Protective immunity of a modified-live cyprinid herpesvirus 3 vaccine in koi (*Cyprinus carpio koi*) 13 months after vaccination

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Objective—To evaluate the long-term protective immunity of a cyprinid herpesvirus 3 (CyHV3) vaccine in naïve koi (*Cyprinus carpio koi*).

Animals—72 koi.

Procedures—Vaccinated koi (n = 36) and unvaccinated control koi (36) were challenge exposed to a wild-type CyHV3 strain (KHVp8 F98-50) 13 months after vaccination.

Results—The CyHV3 vaccine provided substantial protective immunity against challenge exposure. The proportional mortality rate was less in vaccinated koi (13/36 [36%]) than in unvaccinated koi (36/36 [100%]). For koi that died during the experiment, mean survival time was significantly greater in vaccinated than in unvaccinated fish (17 vs 10 days).

Conclusions and Clinical Relevance—The CyHV3 vaccine provided substantial protective immunity against challenge exposure with CyHV3 13 months after vaccination. This provided evidence that koi can be vaccinated annually with the CyHV3 vaccine to significantly reduce mortality and morbidity rates associated with CyHV3 infection. (*Am J Vet Res* 2014;75:905–911)

Cyprinid herpesvirus 3 is a highly contagious pathogen, which causes fatal KHVd of ornamental and food carp species.[1,2] When introduced into an aquaculture facility or ornamental collection, epizootics are devastating to koi (*Cyprinus carpio koi*) and impact breeders, dealers, hobbyists, pet retailers, and aquaculturists.[3–5] Even with proper quarantine protocols and screening tests, CyHV3 can be introduced into aquatic systems as a result of latent or persistent low-level infections in koi, which can be reactivated by temperature stress.[6–8] Exposure can also occur via contaminated water sources, where virus can persist for up to 3 months.[9] Skin, rather than the gills, is the major portal of entry of the virus.[10]

Received March 27, 2014.
Accepted June 19, 2014.
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This manuscript represents a portion of a thesis submitted by Dr. O'Connor to the University of California-Davis School of Veterinary Medicine as partial fulfillment of the requirements for a Master of Preventative Veterinary Medicine degree.
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Abbreviations

CyHV3	Cyprinid herpesvirus 3
KHVd	Koi herpesvirus disease

Recently, a modified-live CyHV3 vaccine was tested in the United States to examine safety and efficacy for use in koi against KHVd.[11] The vaccine was found to have a favorable safety profile, even when used at 10 times the recommended dose, and it provided immunity against CyHV3 following challenge exposure.[11] The objective of the study reported here was to determine whether the modified-live CyHV3 vaccine provided protective immunity in koi against challenge exposure with a wild-type CyHV3 13 months after vaccination. Results could provide the koi industry with information to more effectively protect koi from the destructive effects of KHVd.

Materials and Methods

Animals—Koi (n = 72) were originally purchased for initial vaccine and efficacy experiments to protect against CYHV3 from a closed-system commercial producer[a] with no history of KHV infection at their facility. Sixty fish purchased from the producer at that time were euthanized and screened for CyHV3 DNA or antibodies against CyHV3 by means of quantitative PCR assay[12] and an ELISA[13] and for parasitic or bacterial infections, which were assessed by microscopic analysis of fresh mounts of gill specimens and skin scrapings and microbial culture on sheep blood agar at 22°C. These

fish did not have detectable CyHV3 DNA or serum antibodies against CyHV3 and had low parasitic or bacterial burdens. Before the start of the present study, koi (n = 72) were housed for a total of 14 months in tanks located in the Fish Health Containment Laboratory at the Center for Aquatic Biology and Aquaculture of the University of California-Davis.

For the study reported here, 36 of the 72 koi had been vaccinated 13 months previously with an attenuated CyHV3 modified-live virus vaccine.[14,15] The vaccine[b] was administered as indicated elsewhere.[11] The other 36 koi were not vaccinated and had no prior exposure to CyHV3. For 13 months, the fish were maintained in 6 isolated containment tanks (3 tanks for vaccinated fish and 3 tanks for unvaccinated fish). The tanks (130-L observation tanks) were maintained at 18° to 21°C. Tanks for the vaccinated and unvaccinated fish were physically isolated and separated by another bank of tanks to prevent cross-contamination. The facility used a flow-through water system at a rate of 3.8 L/min. All fish were fed a commercial koi diet[c] at 1% of body weight/d. The study design was reviewed and approved by the University of California-Davis Institutional Animal Care and Use Committee.

Study procedures—The 36 vaccinated koi were reassigned by use of randomization software[d] to 3 tanks (12 fish/tank). At the completion of the study, it was found that one of the vaccinated fish had moved from its original tank to another adjacent tank that contained vaccinated fish, despite the fact the tanks were covered with plastic lids. The 36 unvaccinated koi were also reassigned by use of randomization software[d] to 3 tanks (12 fish/tank). The 2 groups of tanks were separated by a bank of dry tanks, which created a distance barrier of 2 m to prevent cross-contamination between vaccinated and unvaccinated fish.

Challenge exposure with CyHV3—Blood samples were collected from all 72 fish at the beginning of the study and tested with an ELISA[13] for anti-CyHV3 antibodies. Water temperature in the tanks was increased from 21° to 23°C prior to challenge exposure with a wild-type CyHV3. Because temperature can affect antibody production in poikilothermic animals, fish were allowed to acclimatize to the warmer water (21° to 23°C) for 10 days, and blood samples were then collected from 12 fish (6 fish/group) to reexamine ELISA values.

The wild-type CyHV3 challenge exposure virus (KHVp8 F98-50) was maintained as previously described.[16] Culture medium was harvested 15 days after inoculation (viral concentration, 1.4×10^4 plaque-forming units/mL) and immediately used for challenge exposure of the fish.

All 72 fish in each of the 6 tanks subsequently were challenge exposed to the wild-type CyHV3. Tanks were drained until they each contained only 15 L of water; each tank then received 38 mL of wild-type CyHV3 containing 35.5 plaque-forming units/mL. After exposure to the virus for 1 hour, normal water flow was resumed. For the next 21 days, all 6 tanks were monitored twice daily to detect dead and moribund fish. Moribund fish were removed from the tank and euthanized. Samples were collected from moribund fish on the day of removal. All remaining fish were euthanized 21 days after challenge exposure, and samples were then collected from those fish.

Sample collection and analysis—Fish were euthanized with methane tricaine sulfonate[e] (500 µg/mL) buffered with sodium bicarbonate[f] (500 µg/mL). Fish were weighed (within ± 1 g) and measured (total length and fork length). Wet mounts of skin scrapings and gill biopsy specimens were examined for parasites by light microscopy. Aseptically collected swab specimens of the kidneys were submitted for microbial culture on sheep blood agar plates at 22°C. Tissue samples of the gills, trunk kidneys, and spleen were pooled and stored at –20°C in 70% ethanol for PCR assay. Organs were harvested and immersed in neutral-buffered 10% formalin for subsequent histologic evaluation. Samples of the trunk kidney, spleen, and gills were collected from all 72 fish. Samples of the liver, head kidney, intestines, heart, and brain were collected from fish that survived to the end of the study.

Tissue samples for histologic evaluation were routinely embedded in paraffin, sectioned at a thickness of 3 µm, and stained with H&E for light microscopy. After fixation in neutral-buffered 10% formalin, gill specimens were immersed in 10% EDTA solution for 2 days to induce mild demineralization before processing. Histologic evaluation was performed on 6 vaccinated fish that died following viral challenge exposure and 6 vaccinated fish that survived the viral challenge exposure. No histologic evaluation was performed on unvaccinated fish because CyHV3 lesions have been adequately described.[11,17] One pathologist (GDM) performed all histologic evaluations; that investigator did not have knowledge of vaccination history of the koi or the hypotheses being tested. Fish were assessed for condition variables (ie, food in the gastrointestinal tract, glycogen stores in the liver, and mesenteric fat content) and le-

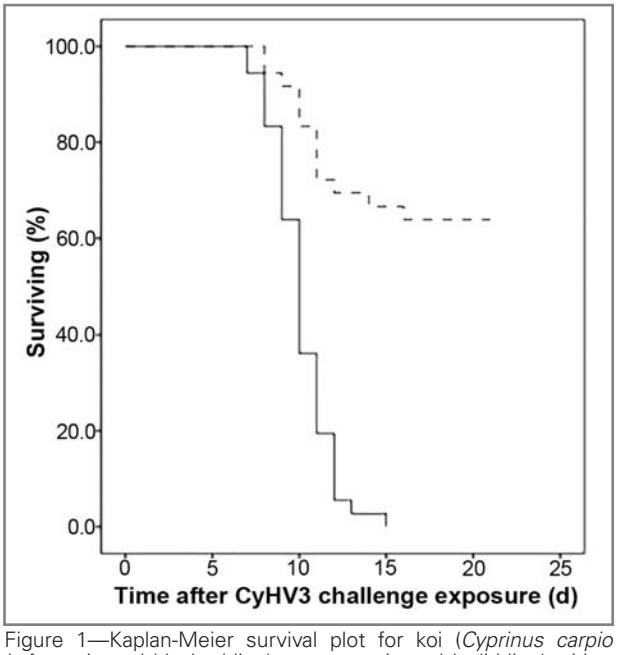

Figure 1—Kaplan-Meier survival plot for koi (*Cyprinus carpio koi*) vaccinated (dashed line) or not vaccinated (solid line) with a CyHV3 vaccine and challenge exposed to a wild-type CyHV3 13 months after vaccination.

sions. Scores were assigned as follows: 0 = none, 1 = mild or small amounts, 2 = moderate, or 3 = severe or abundant. Methods used had been described earlier for systematic histologic evaluation[18] and optimization of photomicrograph illumination and color balance.[19]

Table 1—Results of an ELISA for CyHV3 in koi (*Cyprinus carpio koi*) vaccinated with a CyHV3 vaccine and challenge exposed to a wild-type CyHV3 13 months after vaccination.

Variable	Before challenge exposure (n = 36)	After challenge exposure (n = 23)*
Optical density		
Mean ± SD	0.121 ± 0.060	0.464 ± 0.179
Median	0.104	0.396
Range	0.063–0.280	0.201–0.851
Positive results (No. [%])	5 (14)	23 (100)

*Blood samples were obtained from the 23 fish that survived for 21 days after challenge exposure; samples were not obtained from 13 fish that died following challenge exposure.

Table 2—Results of a quantitative PCR assay to detect CyHV3 in samples obtained from unvaccinated control koi and koi vaccinated with a CyHV3 vaccine and challenge exposed to a wild-type CyHV3 13 months after vaccination.

Viral load category	Cycle threshold	Unvaccinated (n = 36)	Vaccinated (n = 36)
Not detected	—	0	24
Weak positive	39.0–28.0	3	3
Strong positive	27.9–20.0	29	8
Very strong positive	< 20.0	4	1

— = Not applicable.

Genomic DNA was extracted by use of a kit[g] and used for quantitative PCR assay. Concentrations of DNA were normalized to 50 ng/µL for all samples. Quantitative PCR assay was performed by use of specific primers (KHV-86f/KHV-163r) and a CyHV3-specific hydrolysis probe (KHV-109p)[12] that amplifies a 78-bp fragment (GenBank accession No. AF411803). Reactions (12 µL) were performed with a real-time PCR assay system[h] using cycling conditions described elsewhere.[12] A plasmid carrying a 484-bp insert was prepared from University of California-Davis standard CyHV3 strain (KHV-U) as previously described[20] and cloned into a thymine-adenine cloning vector.[i] Serial dilutions (1:10) of plasmid DNA were used to generate a standard curve. Samples were assayed with 10^6 to 10^2 copies of plasmid standards to allow for quantification of CyHV3 DNA per microgram of tissue. The detection limit for the quantitative PCR assay was 10 copies/µg of DNA.[12] The quantitative PCR assay cycle threshold was used to group fish on the basis of viral load as follows: not detected, 39.0 to 28.0 (weak positive), 27.9 to 20.0 (strong positive), and < 20.0 (very strong positive).

Statistical analysis—Means were compared by use of a paired *t* test for independent or paired samples. Proportional mortality rates were compared between vaccinated and unvaccinated koi by use of a Fisher exact test. Mortality rate between groups was compared by use of Kaplan-Meier survival curves and their associated 95% confidence intervals for the survivor function of the 2 groups. Statistical analysis of data was performed with

Table 3—Histopathologic findings in 12 koi vaccinated with a CyHV3 vaccine and challenge exposed to a wild-type CyHV3 13 months after vaccination.

	Died (n = 6)		Survived (n = 6)	
Variable	Mean score	Prevalence (No. [%])	Mean score	Prevalence (No. [%])
Measure of physiologic condition				
Hepatocellular glycogen	0	Inc	2.8	6 (100)
Mesenteric adipose tissue	ND	ND	2.0	6 (100)
Food in gastrointestinal tract	ND	ND	0.7	3 (50)
Pigmented macrophage aggregates				
Kidneys	0.8	5 (83)	1.3	6 (100)
Liver	1.0	Inc	0.8	5 (83)
Spleen	1.2	6 (100)	1.2	6 (100)
Relative area of hematopoietic cells in kidneys	2.0	6 (100)	1.5	6 (100)
Renal tubular generation or regeneration	0.3	2 (33)	1.2	6 (100)
Splenic congestion	1.5	6 (100)	2.4	6 (100)
Lesions and other microscopic findings				
Inflammatory cells in gill arches	0.6	3 (50)	0.7	4 (67)
Ichthyobodo necator in gills	1.2	4 (67)	0	0
Gill lamellar hyperplasia or hypertrophy	1.0	4 (67)	0	0
Monogenean parasites in gills	0.3	3 (33)	0	0
Splenic single cell necrosis	1.2	4 (67)	0	0
Renal tubular epithelial protein	0.7	3 (50)	0	0
Interstitial cell necrosis in kidneys	0.2	1 (17)	0	0
Interstitial cellular phagocytosis in kidneys	0	0	1.3	4 (67)
Endocardial cell hypertrophy	ND	ND	0.7	3 (50)
Lymphohistiocytic endocarditis	0	0	0.3	2 (33)
Intestinal bacterial overgrowth	ND	ND	0.3	1 (17)
Meningoencephalitis	ND	ND	1.0	5 (83)

Fish were categorized on the basis of whether they died within 21 days after challenge exposure or survived for ≥ 21 days after challenge exposure. Scores were assigned as follows: 0 = none, 1 = mild or small amounts, 2 = moderate, or 3 = severe or abundant.

Inc = Incomplete data; samples of the liver were collected from only 1 of the 6 fish, and it had negative results for hepatocellular glycogen but positive results for pigmented macrophage aggregates. ND = Not detected.

statistical software.[J] Values of $P < 0.05$ were considered significant.

Results

The unvaccinated fish became anorectic and developed other nonspecific clinical signs of KHVd within 24 to 48 hours after challenge exposure to the wild-type CyHV3. None of the vaccinated fish were anorectic or had other clinical signs during the first 7 days after challenge exposure. All 36 unvaccinated fish died (mortality rate, 100%); the first fish died 7 days after viral challenge exposure, and mean survival time was 10.1 days (95% confidence interval, 9.5 to 10.6 days; **Figure 1**). In contrast, only 13 vaccinated fish died (mortality rate, 36%); the first vaccinated fish died 8 days after viral challenge exposure, and mean survival time was 17.3 days (95% confidence interval, 15.7 to 19.0 days). Mean survival time differed significantly ($P < 0.001$) between treatment groups. Also, the number of fish that died in each group differed significantly ($P < 0.001$; paired *t* test).

Overall, the vaccine provided significantly ($P < 0.001$; Fisher exact test) better protective immunity against KHVd at 13 months after vaccination, compared with results for unvaccinated control animals. Furthermore, overall health of the 23 surviving vaccinated fish was better than that of the 49 fish that died or became moribund and were euthanized during the study on the basis that a significantly lower proportion of surviving vaccinated fish had external parasites ($P < 0.001$; Fisher exact test) and yielded bacteria during microbial culture of the trunk kidney ($P = 0.002$; Fisher exact test). Regardless of whether fish survived, the proportion of fish with parasites on gill biopsy specimens and skin scrapings was significantly ($P < 0.001$; Fisher exact test) less for the 36 vaccinated fish than for the 36 unvaccinated fish; however, the proportion of fish with positive results for aerobic bacterial cultures did not differ significantly ($P = 0.24$; Fisher exact test) between the vaccinated and unvaccinated fish. There were no significant differences among tanks within each group of fish.

In addition to having better overall health, vaccinated fish had a significant increase in anti-CyHV3 antibody titers following exposure to wild-type CyHV3. Before challenge exposure at 13 months after vaccination, only 5 of 36 (14%) vaccinated koi had positive results

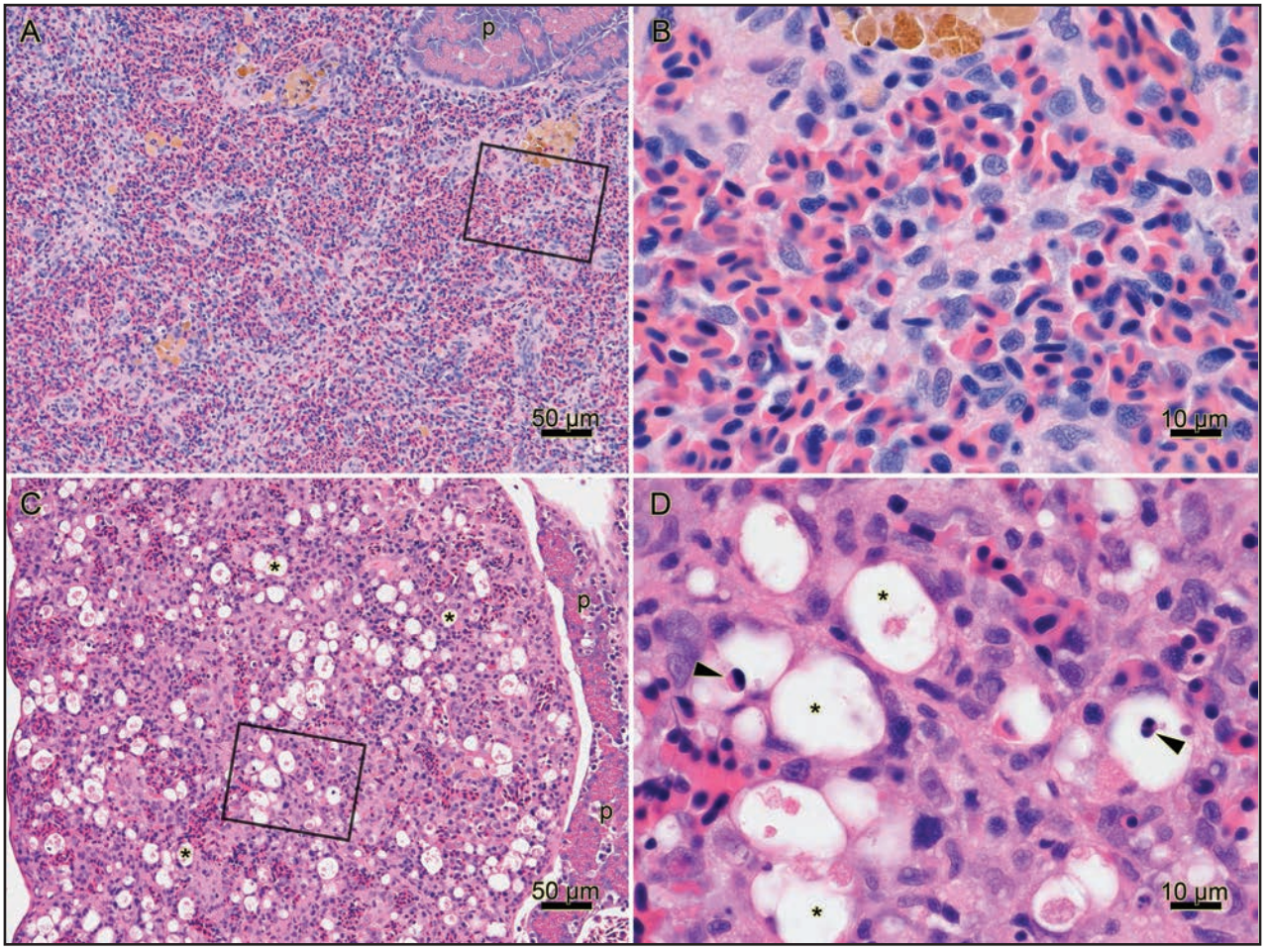

Figure 2—Photomicrographs of tissue sections of spleen from koi that were vaccinated with a CyHV3 vaccine and challenge exposed to wild-type CyHV3 13 months after vaccination. A—Anatomically normal morphology 3 weeks after challenge exposure in a survivor with no detectable CyHV3. Notice the exocrine portion of the pancreas (p). B—Enlargement of the area outlined in the black box in panel A. C—Disseminated single-cell necrosis in a fish that was moribund and euthanized 9 days after challenge exposure (quantitative PCR assay cycle threshold, 25.4). Most nuclei have been lysed, which left only disseminated vacuoles (asterisk). The exocrine portion of the pancreas (p) is indicated. D—Enlargement of the area outlined in the black box in panel C. Notice that a few pyknotic nuclei remain (arrowheads). H&E stain; bar = 50 µm in panels A and C and 10 µm in panels B and D.

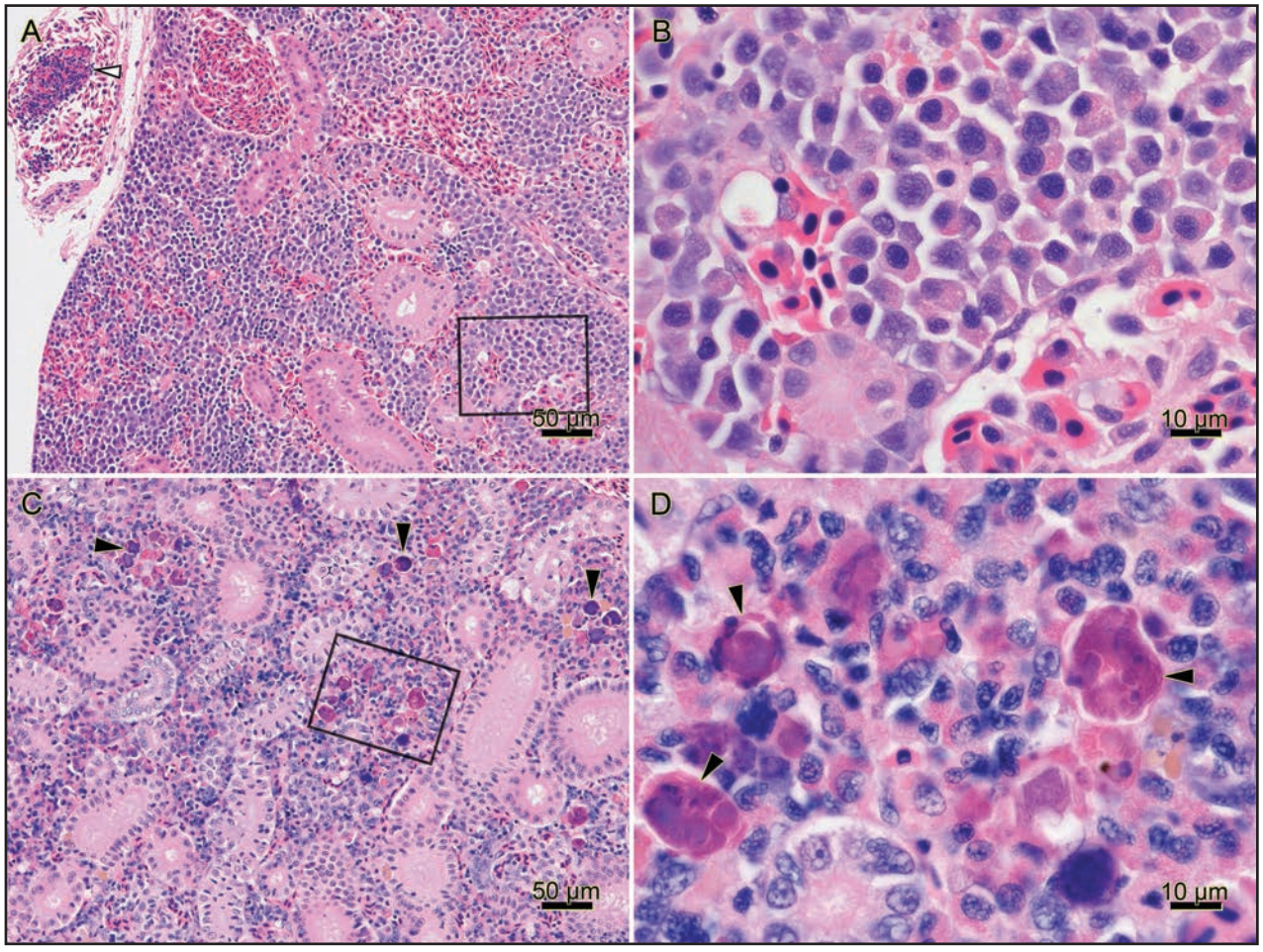

Figure 3—Photomicrographs of tissue sections of trunk kidney from koi vaccinated with a CyHV3 vaccine and challenge exposed to a wild-type CyHV3 13 months after vaccination. A—Notice the moderate interstitial cell hyperplasia but no interstitial cell phagocytosis in a fish that died 12 days after challenge exposure (quantitative PCR assay cycle threshold, 21.5). The vein contains a thrombus (white arrowhead). B—Enlargement of the area outlined in the black box in panel A. C—There is disseminated interstitial cell phagocytosis (black arrowheads) 3 weeks after challenge exposure in a survivor with no detectable CyHV3. D—Enlargement of the area outlined in the black box in panel C. H&E stain; bar = 50 µm in panels A and C and 10 µm in panels B and D.

for the CyHV3 ELISA, and all of these fish had extremely low anti-CyHV3 antibody titers (**Table 1**). For the 23 vaccinated koi that survived, all were seropositive for CyHV3 at the end of the 21-day study period. Antibody titers were not determined for fish that died during the study. Also, the anti-CyHV3 antibody titers at the end of the study were significantly ($P < 0.001$; paired t test) greater than the preexposure titers.

All 23 vaccinated fish that survived to the end of the study were seronegative for CyHV3 on the PCR assay; in contrast, 48 of 49 fish that did not survive until the end of the study were seropositive for CyHV3. Of the 49 fish that did not survive, results for 6 (12%) were weak positive, for 37 (76%) were strong positive, and for 5 (10%) were very strong positive (**Table 2**). All unvaccinated fish had at least weak positive results for CyHV3 on quantitative PCR assay, whereas 24 of 36 (67%) vaccinated fish had negative results for CyHV3 on quantitative PCR assay. Significantly ($P < 0.001$; paired t test) more unvaccinated fish than vaccinated fish had positive results for CyHV3 on the quantitative PCR assay. For fish that died, cycle threshold for unvaccinated fish versus vaccinated fish did not differ significantly ($P = 0.29$; paired t test).

A subset of 12 vaccinated koi (consisting of 6 survivors and 6 fish that died after challenge exposure) was submitted for histologic examination. Microscopically, measures of physiologic condition were mostly considered to be normal for the 6 vaccinated fish that survived the challenge exposure, but the 6 vaccinated fish that died had microscopic evidence of poor health (**Table 3**). Features of healthy fish included moderate to abundant hepatocellular glycogen and mesenteric adipose tissue (fat). Lack of food in the gastrointestinal tract in most of these fish was consistent with the fact that the fish were not fed for approximately 24 hours before they were euthanized and the samples collected. Scores for pigmented macrophage aggregates in these fish were within reference limits for koi of this age and size.[21] Analysis of kidney sections revealed that 4 of the 6 vaccinated fish that died following challenge exposure lacked immature renal tubules (indicative of decreased growth). Scores for erythrocyte stores or congestion in the spleen were lower in those fish than the 6 vaccinated survivors. Also, 5 of 6 vaccinated fish with confirmed CyHV3 infection had microscopic lesions that might have seriously impaired their health: 2 had

moderate or severe single-cell necrosis in the spleen, 2 others had a moderate infestation with *Ichthyobodo necator* in the gills (one of these also had severe lamellar epithelial hyperplasia), and 1 had cells with marginated chromatin consistent with intranuclear inclusion bodies in the trunk kidney and spleen. Four of the 6 vaccinated fish that died had some degree of single-cell necrosis in the spleen, whereas none of the 6 vaccinated fish that survived to the end of the study had that lesion (**Figure 2**). In contrast, 4 of 6 vaccinated survivors had renal interstitial cellular phagocytosis, whereas none of the 6 vaccinated fish that died had this microscopic feature (**Figure 3**).

Discussion

Results of the present study indicated that the modified-live CyHV3 vaccine provided substantial protective immunity to koi against challenge exposure with a wild-type CyHV3 13 months after vaccination. Five vaccinated koi still were seropositive 13 months after vaccination, which is consistent with results of a previous study[14] in which anti-CyHV3 antibody titers slowly decreased during 280 days after vaccination. Despite the lack of a detectable antibody titer in most vaccinated fish prior to challenge exposure, vaccinated koi successfully mounted an immune response to the virus within only 3 weeks after challenge exposure. In addition, 23 of 36 (64%) vaccinated koi challenge exposed to a wild-type CyHV3 had negative results on a quantitative PCR assay for viral DNA at 21 days after exposure, which indicated that the vaccine reduced or prevented viral loads.

One of the most important findings in the present study was that vaccinated koi were able to generate a substantial immune response within 3 weeks after exposure to the wild-type CyHV3. Similar results are common in other vaccinated vertebrate species. For example, vaccinated swine had a secondary immune response following viral challenge exposure 3 weeks after vaccination.[22] In contrast, naïve koi that survived exposure to a wild-type CyHV3 required 9 to 14 weeks to mount a substantial immune response.[23] Antibodies tend to degrade over time, and although 31 of 36 (86%) vaccinated koi in the study reported here did not have detectable amounts of anti-CyHV3 antibodies prior to viral challenge exposure, ultimately 23 of 36 (64%) vaccinated fish mounted a successful immune response and survived the virus challenge exposure at 13 months after vaccination. These findings provided evidence that in addition to the protective immunity of the vaccine, vaccination also resulted in a heightened immune memory and response attributable to previous induction of B cells. Nonetheless, more studies need to be conducted to elucidate the relationship between the use of a modified-live CyHV3 vaccine, antibody production, immunologic responses, and duration of protective immunity in koi.

Although latent virus can be detected by means of quantitative PCR assay in fish infected with wild-type CyHV3,[6] all of the surviving vaccinated koi in the present study had negative results for wild-type CyHV3 on quantitative PCR assay 21 days after challenge exposure. This suggested that the vaccine protected most vaccinated fish and that the wild-type virus was unable to infect and become latent in a proportion of vaccinated koi. Considering that renal interstitial cell phagocytosis (probably of erythrocytes and some leukocytes) was detected only in fish that survived 21 days after challenge exposure, it is possible that phagocytosis might play a role in protective immunity. This finding further demonstrated the potential of the modified-live CyHV3 vaccine.

In the present study, we found that the modified-live CyHV3 vaccine induced substantial protective immunity in koi against exposure to a CyHV3 13 months after vaccination. This information supports a recommendation for annual revaccination on the basis that annual revaccination would significantly reduce mortality and morbidity rates by aiding in the prevention of disease associated with KHVd in koi. On the basis of the results of the present study and another study[11] on the safety and efficacy of the modified-live CyHV3 vaccine, koi caretakers have another means to guard against the introduction of CyHV3 and the severe mortality rates associated with KHVd.

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h. StepOne, Applied Biosystems Inc, Foster City, Calif.
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