Immune and histopathologic responses of DNA-vaccinated hybrid striped bass *Morone saxatilis* × *M. chrysops* after acute *Mycobacterium marinum* infection

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ABSTRACT: The post-challenge immune and histopathologic responses of hybrid striped bass vaccinated with a DNA vaccine encoding the *Mycobacterium marinum* Ag85A gene and subsequently challenged with *M. marinum* were investigated. Juvenile hybrid striped bass *Morone saxatilis* × *M. chrysops* were injected intramuscularly with 25 or 50 µg DNA plasmid and developed significant specific protective responses to live bacterial challenge 120 d post-vaccination. Both vaccine groups demonstrated increased survival, reduced splenic bacterial counts, and reduced granuloma formation compared to the control groups 14 d after challenge with approximately 8×10^5 cfu *M. marinum* g^{-1} fish body wt. The vaccine groups also developed more rapidly and significantly increased antibody and lymphoproliferative responses post-challenge compared to control groups, and these post-challenge immune responses appear to be vital against *M. marinum* infection in vaccinated hybrid striped bass. No significant differences in immune responses were recognized between the 25 and 50 µg vaccination groups, and these groups eventually experienced mortalities, splenic bacterial counts, and granuloma formation 28 d post-challenge comparable to those of the control groups at 14 d post-challenge. Therefore, vaccination of hybrid striped bass with a DNA vaccine encoding the *M. marinum* Ag85A gene provided significant but limited duration of protection against an acute high-dose *M. marinum* challenge.

KEY WORDS: Immunity · DNA · Vaccine · *Mycobacterium marinum* · Fish · Striped bass · *Morone* spp.

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 & Austin 1993, Chinabut 1999). After *M. marinum* enters the fish's body via the gastrointestinal tract, through 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 & Austin 1993). Mechanisms of immunity against *M. marinum* have not been completely elucidated, but previous studies indicate that protective effects in fish are based on both specific and non-specific defenses (Bartos & 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 pro-

vide protection against various piscine pathogens, such as viral hemorrhagic septicemia, infectious hematopoietic necrosis virus, hirame rhabdovirus, and channel catfish virus (Boudinot et al. 1998, Lorenzen et al. 1998, Nusbaum et al. 2002, Takano et al. 2004); 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 & Davis 2000). In mammals, DNA vaccines encoding *Mycobacterium 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 should putatively provide protection through induction via similar specific immune mechanisms.

Pasnik & Smith (2005) determined that a *Mycobacterium marinum* Ag85A DNA vaccine generated significant specific immune responses against *M. marinum* through 70 d after vaccination. The vaccine was found to be protective when striped bass were challenged 90 d post-vaccination, especially in individuals injected intramuscularly (i.m.) with 25 or 50 μg DNA plasmid. However, only basic post-challenge efficacy was determined in that study and mechanisms of protection were not assessed. The DNA vaccine study presented here endeavored to characterize the specific immune and histopathologic responses generated in vaccinated hybrid striped bass after live *M. marinum* challenge and to evaluate vaccine efficacy after a longer duration between vaccination and challenge. Also, because the prior study (Pasnik & Smith 2005) was terminated 36 d post-challenge, the research here was designed to evaluate the protective responses of the vaccinated fish over a longer time frame after challenge. These data will provide further insight into the anti-*M. marinum* immune responses in hybrid striped bass, a widely cultured foodfish.

MATERIALS AND METHODS

Fish. Fingerling hybrid striped bass *Morone saxatilis* × *M. chrysops* weighing approximately 40 to 50 g were obtained from a commercial supplier and housed at the Aquatic Medicine Laboratory of the Viginia-Maryland Regional College of Veterinary Medicine (VMRCVM) in Blacksburg, Virginia, USA. The fish (n = 400) were maintained in multiple 2272 l recircu-

lation aquaculture systems with appropriate biological filtration and aeration. Water temperature was maintained at approximately 24 to 26°C, and the fish were fed daily at 3 to 5% body weight (41% protein; Aqua-Max; Purina). Water quality parameters (ammonia, nitrite, nitrate and pH) were monitored daily using a water analysis kit (HACH Co.). Care of the fish was in compliance with the guidelines of the Institutional Animal Care and Use Committee of the Virginia Polytechnic Institute and State University (VPI&SU).

DNA construct preparation. *Mycobacterium marinum* (American Type Culture Collection, ATCC No. 927) was grown in Middlebrook 7H9 broth (Difco) for 9 d at 28°C. 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) were used for the PCR, and amplification was performed using an Omni Gene thermocycler (Hybaid) at 95°C for 5 min, followed by 30 cycles of 1 min of denaturation at 95°C, 1 min of annealing at 60°C, and 1 min 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) 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^{-1}) 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^{-1}). Plasmids were isolated using Plasmid Maxi and Mega kits according to the manufacturers instructions (Qiagen). 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 *Mycobacterium 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).

Fish vaccination. Hybrid striped bass were separated into 4 experimental groups (n = 100 each group) and given intramuscular (i.m.) injections in the hypaxial muscle. The vaccination groups consisted of fish injected with different doses of the pCMV-85A construct (25 or 50 μg groups) on Days 0 and 14. As controls, other groups of fish were vaccinated with 25 μg empty pcDNA 3.1 plasmid (pCMV-0 group) or sham-vaccinated with sterile phosphate-buffered saline (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, all fish from each control group (Saline and pCMV-0) and vaccination group (25 and 50 µg pCMV-85A) were challenged with live *Mycobacterium marinum* (ATCC No. 927). Fish were individually identified and arbitrarily distributed into three 568 l tanks inside an isolation facility. Challenge was performed by i.m. injection with approximately 8×10^5 cfu *M. marinum* g^{-1} fish body wt, a dose designed to cause onset of mortality at approximately 15 d (Wolf & Smith 1999) and previously used to assess protective effects among DNA-vaccinated striped bass (Pasnik & Smith 2005). The injection challenge route was used because it is a highly reliable and easily reproducible method and ensures challenge of all individual fish with a uniform bacterial dose (Nordmo 1997). The isolate was grown in Middlebrook 7H9 broth at 28°C for 15 d. The resulting bacterial sample was briefly sonicated to break up bacterial aggregates, washed 3 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. The i.m. route of administration was chosen because of its known infectivity potential, because it is a reliable and reproducible challenge method, and because i.m. injection mimics natural exposure via dermal wound invasion (Wolf & Smith 1999, Pasnik & Smith 2005).

During the study, morbid and dead fish were removed immediately and recorded. A relative percent survival (RPS) was determined, and 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.

Splenic bacterial counts. On Days 14 and 28 post-challenge (134 and 148 d post-vaccination), five fish from each unchallenged and challenged group were euthanized by tricaine methanesulfonate (MS-222; Sigma Chemical Co.) 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 in triplicate. Colonies of bacteria were counted after incubation for 10 d at 28°C and confirmed as *Mycobacterium marinum* through assessment of the morphologic characteristics (Gram-positive, acid-fast bacilli) and 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 & Smith (1999). Tissues of 5 fish from each available control and vaccination group were sampled on Days 14 and 28 post-challenge and immersed in 10% neutral buffered formalin for a period of at least 72 h. 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 20× 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 & 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.

Immune responses of vaccinated fish after live bacterial challenge. On Day 42 post-vaccination and on Days 0, 7, 14, 21, and 35 post-challenge, 10 fish from each available group were anesthetized with MS-222 and blood samples taken for ELISA and lymphoproliferative assays.

ELISA. The antibody responses of fish from each group were evaluated for the presence of specific immunoglobulin against the *Mycobacterium marinum* Ag85A using an indirect ELISA (Vemulapalli et al. 2002, Pasnik et al. 2003). Recombinant *M. marinum* Ag85A was generated for the ELISA and lymphoproliferative assay as described in Pasnik & Smith (2005). 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) with 50 µl well^{-1}. The plates were incubated at 4°C overnight, washed 4 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 h 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^{-1}. A positive control serum sample and a diluent- only sample were tested in the same manner. The plates were incubated for 4 h at room temperature and then washed 4 times with wash buffer.

The secondary antibody solution, a protein A-peroxidase conjugate (Sigma), was added at 100 µl well^{-1} at a 1:500 dilution. After 1 h incubation at room temperature, the plates were washed 4 times and 100 µl of substrate solution (TMB Microwell Peroxidase Substrate; Kirkegaard and Perry Laboratories) were added to each well. After 20 min incubation at room temperature, 100 µl of stop solution (0.185 M sulfuric acid) were added. The absorbance at 450 nm was then recorded with a microplate reader (Molecular Devices) and the mean absorbance of each sample serum compared against that of the control well.

Lymphoproliferative assay. Peripheral blood lymphocytes of fish from each group were evaluated for the

presence of specific immunoglobulin against ConA and against *Mycobacterium marinum* Ag85A using a lymphoproliferative assay (Marsden et al. 1996, Pasnik et al. 2003). Blood from fish in each control and vaccination group was collected and diluted with sterile RPMI-1640 medium (Mediatech, Cellgro). The resulting cell suspension was washed twice in RPMI-1640 medium and resuspended in 3 ml RPMI-1640 medium. Using an aseptic technique, phagocytes were placed over the Lymphoprep separation medium (1.077; Nycomed) and centrifuged at $400 \times g$. 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). The cells were then adjusted to 1×10^6 cells ml^{-1} with RPMI-1640 medium supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (Sigma), 50 IU ml^{-1} penicillin, and 50 mg ml^{-1} streptomycin (Cellgro). Aliquots of cells were cultured in triplicate in 96-well plates (Corning) with supplemented RPMI-1640 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 h. After 24 h, 20 µl Alamar Blue (Trek Diagnostic) was added to the wells, and the cells were incubated for an additional 48 h. Absorbance was then measured at 570 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.

Statistical analysis. Statistical analysis was performed with SAS software (SAS Institute). 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 a 1-way analysis of variance; Bonferroni correction was performed to compare the various experimental dose groups within each sampling day. The data for each test is reported as means with SEM determined from pooled means. An overall significance level of $p < 0.05$ was accepted.

RESULTS

Protective effects following live bacterial challenge

Vaccinated fish were exposed to live *Mycobacterium marinum* at a dose designed to cause the onset of mortality at approximately 15 d post-challenge. When fish were challenged 120 d post-vaccination, the Saline control and pCMV-0 group fish experienced onset of mortalities early in the experiment, reaching 100% mortality within 21 d (Fig. 1). In contrast, the 25 and 50 µg groups showed a delayed onset of mortality, with the first mortalities occurring on Days 22 and 21 post-challenge, respectively. The vaccination groups also exhibited a significantly increased mean survival time (Table 1), although all of the fish in the vaccination groups eventually died within 46 d. After the first mortalities in each group, all groups experienced a fairly rapid progression of disease to 100% mortality. However, while all the fish in the control groups died within approximately 15 d of the onset of mortalities, those in the vaccinated groups died within approximately 25 d of the first mortalities.

Splenic bacterial cultures were obtained on Days 14 and 28 post-challenge and incubated for 10 d. Colonies present after incubation were identified as *Mycobacterium marinum* through assessment of morphologic characteristics and PCR. On Day 14 post-challenge, both control groups exhibited significantly higher mycobacterial growth in the spleen samples than either the 25 or 50 µg pCMV-85A groups. The RPS on Day 14 post-challenge for the pCMV-0 group was 0%, while the RPS values for the vaccination groups were 100% each. Later, the RPS on Day 28 post-challenge showed vaccine efficacies for the 25 and 50 µg groups of 91 and 88%, respectively, compared to 0% for the other group (Table 2). On Day 28 post-challenge, splenic samples from the 25 and 50 µg groups also indicated increased growth of bacteria within the fish, although these bacterial counts were still not as high as the bacterial counts from the control groups on Day 14 post-challenge.
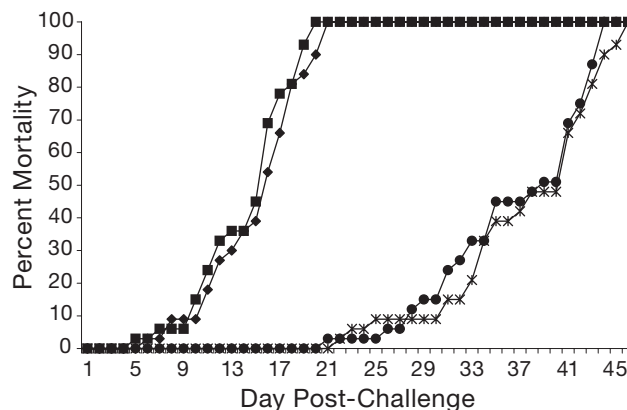

Fig. 1. *Morone saxatilis × M. chrysops* challenged by *Mycobacterium marinum.* Cumulative mortality for pCMV-85A-vaccinated, hybrid striped bass, following challenge with approx. 8×10^5 cfu *M. marinum* g^{-1} fish body wt on Day 120 post-vaccination. (♦) Saline control; (■) pCMV-0 control; (✳) 25 µg pCMV-85A; (●) 50 µg pCMV-85A

Table 1. *Morone saxatilis* × *M. chrysops* challenged by *Mycobacterium marinum*. Comparison of mean survival time, relative percent survival (RPS) and splenic bacterial counts (log *M. marinum,* cfu) on Day 14 post-challenge following live bacterial challenge of pCMV-85A-vaccinated hybrid striped bass. Data are mean ± SEM. Different superscripts indicate significant differences (p < 0.05) between groups within each measured category determined according to Bonferroni-corrected data. RPS = (1 – % mortality vaccinated fish/% mortality Saline control fish) × 100. na: not applicable

Vaccination group	Survival time (d)	Log *M. marinum* (cfu)	RPS (%)
Controls			
Saline	15.3 ± 0.68[a]	5.0690 ± 0.09732[a]	na
pCMV-0	14.6 ± 0.65[a]	5.1495 ± 0.09732[a]	0
pCMV-85A			
25 µg	37.7 ± 1.12[b]	3.8122 ± 0.09732[b]	100
50 µg	36.9 ± 1.09[b]	3.8215 ± 0.09732[b]	100

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. The splenic tissues of the 25 and 50 µg groups exhibited only a low number of granulomas and appeared largely unaffected; mean splenic scores for the 25 and 50 µg groups were 1.0

Table 2. *Morone saxatilis* × *M. chrysops* challenged by *Mycobacterium marinum*. Comparison of mean survival time, relative percent survival (RPS) and splenic bacterial counts (log *M. marinum,* cfu) on Day 28 post-challenge following live bacterial challenge of pCMV-85A-vaccinated hybrid striped bass. No significant differences (p < 0.05) between vaccination groups within each measured category were found according to Bonferroni-corrected data. Data presentation as in Table 1

Vaccination group[a]	Survival time (d)	Log *M. marinum* (cfu)	RPS (%)
25 µg	37.7 ± 1.12	4.7227 ± 0.05385	91
50 µg	36.9 ± 1.09	4.6709 ± 0.05385	88

[a]All fish in Saline and pCMV-0 control groups were dead by Day 28 post-challenge and are thus not included

and 0.3, respectively, and were significantly lower than those of the control groups. However, on Day 28 post-challenge, the 25 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.

Ag85A-specific antibody production

The concentration of *Mycobacterium marinum* Ag85A-specific antibodies were measured by ELISA post-vaccination and post-challenge. 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 significantly increased levels of antibodies (25 µg group = 0.469 ± 0.011; 50 µg group = 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. 2). However, rapidly increasing concentrations of antibodies were generated by the vaccination groups up to Day 28 post-challenge. Significantly
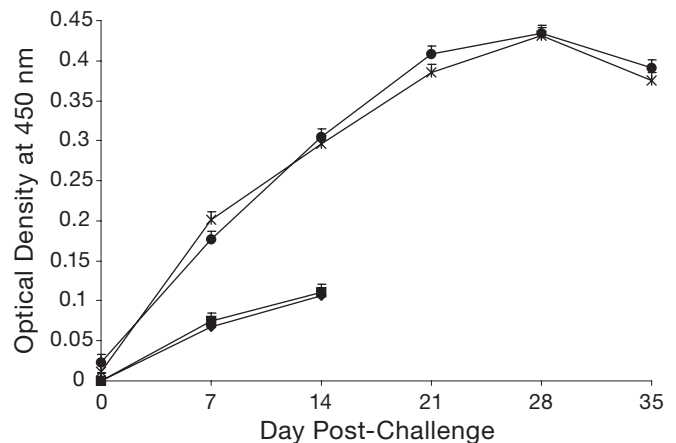

Fig. 2. *Morone saxatilis* × *M. chrysops* challenged by *Mycobacterium marinum*. Detection of Ag85A-specific antibodies by ELISA from serum of pCMV-85A-vaccinated, *M. marinum*-challenged hybrid striped bass. Sera were collected from available vaccine groups on Days 0, 7, 14, 21, 28, and 35 post-challenge, diluted 1:100, and assayed for presence of antibodies to *M. marinum* Ag85A using absorption spectrophotometry. (●) Saline control; (■) pCMV-0 control; (✳) 25 µg pCMV-85A; (●) 50 µg pCMV-85A. Both control groups experienced expected 100% mortalities during experiment and thus samples were not available for all time points. Data are mean + SEM of OD$_{450}$ of developed color. pCMV-85A vaccination groups were significantly different (p < 0.05) from the control groups at all time points after Day 0 post-challenge, but no significant differences were noted between the 25 and 50 µg groups at any time point

Table 3. *Morone saxatilis* × *M. chrysops* challenged by *Mycobacterium marinum*. Comparison of mean granuloma counts following live bacterial challenge of pCMV-85A-vaccinated hybrid striped bass. Day p-c: day post-challenge. *Significant differences (p < 0.05) between groups and Saline control indicated for each sampled internal organ; mean granuloma counts for vaccination groups on Day 28 were compared to those of the Saline control group on Day 14

Vaccination group[a]	Day p-c	Spleen	Posterior kidney	Liver	Heart
Controls					
Saline	14	13.6	4.2	2.6	4.2
pCMV-0	14	15.2	5.1	2.4	4.5
pCMV-85A					
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

[a]All fish in Saline and pCMV-0 control groups were dead by Day 28 post-challenge and are therefore not included

greater responses were seen among the vaccination groups than the control groups, although there were no significant differences between the 2 vaccination groups. After Day 28 post-challenge, the concentrations of antibodies began to decrease, but the fish continued to maintain significantly elevated levels of antibodies. Specific antibody responses were detected in the Saline and pCMV-0 and groups after challenge, although these specific responses were minimal and increased slowly.

Lymphoproliferative responses

Post-vaccination and post-challenge samples of peripheral lymphocytes were cultured *in vitro* in the presence of ConA or Ag85A. On Day 42 post-vaccination, ConA-stimulated cells from the vaccination groups (25 µg group = 0.504 ± 0.018; 50 µg group = 0.538 ± 0.018) demonstrated significantly increased proliferation compared to those of the control groups (Saline = 0.372 ± 0.018; pCMV-0 = 0.332 ± 0.018). This significant difference was largely diminished on Day 0 post-challenge (Day 120 post-vaccination). After live bacterial challenge, all groups still exhibited proliferative lymphocyte responses after ConA stimulation (Fig. 3). However, the 25 and 50 µg groups demonstrated significant responses above those of the control groups, and these responses continued to increase after the controls had experienced the expected 100% mortalities.

On Day 42 post-vaccination, Ag85A-stimulated cells from the vaccination groups (25 µg group = 0.368 ± 0.012; 50 µg group = 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 largely 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 (Fig. 4). Both the 25 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

Prior experiments have indicated that a vaccine with the *Mycobacterium marinum* Ag85A can provide protection against *M. marinum* infection in striped bass, but only the post-vaccination, pre-challenge immunostimulatory effects were studied (Pasnik et al. 2003, Pasnik & Smith 2005). The study presented herein characterizes the immune mechanisms involved in protection against *M. marinum* after challenge and indicates the importance of Ag85A-specific immune responses generated post-challenge. Vaccinated fish (25 and 50 µg groups) exhibited very low
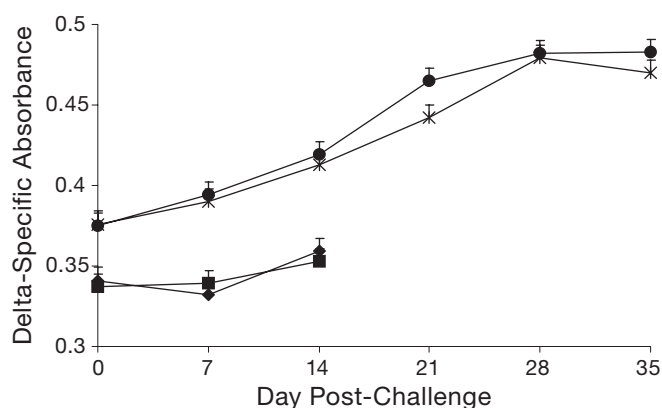

Fig. 3. *Morone saxatilis* × *M. chrysops* challenged by *Mycobacterium marinum*. Proliferation of peripheral lymphocytes from pCMV-85A-vaccinated, *M. marinum*-challenged hybrid striped bass after *in vitro* stimulation with ConA. Cells were harvested from available vaccine groups on Days 0, 7, 14, 21, 28 and 35 post-challenge, cultured for 3 d, and lymphocyte proliferation determined by Alamar blue assay. Data are mean + SEM of the delta-specific absorbance of the developed color. Symbols and further details as in Fig. 2
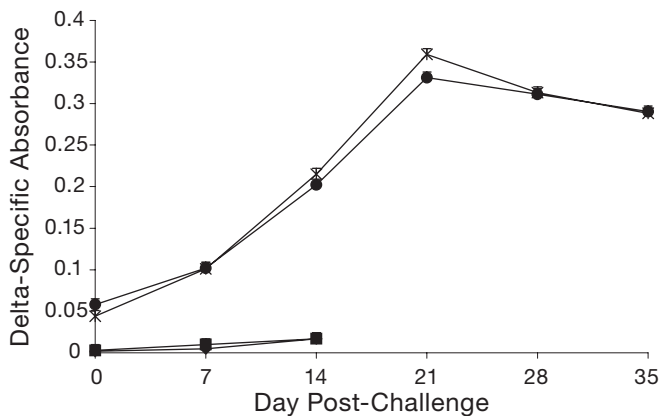
Fig. 4. *Morone saxatilis* × *M. chrysops* challenged by *Myco-bacterium marinum*. Proliferation of peripheral lymphocytes from pCMV-85A-vaccinated, *M. marinum*-challenged hybrid striped bass after *in vitro* stimulation with Ag85A. Symbols and further details as in Figs. 2 & 3

levels of specific responses at Day 0 post-challenge (Day 120 post-vaccination), suggesting that vaccine efficacy was not conferred by pre-existing elevated levels of immune responses induced by vaccination. Instead, the protective effects were most probably conferred by immunologic memory and the rapidly increasing specific immune responses following challenge. These post-challenge immune responses included rapidly increasing specific antibody concentrations and lymphocyte responsiveness. These immune responses appear to be vital against *M. marinum* infection in hybrid striped bass. In contrast, 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 control group 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 and 50 µg groups. By Day 21 post-challenge, both control groups had reached 100% mortalities, while mortalities among the vaccine groups were 0 to 3%. However, in contrast to our previous study in which striped bass were challenged 90 d post-vaccination (Pasnik & Smith 2005), the protective effects in this study were diminished, although still significant, 35 d after challenge on Day 120 post-vaccination. In the present study, the 25 and 50 µg groups showed relative percent survival of 61 and 55%, respectively, 35 d after challenge. These findings indicate a significant, but limited protective effect of the DNA vaccine.

Previous studies by other researchers have shown protective effects in fish following DNA vaccine administration. DNA vaccines for viral piscine pathogens have been 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, Takano et al. 2004). 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 in rainbow trout; 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, although Gomez-Chiarri et al. (1996) were able to generate significant protection with DNA vaccines against *Renibacterium salmoninarum*. While these studies offer promising results, most have not assessed the long-term efficacy and the post-challenge protective immune response provided by these DNA vaccines.

Our previous work found that a recombinant vaccine expressing a mammalian *Mycobacterium* sp. Ag85A was significantly immunostimulatory in *Morone* sp. post-vaccination, pre-challenge (Pasnik et al. 2003). However, no protective effects were conferred when fish were challenged with a high dose of *Mycobacterium marinum*. In the Pasnik & Smith (2005) study and the present study, significant protection was most probably conferred by the utilization of a DNA vaccine as the method of delivery for the *M. marinum* Ag85A. These factors putatively identify sufficiently primed, immune responses that were significantly stimulated post-challenge to provide protection. In addition, the vaccine was protective even although *Morone* spp. are highly susceptible to disease caused by *Mycobacterium* spp. (Wolf & Smith 1999, Gauthier et al. 2003, Harms et al. 2003). The data from the present study nonetheless 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 increased mortalities, while the vaccination groups had low bacterial growth, minimal lesion development and 0% mortalities. However, at Day 28 post-challenge, samples of the vaccination groups exhibited bacterial growth and lesions similar in magnitude and severity to those of the control groups at Day 14 post-challenge. Although the 25 and 50 µg groups had only 9 to 12% mortalities 28 d post-challenge, the increasing bacterial counts and developing granulomas could account for the subsequent increase in mortalities.

The high-challenge i.m. dose of 8×10^5 cfu *Mycobacterium marinum* g^{-1} fish body wt was designed to cause acute mortalities among naïve striped bass (Wolf & Smith 1999, Pasnik & Smith 2005). The post-challenge immune responses in vaccinated individuals may hinder the immediate dissemination and/or replication of *M. marinum* in the host. However, these post-challenge responses only appear to delay development of clinical signs and mortalities instead of preventing the disease. Long-term immunity to the administered *M. marinum* may have been deterred by immunosuppression of the fish through the use of an unnaturally high bacterial challenge dose and/or induction of an unsuitable immune response. The high *M. marinum* dose, presumably overwhelmed the induced immune responses or immunosuppressed the vaccinated fish, eventually allowing unhindered disease development. *Mycobacterium* spp. themselves are known to be immunosuppressive (Geijtenbeek et al. 2003) and to resist host immune activity (Chan et al. 2002, Ruley et al. 2004), thereby encumbering immunity. Immunosuppression and/or induction of inappropriate immune responses are important factors in the development of piscine mycobacteriosis (Barker et al. 1997, Talaat et al. 1998, Gauthier et al. 2003, Harms et al. 2003). Based upon the post-challenge ELISA and lymphoproliferative assays, the decrease in vaccinated fish immunity occurred after the measured immune response peaks at 21 to 28 d post-challenge. The subsequent decreases in immune responses coincided with the onset of mortalities among vaccinated fish.

These factors may have prevented long-term vaccine-induced protection against the experimental high-dose exposures and allowed the eventual development of 100% mortality in the vaccination groups. However, in nature, the fish would most likely be naturally exposed to mycobacterial organisms at significantly lower levels, and would not naturally experience high-dose exposure by direct mycobacterial i.m. injection. As such, the vaccine produced as a result of this research may confer better protective effects against natural routes of infection (i.e. ingestion, penetration of intact mucus membranes, or dermal wound invasion) by inducing post-vaccination or post-challenge immune responses capable of controlling environmentally relevant numbers of mycobacteria.

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