Fish & Shellfish Immunology 23 (2007) 657–669

Fish & Shellfish Immunology

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Characterization of serum and mucosal antibody responses in white sturgeon (*Acipenser transmontanus* Richardson) following immunization with WSIV and a protein hapten antigen

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Received 28 August 2006; revised 19 January 2007; accepted 22 January 2007
Available online 1 February 2007

Abstract

Serum and cutaneous mucus antibodies were monitored in white sturgeon for 15 weeks following intraperitoneal immunization. Ten fish were immunized (50 μg) with white sturgeon iridovirus (WSIV) or white sturgeon gonad (WSGO) tissue culture cells emulsified with or without FCA. An additional group was immunized with FITC:KLH + FCA. Fish were booster immunized at 6 weeks. Fish immunized with FITC:KLH + FCA produced significant serum antibodies to FITC by 6 weeks and this response peaked at 12 weeks (average titer 31,000). Mucosal antibodies to FITC were first detected at 12 weeks and significantly elevated by 15 weeks (average titer 18). Anti-WSIV antibody titers were detected in the serum by 9 weeks in fish immunized with WSIV and WSIV + FCA, but only a small number responded to immunization. At 15 weeks, four fish immunized with WSIV produced serum antibodies (average titer 838) and one fish immunized with WSIV + FCA had a serum titer of 1600. Mucosal anti-WSIV antibody titers of 8 and 16 were observed in two fish from the WSIV group at 12 weeks while four different fish from this group responded at 15 weeks (average titer 4). Western Blot using a monoclonal antibody confirmed immunoglobulin in mucus, and specificity to WSIV was further demonstrated by immunocytochemistry using serum from fish immunized with WSIV. Specific antibody was not detected in mucus of fish immunized with WSIV + FCA, WSGO, or WSGO + FCA. Collectively, these experiments demonstrate that white sturgeon can generate a specific antibody response following immunization, and is the first report showing mucosal immunoglobulin is present in this species.

Keywords: Sturgeon; White sturgeon iridovirus; WSIV; FITC; Vaccination; Antibodies; Mucus; Serum, Immunocytochemistry

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doi:10.1016/j.fsi.2007.01.016

1. Introduction

White sturgeon, *Acipenser transmontanus* (Richardson), are commonly cultured for production of caviar and meat as well as for recovery of depleted wild populations. In the United States, the major commercial producers of sturgeon are located in California and Idaho and production has increased steadily over the past decade. Approximately 544 metric tons of farm-raised white sturgeon were processed for meat and 3.6 metric tons of caviar were produced in 2000 [1]. Furthermore, white sturgeon aquaculture for conservation enhancement purposes has increased. One such program is aimed at the recovery of the endangered Kootenai River white sturgeon in northern Idaho [2,3].

The establishment of culture techniques and subsequent intensive production of white sturgeon has resulted in the identification of previously unknown viral pathogens capable of causing high mortality. Most notably, these include white sturgeon iridovirus (WSIV) [4], Acipenserid herpesvirus-1 [5], Acipenserid herpesvirus-2 [6], and to a lesser degree white sturgeon adenovirus [7]. Of these newly identified viruses, WSIV has gained much attention due to its ability to impair growth and cause serious losses, upwards of 95% mortality, in sturgeon fry and fingerlings in both commercial and conservation aquaculture programs [4,8]. Sturgeon can also be impacted by bacterial diseases such as *Aeromonas salmonicida* [9], *Aeromonas hydrophila* [10], as well as *Flavobacterium* spp. [11]. The increase in sturgeon aquaculture presents new challenges to successfully rear these fish while minimizing disease outbreaks and mortality associated with these pathogens.

Vaccination is a tool that could be added to a fish health management program to protect sturgeon from disease outbreaks. However, the ontogeny and components of the sturgeon adaptive immune system have not been well characterized in comparison to Chondrichthyes (sharks) and teleost fishes [12–14]. The sturgeon immune system is unique in that it possesses lymphomyeloid tissue around the heart and cranium in addition to hematopoietic tissues of the kidney, spleen, and thymus as in teleost fish [15]. The predominant immunoglobulin (Ig) isotype is the IgM class and it has been shown to have a tetrameric structure [16].

A few past studies have demonstrated the ability of sturgeon to elicit specific immune responses against pathogens. Complete protection against motile *Aeromonas* septicemia in Persian sturgeon *Acipenser persicus* (Borodin) was shown following immersion vaccination with heat-killed *Aeromonas hydrophila* [10]. White sturgeon experimentally immersion challenged with Acipenserid herpesvirus-2 developed serum anti-viral neutralization activity [6]. Also, Siberian sturgeon *Acipenser baeri* (Brandt) have been shown to produce an increase of serum anti-*Aeromonas salmonicida* agglutinating titers following immunization [9]. These studies show sturgeon are capable of mounting an adaptive immune response following immunization or challenge. This information also suggests vaccination could be an important tool for use in sturgeon aquaculture to provide protection from certain pathogenic diseases.

As aquaculture of white sturgeon increases, so does the need to better understand their ability to mount a specific immune response(s) to pathogens, which may aid in the development of better disease control strategies and will determine the feasibility of vaccination for disease prevention. The recent development of a monoclonal antibody specific for sturgeon immunoglobulin (Ig) has given us the opportunity to more closely investigate the adaptive humoral immune response in white sturgeon. Previous application of this monoclonal antibody using an ELISA assay demonstrated white sturgeon generated increased serum antibodies to dinitrophenyl (DNP) after 3 and 6 weeks following immunization with a hapten carrier molecule (dinitrophenyl-keyhole limpet hemocyanin) DNP-KLH [16]. The objective of this study was to further characterize this type of response in white sturgeon by mapping serum and cutaneous mucosal antibody responses over a 15-week period following immunization against WSIV. Because of difficulties in obtaining large quantities of virus and limited knowledge on the necessary dose needed to elicit an antibody response, white sturgeon were also immunized with fluorescein isothiocyanate (FITC) conjugated to KLH since past studies have shown this antigen complex to be highly immunogenic in rainbow trout *Oncorhynchus mykiss* (Walbaum) [17]. Serum and mucosal antibody titers from individual fish were quantified by the development of two different indirect ELISAs to capture anti-WSIV or anti-FITC antibodies. Results from this study further demonstrate that white sturgeon are capable of generating specific serum antibodies to different antigens following immunization and reveal for the first time that the adaptive immune response can elicit the production of specific antibodies in cutaneous mucus.

2. Materials and methods

2.1. Fish and rearing conditions

Two-year-old white sturgeon (mean weight approximately 125 g) were maintained on pathogen free de-chlorinated municipal water at 15 °C in a 0.28-m^3 (0.22 m × 0.053 m × 0.023 m) flow-through trough supplemented with air-stones for aeration. The fish were fed a commercial diet (Rangen Inc., Buhl, ID, USA) at 1% body weight per day. These fish originated from the Kootenai Tribal Conservation Sturgeon Hatchery (Bonners Ferry, ID, USA) as eggs from a single mating cross. Prior to transport to the University of Idaho in 2002, eggs were surface disinfected with iodine (Argentyne, Argent Chemical Laboratories, Redmond, WA, USA) for 10 min. The eggs were incubated and hatched in virus-free water and the resulting progeny were monitored for the presence of WSIV. Previous transmission studies showed that these fish were not infected with WSIV [18]. Histopathology examinations performed on these fish showed no evidence of lesions associated with WSIV infection and no disease outbreaks occurred during this study.

2.2. Antigen preparation

White sturgeon iridovirus, white sturgeon gonad (WSGO) tissue culture cells, and FITC:KLH antigen were used for the immunization of white sturgeon. The protein concentration of these antigens was determined using the Micro BCA™ Protein Assay according to the manufacturer's instructions (Pierce, Rockford, IL, USA) and diluted in phosphate buffered saline (PBS) to the appropriate concentrations. Immunizations involving the addition of Freund's complete adjuvant (FCA) were prepared by mixing equal volumes of antigen and FCA through two connected syringes until fully emulsified.

2.2.1. Virus

White sturgeon iridovirus (Abernathy isolate) was originally isolated from a diseased juvenile white sturgeon from the Abernathy Fish Technology Center (Longview, WA, USA) and repeatedly passed by previously described methods [19]. Viral antigen was obtained by inoculating two 150-cm^2 flasks (Falcon, BD Biosciences, Bedford, MA, USA) containing WSGO tissue culture cell monolayers with pass 3 of WSIV. Complete cytopathic effect (CPE) was observed after 113 days of incubation at 15 °C. Cells were harvested using a scraper and combined with supernatant (media). Contents were transferred to a 250-ml centrifuge bottle, freeze-thawed one time at −80 °C, homogenized (Tissue Tearor, Model 398, Biospec Products Inc., Bartlesville, OK, USA) for 2 min, and centrifuged at 5800 × g in a GSA rotor (Sorvall) for 30 min at 4 °C. The pelleted material was discarded. Virus remaining in the supernatant (approximately 127 ml) was then concentrated by high-speed centrifugation at 27,500 rpm for 60 min at 4 °C using two swinging buckets from a SW 28 rotor (Beckman Coulter, Fullerton, CA, USA). Following centrifugation, all but 0.5 ml of supernatant was discarded from each bucket and incubated at 4 °C overnight to soften the pelleted virus. Virus was then pooled and resuspended by titration and heated to 56 °C for 60 min to inactivate virus [20]. Viral antigen was then stored at −80°C until used for the immunizations.

2.2.2. WSGO cells

Since propagation of WSIV was performed using WSGO cells and virus was considered only partially purified by centrifugation, we included WSGO cells alone as a negative control. Confluent WSGO cells from one 150-cm^2 flask were disrupted using a scraper, combined with media (MEM-10; minimal essential medium with Earle's salts supplemented with 10% fetal bovine serum, 50 IU ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and 2 mM L-glutamine) and processed in a similar manner previously described for preparing the virus, except that centrifugation was not performed.

2.2.3. FITC

The hapten fluorescein isothiocyanate (FITC) (Sigma-Aldrich, St. Louis, MO, USA) was conjugated to KLH (Sigma-Aldrich) following previously described methods [21]. Briefly, 20 mg of KLH was combined with 10 mg of FITC-cellite in 400 μl of 0.25 M carbonate/bicarbonate (pH 9.0) and mixed for 12 h at 4 °C. Cellite was removed by centrifugation at 10,000 × g and the remaining supernatant was loaded onto a Sephadex G-25 fractionation column

and eluted with 10 ml of PBS + 0.02% sodium azide to remove unbound FITC. The conjugate was dialyzed against PBS overnight at 4 °C. The FITC:KLH ratio was determined to be 3.2:1 based on the equation; FITC:protein = $2.87 \times OD_{495}/OD_{280} - (0.35 \times OD_{495})$.

2.3. Immunization

White sturgeon ($N = 50$) were anaesthetized by immersion in 4 l of water containing 150 mg l^{-1} tricaine methane sulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA, USA) for approximately 1 min before immunization and sampling. Each fish was individually labeled with a passive integrated transponder by intradermal placement behind the 3rd scute from the left pectoral fin for identification. Ten sturgeon were immunized by intraperitoneal injection with 100 μl containing 50 μg of either WSIV + FCA, WSIV only, WSGO cells + FCA, WSGO cells only, or FITC:KLH + FCA. A 1 cm^3 tuberculin syringe with a 23-gauge needle was used for all injections. Sturgeon from each treatment group received a booster immunization 6 weeks following primary immunization using identical antigen preparations.

2.4. Sampling mucus and serum

Cutaneous mucus and blood were collected from individual sturgeon from each treatment group prior to immunization and at 3-week intervals for the duration of the 15-week study. After being anaesthetized, each fish was placed into a plastic bag and mucus was allowed to accumulate for approximately 1 min and then collected. Mucus was then transferred to a 2.0-ml microfuge tube, centrifuged at 15 000 × *g* for 10 min, and the supernatant was then collected. Blood samples (200 μl) from each fish were obtained by caudal puncture using a 1-cm^3 tuberculin syringe with a 23-gauge needle and transferred to a sterile 1.5-ml microfuge tube. Blood was allowed to clot overnight at 4 °C. Serum was collected from blood samples by centrifugation at 10 000 × g for 10 min at 4 °C. Both mucus and serum samples from each sturgeon were frozen at −20 °C until needed for ELISA, Western blot analysis, or immunocytochemistry.

2.5. FITC ELISA

Fluorescein isothiocyanate conjugated to bovine serum albumin (BSA) was prepared in a similar manner as previously described [21] and was used as coating antigen to capture FITC-specific antibody from serum or mucus samples by ELISA. Immulon II high binding 96-well plates (Thermo Labsystems, Franklin, MA, USA) were coated with 0.3 μg per well FITC:BSA in 100 μl of carbonate/bicarbonate buffer (pH 9.6) for 8 h at 15 °C. Following incubation, buffer was removed and wells were blocked with 200 μl of PBS + 1% BSA for 60 min at room temperature. Serum or mucus samples from sturgeon immunized with either FITC:KLH + FCA or WSGO cells + FCA were initially diluted 1/800 or 1/2, respectively, in PBS + 0.02% sodium azide and serially diluted (2-fold) into wells (100 μl per well) in duplicate. Samples were incubated overnight at 15 °C. Positive and negative controls were also included on each ELISA plate. The positive control was serum from a FITC:KLH + FCA immunized sturgeon sampled on week-15 exhibiting a high antibody endpoint titer of 102,800. Equal volumes of serum or mucus collected from sturgeon prior to immunization were pooled and served as the negative control. Following overnight incubation, wells were rinsed five times with PBS + 0.05% $Tween_{20}$ (PBS + T) to remove unbound antibody and 100 μl of monoclonal anti-sturgeon Ig diluted 1/100 in PBS-T was added to each well and incubated for 60 min at room temperature. Wells were rinsed in a similar manner as previously described and then incubated with 100 μl of horseradish peroxidase (HRP) conjugated goat anti-mouse Ig (Calbiochem, San Diego, CA, USA) diluted 1/2000 in PBS + T for 60 min at room temperature. After another rinse, ABTS® peroxidase substrate system was mixed following the manufacturer's protocol (Kirkegaard & Perry Laboratories, Gaitherburg, MD, USA) and 100 μl per well was added for colorimetric development. Serum and mucus ELISA assays were allowed to develop for 15 and 75 min at room temperature, respectively. The optical density (OD) of each well was determined by using a microplate autoreader (Bio-Tek Model EL 312E, Bio-Tek Instruments Inc., Winooski, VT, USA) at 405 nm. The reciprocal of the highest dilution having an OD at least two times the negative control was defined as the antibody endpoint titer for each serum or mucus sample.

2.6. WSIV ELISA

An ELISA assay was developed to quantify specific anti-WSIV antibody in serum and mucus of sturgeon from treatment groups immunized with WSIV + FCA, WSIV only, WSGO cells + FCA or WSGO cells only. Sturgeon treatment groups immunized with WSGO cells + FCA, or WSGO cells served as negative controls. Additionally, equal volumes of serum or mucus collected from fish prior to immunization from all four treatment groups was pooled and served as additional negative controls. One 150-cm^2 flask containing WSGO cells infected with WSIV (pass 6) was used for the development of the ELISA. Following CPE, approximately 44 ml of infected cells and supernatant was harvested and contents were sonicated using a Fisher Scientific model 50 Ultrasonic Dismembrator (Santa Clara, CA, USA). Sonication was performed on ice and involved three 10-s on/off pulses using 2.5-mm diameter tapered microtip with the instrument set at 40% amplitude followed by two 30-s on/off pulses at 50% and 60% amplitude. Protein concentration of sonicated WSIV was determined and wells of a 96-well ELISA plate were coated with 6.2 μg in 100 μl of carbonate/bicarbonate buffer for 8 h at 15 °C. Incubations, wash steps and application of both monoclonal anti-sturgeon Ig and goat anti-mouse-HRP were similar to previously described steps for the development of the FITC ELISA. Serum or mucus samples were initially diluted at 1/50 or 1/2, respectively, in PBS + 0.02% sodium azide and then serially diluted (2-fold) into wells (100 μl per well) in duplicate. For the development of serum and mucus ELISA plates, plates were incubated for 30 and 45 min, respectively, with ABTS® peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaitherburg, MD, USA) for colorimetric development to determine antibody endpoint titer for each sturgeon. The reciprocal of the highest dilution having an OD at least two times the negative control was defined as the antibody endpoint titer for each serum and mucus sample.

2.7. SDS-PAGE and Western blot analysis

Serum and cutaneous mucus samples collected at 15 weeks post-immunization from one white sturgeon immunized with FITC:KLH + FCA were separated by SDS-PAGE according to the method of Laemmli [22] in a Mini-Protean 3 electrophoresis apparatus (Bio-Rad Laboratories, Hercules, CA, USA) as outlined in the manufacturer's instructions. Prior to electrophoresis, approximately 600 μl of mucus was concentrated to a final volume of 100 μl by evaporation (Labconco CentriVap Concentrator, Kansas, MO, USA). Serum and concentrated mucus were diluted in Laemmli sample buffer containing a reducing agent (40 mM DTT) and boiled for 5 min. Following boiling, approximately 250 μg and 180 μg of serum and mucus protein, respectively, were loaded into pre-cast 10–20% polyacrylamide gradient gels (Bio-Rad Laboratories, Hercules, CA, USA) and were electrophoresed for 90 min at a constant 125 V.

Proteins resolved by SDS-PAGE were transferred to a nitrocellulose membrane by electrophoresis for 60 min at 100 V constant using a Mini Trans-blot electrophoretic transfer cell (Bio-Rad, Hercules Laboratories, CA, USA) according to the manufacturer's instructions. Following transfer, the nitrocellulose membrane was blocked in PBS containing 4% non-fat dry milk (Bio-Rad Laboratories, Hercules, CA, USA) for 2 h at 15 °C and incubated overnight at 15 °C with a monoclonal anti-sturgeon Ig diluted 1:100 in PBS + T containing 0.02% sodium azide. After incubation, the membrane was washed three times with PBS + T and incubated for 60 min at room temperature with alkaline-phosphatase conjugated goat anti-mouse Ig (Bio-Rad Laboratories, Hercules, CA, USA) diluted 1:500 in PBS + T. Following three more washes with PBS + T to remove unbound antibody, the membrane was incubated with the substrate 5-bromo-4-chloro-3-indolylphosphate toluidine salt and 4-nitro blue tetrazolium chloride as outlined in the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA) to visualize immunoreactivity. The membrane was washed in distilled water to stop color development.

2.8. Immunocytochemistry

White sturgeon gonad tissue culture cells infected with the Abernathy isolate of WSIV (pass 6) and showing 95% CPE after 135 days of incubation were harvested from a single 150-cm^2 tissue culture flask (Falcon) using a cell scraper and transferred to a 50-ml conical tube. Uninfected WSGO cells were harvested in a similar manner. Approximately 1 ml of each cell suspension was transferred to a 1.5-ml microfuge tube. Cells were washed three times by centrifugation at 5000 × *g* for 10 min at 4 °C followed by resuspension of the cell pellets in 1 ml of PBS by titration. After the third wash, cells were resuspended in 500 μl of PBS and quantified using a hemacytometer. Approximately 3.2×10^3 cells (10 μl) were aliquoted onto Probe-On Plus microscope slides (Fisher Scientific) and allowed to dry for

60 min at room temperature. Cells were then fixed with chilled in acetone for 3 min and allowed to dry for at least 2 h. Dried cell preparations on each microscope slide were circled using a PAP-PEN (Daido Sangyo Co. Ltd., Tokyo, Japan) and hydrated by submersion in PBS for 4 min.

Serum collected 15 weeks post-immunization from white sturgeon immunized with WSIV and showing an elevated antibody response to WSIV by ELISA or serum collected 15 weeks post-immunization from white sturgeon immunized with WSGO cells were used to probe cell preparations. Additionally, both cell preparations were incubated with pre-immune serum collected from white sturgeon prior to immunization with WSIV. Cell preparations were probed with 100 μl of each serum sample diluted 1:20 in PBS and incubated for 60 min at 15 °C. Unbound antibody was removed by three rinses in PBS for 4 min each. Cell preparations were then probed with 100 μl of the anti-sturgeon Ig monoclonal antibody diluted 1:10 in PBS for 60 min at 15 °C. Unbound antibody was removed with three rinses in PBS for 4 min each. Colorimetric detection was done using a Dako Envision system (AEC) kit (Dako Corporation, Carpinteria, CA, USA) following the manufacturer's instruction. This kit utilizes HRP labeled goat anti-mouse antibodies and positive detection is visualized by the appearance of a red stain following incubation of substrate for 5 min and 30 s. In addition to the serum samples previously mentioned, both cell preparations were probed with a monoclonal antibody that was developed by Mark Adkison (California Department of Fish and Game, Rancho Cordova, CA, USA) and kindly provided by Dr Ronald Hedrick (University of California, Davis, CA, USA). This monoclonal antibody was made against WSIV and designated III-A11. It recognizes viral protein bands in the 25–37 kDa range by Western blot analysis (data not shown). This monoclonal antibody was diluted 1:5000 in PBS and served as a positive control for the detection of virus infected cells. Also, both cell preparations were probed with the anti-sturgeon Ig monoclonal antibody diluted 1:10 in PBS or HRP conjugated goat anti-mouse antibody supplied from the DAKO kit to evaluate non-specific immune reactivity.

2.9. Statistical analysis

Statistical significance of serum and mucosal antibody responses specific for WSIV antigen between immunization treatment groups at each sampling time point were assessed by one-way analysis of variance (ANOVA) using GraphPad Prism statistical software (GraphPad Software Inc., San Diego, CA, USA). If differences were detected, additional post-hoc analysis was performed using paired comparisons between treatment groups using Tukey's test. Statistical analysis of serum and mucosal antibody responses specific for FITC antigen at each sampling time point between sturgeon immunized with FITC + FCA or WSGO + FCA was performed by two-sample *t*-test. Values were considered significantly different at *p*-values <0.05.

3. Results

3.1. FITC specific serum and mucus antibodies

Throughout the 15-week study, no mortality occurred and sturgeon from all treatment groups appeared healthy and increased in weight (data not shown). Sturgeon immunized with FITC:KLH + FCA produced a strong serum antibody response to FITC antigen over the course of the 15-week study (Fig. 1). Results from the ELISA assay showed that sturgeon from this treatment group generated an average antibody endpoint titer that was significantly elevated at 6, 9,12, and 15 weeks ($p < 0.05$) compared to sturgeon immunized against WSGO + FCA (controls). Prior to the booster immunization at 6 weeks, only four sturgeon showed an antibody response with titers averaging 800. However, all ten sturgeon responded following booster immunization. Average serum antibody titer of 31 000 was highest at 12 weeks post-immunization with only a slight decrease to 29 700 observed at 15 weeks. Individual sturgeon antibody titers during these last two sampling periods ranged from 1000 to 102 400. No detectable antibody response to FITC was observed in the controls throughout the course of this study, except during week 3 where two sturgeon exhibited background titers of 800.

Specific anti-FITC antibody titer of 8.2 in the mucus of white sturgeon immunized with FITC:KLH + FCA was observed at 12 weeks post-immunization (Fig. 1). This increase in antibody production was detected in seven sturgeon during this sampling period. At 15 weeks post-immunization, mucosal anti-FITC antibodies were observed in all ten sturgeon and the average titer of 17.6 was significantly elevated ($p < 0.05$) from the control group. Individual antibody responses ranged from 2 to 64. No detectable antibody response to FITC was observed in the mucus from the controls

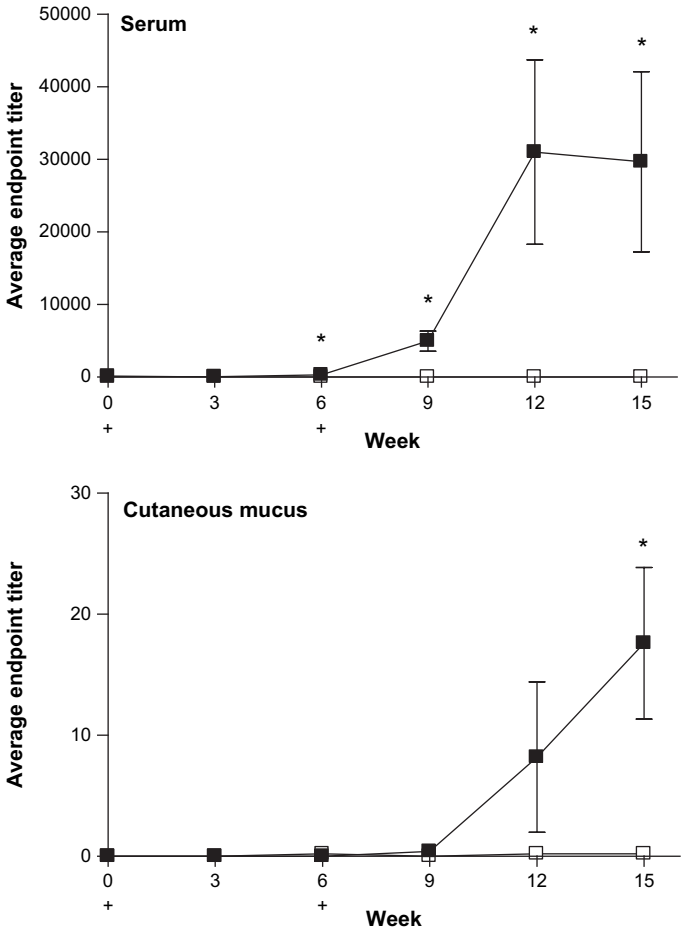

Fig. 1. FITC-specific antibody titers in serum and cutaneous mucus of white sturgeon immunized by intraperitoneal injection with FITC:KLH (■) emulsified with Freund's complete adjuvant (FCA) or white sturgeon gonad cells (WSGO) + FCA (□). Antibody titers are given as the reciprocal of the dilution producing 2× the optical density of pre-immune sera or mucus. Mean antibody endpoint titers ± SEM ($N = 10$) are shown. Primary and booster immunizations (+) were administered for each treatment at 0 and 6 weeks. Asterisks indicate significant differences ($p < 0.05$) between treatment groups.

throughout the course of this study, except in one fish at 12 weeks and one fish at 15 weeks that exhibited background titers of 2.

3.2. WSIV specific serum and mucus antibodies

An increase in the production of serum antibodies specific for WSIV antigen was observed in sturgeon from groups immunized with WSIV or WSIV + FCA (Fig. 2). However, no significant differences of average antibody titers were observed between any of the immunization groups. These results are most likely due to the low number of sturgeon responding to immunization. During the first 6 weeks after initial immunization, a serum antibody response of 50 was detected in one fish immunized with WSIV (without FCA). Following booster immunization, antibody endpoint titers of 400, 100, and 50 were detected in three other fish from this group at 9 weeks post-immunization. At 12 weeks post-immunization these same fish had titers of 400, 100, and 800, respectively, and the fish having a titer of 50 at 3 weeks had a titer of 800. At 15 weeks post-immunization only these four fish had detectable endpoint titers of 100, 50, 1600, and 1600, respectively. In the group immunized with WSIV + FCA, only one fish had a detectable endpoint titer of 200 at 9 weeks post-immunization. The titer of this fish increased to 1600 at 12 weeks and remained at this level at 15 weeks post-immunization. Another fish from this group had a detectable titer of 200 at 12 weeks that increased to

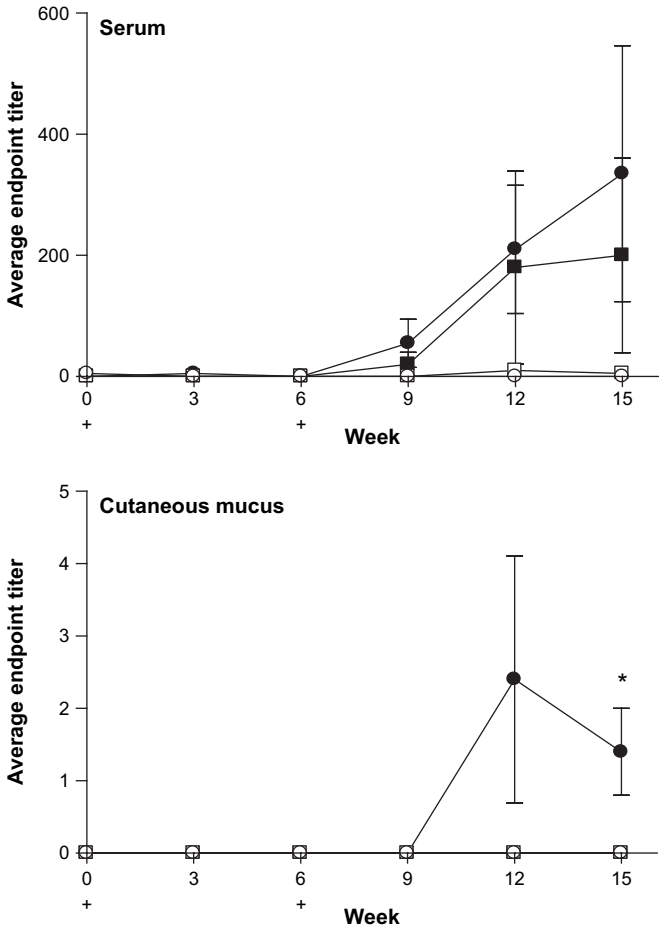

Fig. 2. Detection of WSIV-specific antibody titers in serum and cutaneous mucus of white sturgeon immunized by intraperitoneal injection with WSIV (●), WSIV + FCA (■), WSGO cells (○), or WSGO cells + FCA (□). Antibody titers are given as the reciprocal of the dilution producing 2× the optical density of pre-immune sera or mucus. Mean antibody endpoint titers ± SEM ($N = 10$) are shown. Primary and booster immunizations (+) were administered for each treatment at 0 and 6 weeks. Asterisks indicate significant differences ($p < 0.05$) when compared to other treatment groups.

400 at 15 weeks post-immunization. Sturgeon immunized with WSGO cells showed no detectable antibody titers throughout the study, except one sturgeon had an endpoint titer of 50 prior to initial immunization. Additionally, one sturgeon from the treatment group immunized with WSGO cells + FCA produced antibody endpoint titers of 100 and 50 at 12 and 15 weeks, respectively.

Only sturgeon immunized with WSIV generated a mucus antibody response specific for WSIV antigen during the 15-week study (Fig. 2). Antibody endpoint titers of 8 and 16 were first observed in two fish at 12 weeks post-immunization. Interestingly, no detectable antibody titers were observed in these two fish at 15 weeks but titers of 4, 4, 4, and 2 were observed in four different fish at 15 weeks post-immunization. The average mucus antibody titer at 15 weeks was significantly higher ($p < 0.05$) than other immunization treatment or control groups. Of the six sturgeon that had detectable mucus antibody titers at 12 and 15 weeks post-immunization, only three also had detectable serum antibody titers towards WSIV.

3.3. Western blot of serum and mucus

Serum and concentrated cutaneous mucus from a single white sturgeon were probed with a monoclonal anti-sturgeon Ig by Western blot assay. The results from this assay revealed the presence of a 27–30 kDa band in both

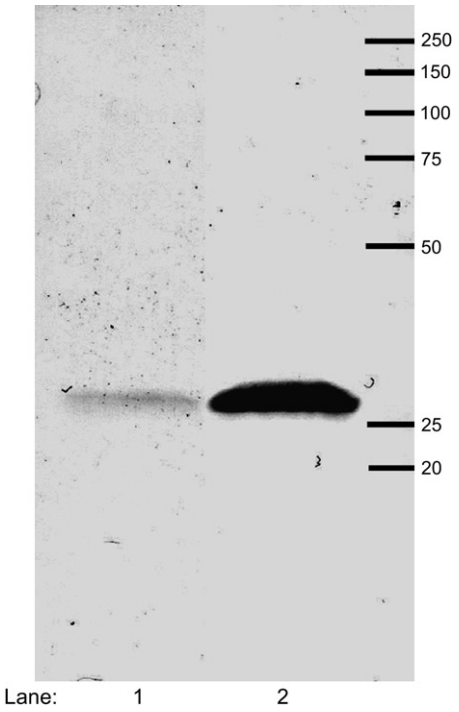

Fig. 3. Western blot analysis of white sturgeon cutaneous mucus (lane 1) and serum (lane 2) probed with monoclonal antibody II-32 specific for white sturgeon immunoglobulin light chain. Molecular mass markers (kDa) are indicated on the right.

samples corresponding to the light chain of white sturgeon IgM (Fig. 3). These results confirm the presence of Ig in cutaneous mucus of white sturgeon.

3.4. Immunocytochemistry

Infection of WSGO tissue culture cells with WSIV was determined by positive staining of numerous cells when virus infected cell preparations were probed with the anti-WSIV monoclonal antibody (Fig. 4). Detection of viral antigen appeared to be localized around the outer edges of infected cells. A similar pattern of staining was observed when infected cells were probed with serum collected from white sturgeon immunized with WSIV at 15 weeks post-immunization. This demonstrated the specificity of the immune response, which was further demonstrated by the absence of staining when uninfected WSGO cell preparations were probed using the same serum sample or the anti-WSIV monoclonal antibody. Additionally, no staining was observed when using serum collected at 15 weeks post-immunization from control sturgeon immunized with WSGO cells or pre-immune serum collected from fish prior to immunization with WSIV. Staining was also absent when both cell preparations were probed with the monoclonal against sturgeon Ig or HRP conjugated goat anti-mouse detection antibody supplied from the DAKO kit.

4. Discussion

In this study, generation of specific serum and cutaneous mucus antibody responses against FITC or WSIV in white sturgeon was investigated for 15 weeks following immunization. Fish receiving a primary immunization with FITC:KLH + FCA followed by booster immunization at 6 weeks generated a strong serum antibody response against the FITC molecule that continued to increase until peaking at 12 weeks when it remained elevated until the end of the study (Fig. 1). The observed heightened antibody response following booster immunization increased almost 16-fold between 6 and 9 weeks. This observation is suggestive of a secondary antibody response [23]. In addition, the antibody response peaked at around 12 weeks, which agrees with responses observed in rainbow trout immunized against

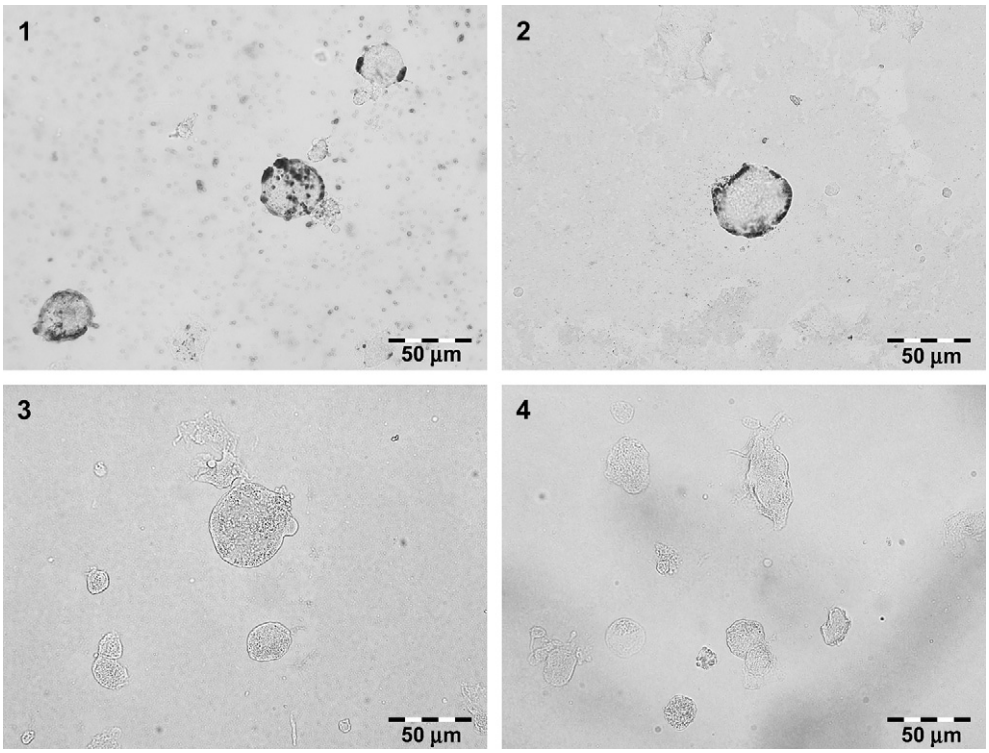

Fig. 4. Detection of white sturgeon iridovirus (WSIV) in infected white sturgeon gonad (WSGO) tissue culture cells by immunocytochemistry. Virus infected cells were detected when probed with (1) the monoclonal antibody (III-A11) made against WSIV and (2) serum collected at 15 weeks from white sturgeon immunized with WSIV. No staining was observed when infected cells were probed with (3) serum collected after 15 weeks from white sturgeon immunized with WSGO cells or (4) serum collected from white sturgeon prior to immunization with WSIV.

various antigens and held at similar temperatures [17,24,25]. These results support the idea that these primitive cold-water fish species can generate similar adaptive humoral immune responses to their less primitive counterparts.

Although antibodies were detected, a low number of white sturgeon responded to immunization with WSIV or WSIV + FCA groups. This low number of responders likely resulted in the lack of statistical significance observed and makes it somewhat difficult to draw conclusions on the generation of anti-WSIV antibodies over time. However, serum antibody titers against WSIV following booster immunization were detected in a greater number of fish. This response was similar to that observed for fish immunized with FITC:KLH + FCA, and demonstrates that an adaptive humoral immune response to WSIV was triggered.

In the present study, detection of specific antibodies measured by ELISA was observed in the cutaneous mucus of white sturgeon following immunization. The mucus layer on the epithelial surfaces of fish forms a physical barrier to the surrounding environment and is considered the first line of defense against horizontally transmitted pathogens [26–28]. The first report demonstrating the presence of antibody in the mucus of teleost fish was in 1969 following immunization of plaice *Pleuronectes platessa* (Linnaeus) with various antigens [29]. A few years later (1971), the presence of IgM antibodies in the mucus of gar *Lepisosteus platyrhincus* (DeKay) was observed [30]. Since then, investigations of the mucosal immune system of fish have demonstrated an increase in specific mucosal antibodies following immunization in a number of fish species (mainly teleost fishes) including rainbow trout [31], catfish *Ictalurus punctatus* (Rafinesque) [32], carp *Cyprinus carpio* (Linnaeus) [33], tilapia *Oreochromis mossambicus* (Peters) [34], brook trout *Salvelinus fontinalis* (Mitchill) [35], ayu *Plecoglossus altivelis* (Temminck & Schlegel) [36], grouper *Epinephelus coioides* (Hamilton) [37] and sea bass *Dicentrarchus labrax* (Linnaeus) [38]. Specific antibody against FITC and WSIV were detected in the mucus of white sturgeon in this study. In addition, Western blot analysis of cutaneous mucus using a monoclonal antibody against sturgeon Ig confirmed the presence of Ig and resulted in the detection of a single 27–30 kDa protein band that is consistent with the light chain of sturgeon Ig found in serum [16].

Results from the ELISA assays identified a delay in the mucus antibody response when compared to the serum antibody response. Mucus anti-FITC antibodies did not increase until approximately 6 weeks after detection of serum anti-FITC antibodies in fish immunized with FITC:KLH + FCA. Similarly, mucus anti-WSIV antibodies were not detected until approximately 3 weeks after detection of serum anti-WSIV antibodies. Taken as a whole, these results suggest that specific mucosal antibodies may be disseminated from the circulatory system following a systemic immune response, as put forth by Cain et al. [39]. However, labeling each white sturgeon with a passive integrated transponder allowed us to monitor the antibody response of individual fish throughout the course of the study. This approach showed there was no correlation between the observed serum antibody titers in fish immunized with FITC:KLH + FCA to their observed mucus antibody titers, meaning that the highest serum titers did not necessarily result in the highest mucosal titers. In addition, mucus anti-WSIV antibodies were present in a total of six white sturgeon immunized with WSIV (without FCA) but only three of these fish had detectable serum antibody titers against WSIV. These results suggest antibodies in the cutaneous mucus (even following intraperitoneal immunization) do not arrive by passive transfer from the blood and that an independent process for the stimulation of mucosal and serum immune responses exist in sturgeon as speculated by Maki and Dickerson [40] for channel catfish *Ictalurus punctatus* (Rafinesque).

The results from immunocytochemistry demonstrate that anti-WSIV antibodies from white sturgeon immunized against WSIV are indeed specific for WSIV. Since WSIV was only partially purified for immunization and the virus used to coat wells in the ELISA assay was from sonicated WSIV-infected WSGO tissue culture cells, it was possible that antibody may have been generated to components other than viral antigen (such as WSGO cellular material). However, serum collected at 15 weeks post-immunization from white sturgeon immunized with WSIV (without FCA) and showing an antibody endpoint titer of 1600 by ELISA was specific for cells infected with virus and showed no immune reactivity to uninfected WSGO cells. Apparently, white sturgeon used in this study did not develop an immune response against WSGO cells possibly due to closely related (self-antigen) properties of these sturgeon derived cell lines. To look at this closer, proteins of WSIV-infected WSGO tissue culture cells and uninfected cells were separated by SDS-PAGE and analyzed by Western blotting using the same serum samples used as the immunocytochemistry study. This was done to see if an antibody response may be directed towards internal WSGO cellular antigen and to potentially identify WSIV antigens recognized by immune serum. Both infected and uninfected cells preparations were also probed with the monoclonal against WSIV as a positive control. No immune reactivity was observed on blots containing proteins of uninfected WSGO cells when probed with 15-week serum from white sturgeon immunized with WSGO cells or 15-week serum from white sturgeon immunized with WSIV (without FCA), indicating that no antibody response was directed towards WSGO cellular material (internally or externally). In addition, no immune reactivity was observed on the WSIV infected cell blot when probed with serum collected from white sturgeon immunized with WSGO cells. However, both the monoclonal against WSIV and serum collected at 15 weeks post-immunization from the white sturgeon immunized with WSIV reacted with blots containing virus infected cells (data not shown). The monoclonal antibody recognized a series of bands between 25 and 37 kDa in size and the serum recognized just a single band within the same region. This supports the immunocytochemistry results that show that the immune response in fish immunized with WSIV generated specific anti-WSIV antibodies. It also provides evidence for a possible immunogenic antigen of WSIV.

White sturgeon iridovirus is considered an epitheliotropic virus where infection is non-systemic. Infection is limited to epithelial tissues of the skin, gills, oropharynx, and olfactory organs, such as the barbels and nares [4]. The virus has not been detected in internal organs. In a previous study, our laboratory challenged white sturgeon (mean weight 2.5 g) by intraperitoneal injection or immersion with WSIV [19]. After 126 days at 15 °C the injected groups had a cumulative mortality of 58% and the immersion groups 88%. Histological examination confirmed the presence of WSIV infection in fish challenged with virus. Fifteen survivors of the injected groups and seven survivors of the immersion challenged groups were euthanized and serum was collected from each fish. In addition, serum was also collected from seven sturgeon from groups mock infected by intraperitoneal injection with MEM-10 and having a cumulative mortality of 3% from the same study. To further confirm the ability of fish to mount an immune response to WSIV, serum samples from these fish were tested for the presence of anti-WSIV antibodies using the previously described ELISA assay. No antibodies against WSIV were present in the mock-infected group. However, antibodies were detected and differences did exist between sturgeon surviving challenge with WSIV by injection or immersion (data not shown). Twelve sturgeon from the injected groups had antibody endpoint titers against WSIV ranging from 50 to 6400 (average titer ± SD; 817 ± 1777). This was significantly different ($p < 0.001$; ANOVA) from serum sampled

from mock-infected sturgeon and survivors of the immersion challenge, where only one fish had a detectable antibody titer of 100. Since immersion challenge resulted in only a minimal serum antibody response, it is possible that the epitheliotropic nature of WSIV favors stimulation of mucosal immunity in white sturgeon and not a systemic response. This has been demonstrated to occur in other fish following immersion vaccination [41], but further studies are needed to confirm this in sturgeon and clarify our understanding of the protective nature of immune responses against WSIV.

In summary, the results from this study demonstrate the ability of white sturgeon to generate specific antibodies to a defined antigen as well as viral antigen following immunization and challenge. To our knowledge, this is the first study that has examined antibody kinetics in sturgeon over time at a constant temperature following immunization and the first study to demonstrate mucosal Ig in sturgeon. The kinetics of the observed antibody responses in both serum and cutaneous mucus suggests that vaccination strategies may be feasible in the future to minimize disease due to microbial pathogens in an aquaculture setting. Results presented here showed a high level of variability in responses of white sturgeon to WSIV following immunization. Further work should focus on temperature effects on the immune response in white sturgeon and the role of specific and non-specific immunity in providing protection from WSIV disease.

Acknowledgements

The Kootenai Tribe of Idaho and the Bonneville Power Administration funded this project (BPA Project Number 198806400). The authors would like to thank Dr Ronald Hedrick (University of California, Davis, CA, USA) and Mark Adkison (California Department of Fish and Game, Rancho Cordova, CA, USA) for providing the monoclonal antibodies. The authors also want to thank Jack Siple at the KTOI Hatchery and Pete Taylor at the Abernathy Fish Technology Center for their assistance.

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