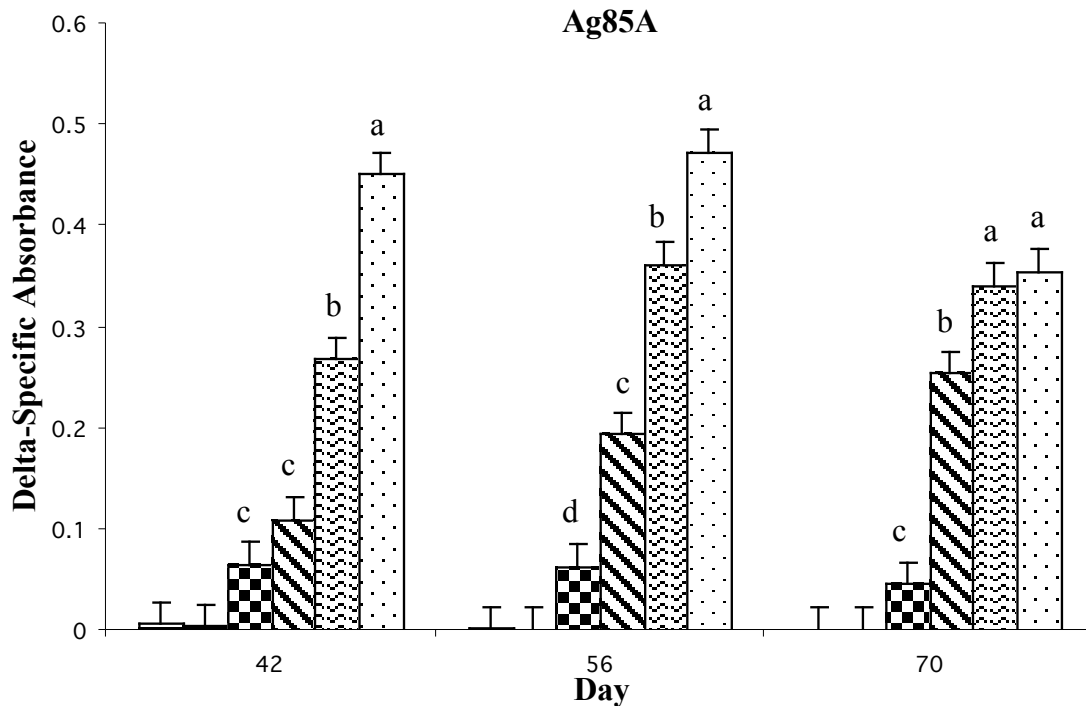
Fig. 5. Proliferation of anterior kidney lymphocytes from pCMV-85A-vaccinated hybrid striped bass (*Morone saxatilis* x *M. chrysops*) after *in vitro* stimulation with Ag85A. Cells were harvested on Day 0, 14, 28, 42, 56, and 70, cultured for 3 days, and lymphocyte proliferation determined by Alamar blue assay. Vaccination groups included were: 50 μg i.m. (spotted bar), 25 μg i.m. (wavy bar), 25 μg i.p. (diagonal striped bar), and 5 μg i.m. (checkered bar) pCMV-85A. The Saline (horizontal striped bar) and pCMV-0 (solid black bar) control groups are also included. Results are shown as the mean + SEM of the delta-specific absorbance of the developed color. Significant differences ($p < 0.05$) among vaccination groups within each sampling day are indicated on the graph by alphanumeric letters.
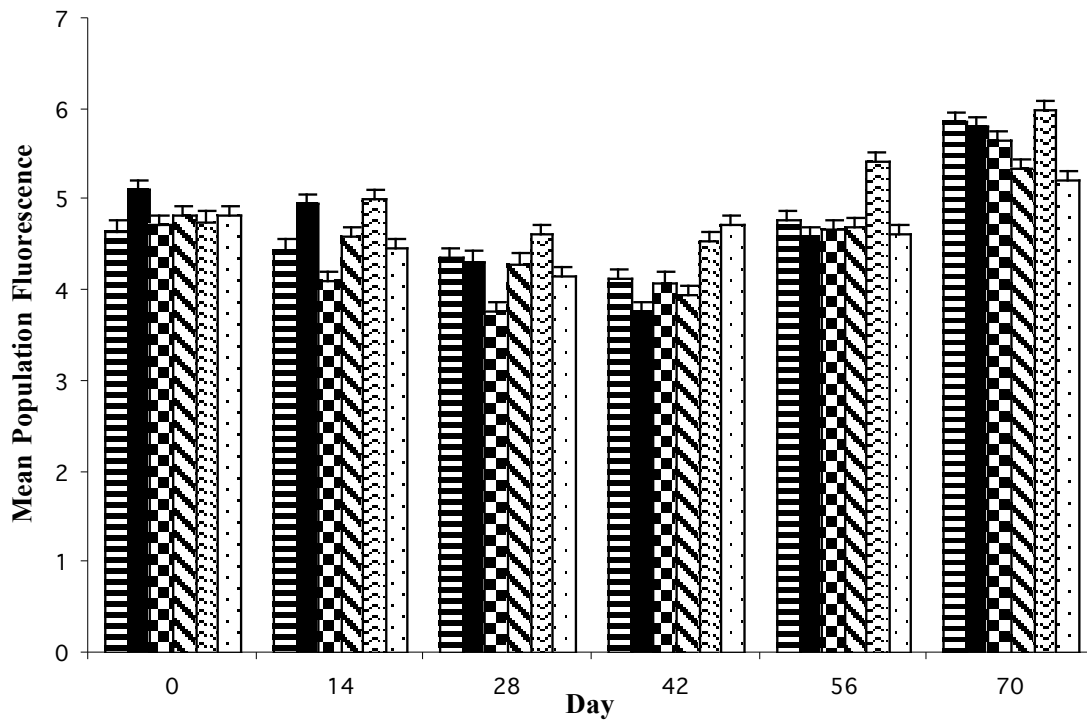
Fig. 6. Phagocytic uptake by macrophages from pCMV-85A-vaccinated hybrid striped bass (*Morone saxatilis* x *M. chrysops*). Cells were harvested on Day 0, 14, 28, 42, 56, and 70, incubated overnight with microspheres, and analyzed by flow cytometry. Vaccination groups included were: 50 µg i.m. (spotted bar), 25 µg i.m. (wavy bar), 25 µg i.p. (diagonal striped bar), and 5 µg i.m. (checkered bar) pCMV-85A. The Saline (horizontal striped bar) and pCMV-0 (solid black bar) control groups are also included. Results are shown as the mean + SEM of the total fluorescence.
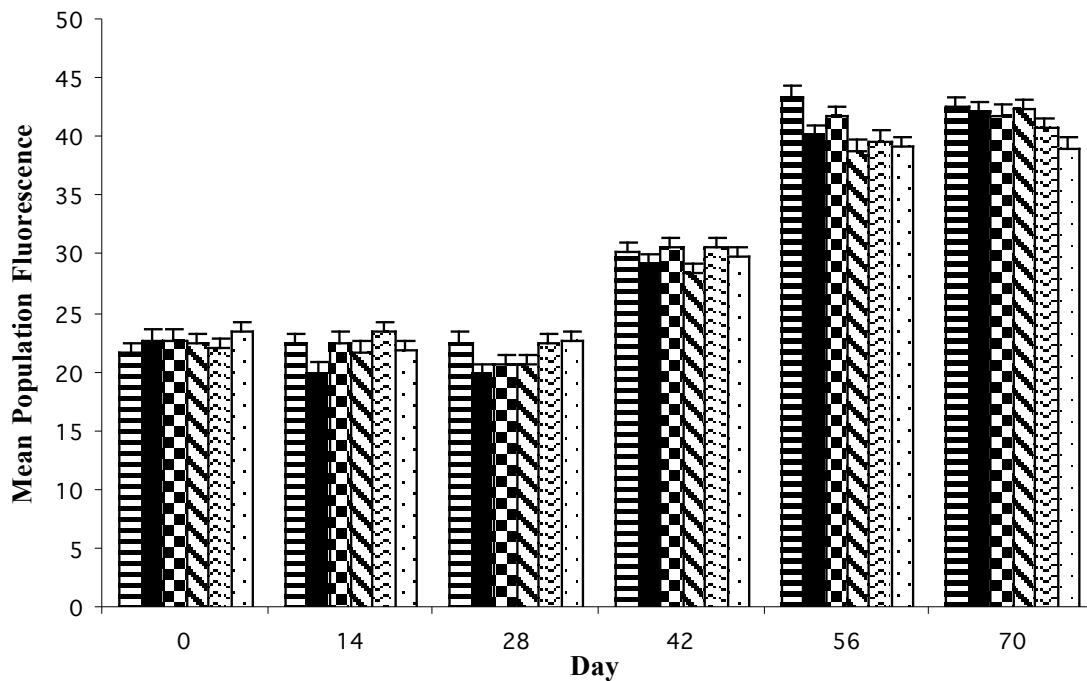
Fig. 7. Macrophage respiratory burst from pCMV-85A-vaccinated hybrid striped bass (*Morone saxatilis* x *M. chrysops*). Cells were harvested on Day 0, 14, 28, 42, 56, and 70, incubated with dichlorofluorescein diacetate, stimulated with phorbol-12-myristate 13-acetate, and analyzed by flow cytometry. Vaccination groups included were: 50 µg i.m. (spotted bar), 25 µg i.m. (wavy bar), 25 µg i.p. (diagonal striped bar), and 5 µg i.m. (checkered bar) pCMV-85A. The Saline (horizontal striped bar) and pCMV-0 (solid black bar) control groups are also included. Results are shown as the mean + SEM of the total fluorescence.
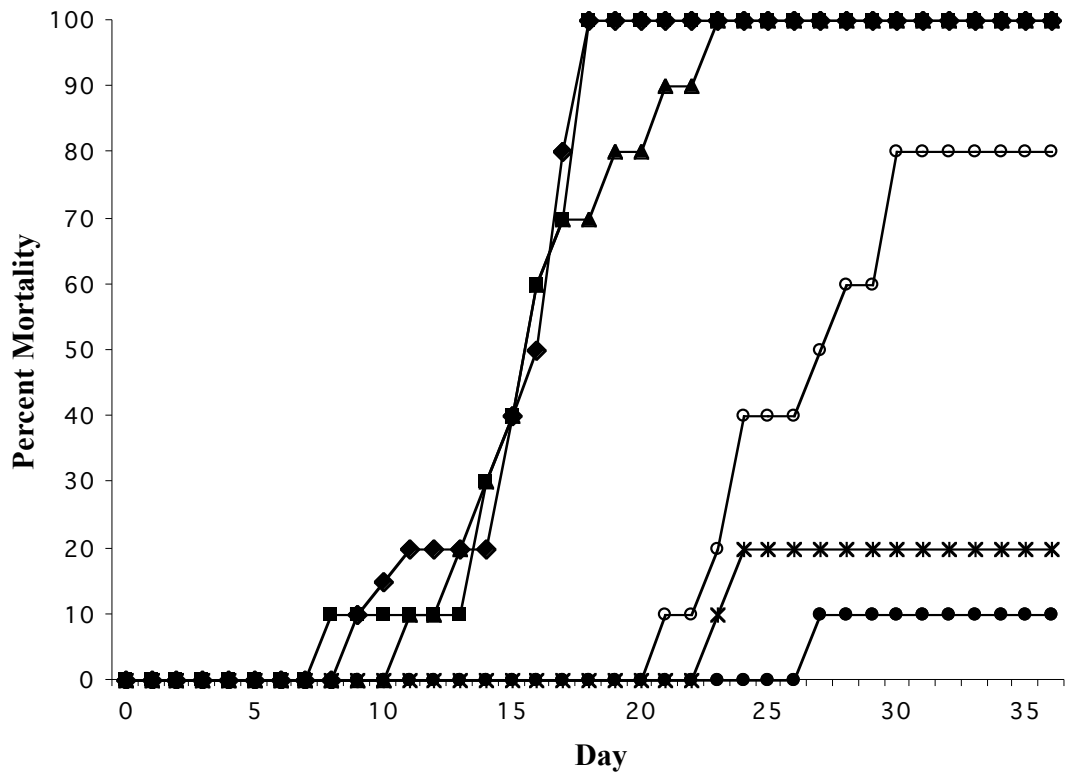
Fig. 8. Cumulative mortality for pCMV-85A-vaccinated hybrid striped bass (*Morone saxatilis* x *M. chrysops*) following challenge with approximately 8 x 10^5 cfu *M. marinum*/g body weight of fish on Day 90 post-vaccination. Vaccination groups included were: 50 µg i.m. (closed circle), 25 µg i.m. (asterik), 25 µg i.p. (open circle), and 5 µg i.m. (triangle) pCMV-85A. The Saline control (diamond) and the pCMV-0 (square) vaccination groups are also included.