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Vaccination-Induced Systemic Autoimmunity in Farmed Atlantic Salmon

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Over half of the salmon consumed globally are farm-raised. The introduction of oil-adjuvanted vaccines into salmon aquaculture made large-scale production feasible by preventing infections. The vaccines that are given i.p. contain oil adjuvant such as mineral oil. However, in rodents, a single i.p. injection of adjuvant hydrocarbon oil induces lupus-like systemic autoimmune syndrome, characterized by autoantibodies, immune complex glomerulonephritis, and arthritis. In the present study, whether the farmed salmon that received oil-adjuvanted vaccine have autoimmune syndrome similar to adjuvant oil-injected rodents was examined. Sera and tissues were collected from vaccinated or unvaccinated Atlantic salmon (experimental, seven farms) and wild salmon. Autoantibodies (immunofluorescence, ELISA, and immunoprecipitation) and IgM levels (ELISA) in sera were measured. Kidneys and livers were examined for pathology. Autoantibodies were common in vaccinated fish vs unvaccinated controls and they reacted with salmon cells/Ags in addition to their reactivity with mammalian Ags. Diffuse nuclear/cytoplasmic staining was common in immunofluorescence but some had more specific patterns. Serum total IgM levels were also increased in vaccinated fish; however, the fold increase of autoantibodies was much more than that of total IgM. Sera from vaccinated fish immunoprecipitated ferritin and ~50% also reacted with other unique proteins. Thrombosis and granulomatous inflammation in liver, and immune-complex glomerulonephritis were common in vaccinated fish. Autoimmunity similar to the mouse model of adjuvant oil-induced lupus is common in vaccinated farmed Atlantic salmon. This may have a significant impact on production loss, disease of previously unknown etiology, and future strategies of vaccines and salmon farming. The Journal of Immunology, 2008, 181: 4807–4814.

Atlantic salmon farming is a rapidly expanding industry as indicated by an increase in production from around 50,000 tons in 1986 to nearly 641,000 tons in 2005 in Norway alone (1, 2). Over half of the salmon consumed globally are farm-raised in northern Europe, Chile, Canada, and the United States (3). Intensive salmonid aquaculture relies on the use of i.p. injection of oil-adjuvanted vaccines to prevent infectious diseases. The introduction of oil-adjuvanted vaccines into Atlantic salmon aquaculture in the early 1990s made large-scale production feasible by resolving critical issues such as mortality from bacterial infection and an excessive use of antibiotics (1, 4). Present practice in the salmon industry is to inject 0.1 ml of oil-adjuvanted vaccine i.p. to anesthetized salmon, when the fish are 30–40 g in weight during the late freshwater period before transfer to the marine environment (4). The vaccines commonly used contain an oil-based adjuvant (4), such as mineral hydrocarbon oil and emulsifier (same as IFA, containing mineral oil Bayol F and emulsifier) or animal/vegetable oil that contains pristane (2,6,10,14-tetramethylpentadecane) (5) or squalene (6). A number of undesirable side effects have been attributed to the vaccination of salmon, including retarded growth, chronic peritonitis with pigmentation and adhesion of internal organs (7–9), granulomatous uveitis (10), and spinal deformities (11).

Adjuvant is an essential component of a vaccine to induce efficient protection against pathogens. However, in nonautoimmune mice, a single i.p. injection of adjuvant hydrocarbon oil such as IFA, squalene, or pristane induces lupus-like systemic autoimmune syndrome characterized by production of antinuclear Abs (ANA)4 including anti-double-stranded (ds) DNA, -chromatin, and -small nuclear ribonucleoproteins (snRNPs), immune complex glomerulonephritis, and arthritis (12–15). In this study, we report the first evidence of systemic autoimmunity of fish in vaccinated, farmed Atlantic salmon, characterized by various serum autoantibodies, immune complex glomerulonephritis, and liver thrombosis.

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† Abbreviations used in this paper: ANA, antinuclear Ab; snRNP, small nuclear ribonucleoprotein; IIF, immunofluorescence; IPP, immunoprecipitation; PAS, periodic acid-Schiff; MSB, Martius scarlet blue; EM, electron microscopy; ds, double-stranded; ss, single-stranded; RF, rheumatoid factor; GBM, glomerular basement membrane; IFN, type 1 interferon.

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Table I. Summary of demographic information on samples

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Mean Weight</th>
<th>Location</th>
<th>Hatched/ Vaccinated/Sampled</th>
<th>Vaccine</th>
<th>Adjuvant Oil (Dose)</th>
<th>Antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (n = 10) 2626 g</td>
<td>Fish tank, EWOS Research Station, Lønningsdal, Os, Norway, Experimental Setting</td>
<td>Winter 2003/NA/ Summer 2005</td>
<td>None</td>
<td>Mineral oil, emulsifiers (0.1 ml)</td>
<td>A. salmonicida, V. anguillarum 01/02, V. salmonicida, M. viscosa*</td>
<td>NA</td>
</tr>
<tr>
<td>B (n = 10) 2376 g</td>
<td>Same as above</td>
<td>Winter 2003/ Fall 2005</td>
<td>Tetra Forte Vet</td>
<td>Mineral oil, emulsifiers (0.1 ml)</td>
<td>Same bacteria as group B plus IPN virus (formalin inactivated)</td>
<td>NA</td>
</tr>
<tr>
<td>C (n = 20) 3600 g</td>
<td>Hauvik living genebank for wild salmon, Norway, in running freshwater</td>
<td>Winter 1999/NA/ Summer 2006</td>
<td>None</td>
<td>Mineral oil, emulsifiers (0.1 ml)</td>
<td>Same bacteria plus IPN virus (formalin inactivated)</td>
<td>NA</td>
</tr>
<tr>
<td>D (n = 55) 3100 g</td>
<td>Commercial farm, sea cages, western Norway, Farm #1</td>
<td>Winter 2003/ Fall 2005</td>
<td>AlphaJect 6-2</td>
<td>Mineral oil, emulsifiers (0.1 ml)</td>
<td>Same bacteria plus IPN virus (formalin inactivated)</td>
<td>NA</td>
</tr>
<tr>
<td>E (n = 20) 5461 g</td>
<td>Commercial farm, sea cages, western Norway, Farm #2</td>
<td>Winter 2003/ Winter 2004/ Winter 2006</td>
<td>AlphaJect 6-2</td>
<td>Mineral oil, emulsifiers (0.1 ml)</td>
<td>Same bacteria plus IPN virus (formalin inactivated)</td>
<td>NA</td>
</tr>
<tr>
<td>F (n = 19) 5400 g</td>
<td>Commercial farm, sea cages, northwestern Norway, Farm #3</td>
<td>Winter 2003/ Fall 2003/ Winter 2006</td>
<td>Pentium Forte Vet</td>
<td>Mineral oil, emulsifiers (0.1 ml)</td>
<td>Same bacteria plus IPN virus (formalin inactivated)</td>
<td>NA</td>
</tr>
<tr>
<td>G (n = 20) 1963 g</td>
<td>Commercial farm, sea cages, western Norway, Farm #4</td>
<td>Winter 2004/ Spring 2005/ Summer 2006</td>
<td>Pentium Forte Vet</td>
<td>Mineral oil, emulsifiers (0.1 ml)</td>
<td>Same bacteria plus IPN virus (formalin inactivated)</td>
<td>NA</td>
</tr>
<tr>
<td>H (n = 20) 5194 g</td>
<td>Commercial farm, sea cages, western Norway, Farm #5</td>
<td>Winter 2004/ Summer 2004/ Spring 2006</td>
<td>Norvax Compact 6 Vet</td>
<td>Animal/Vegetable oil (0.1 ml)</td>
<td>Same bacteria plus recombinant surface protein from IPN virus (formalin inactivated)</td>
<td>NA</td>
</tr>
<tr>
<td>I (n = 20) 5322 g</td>
<td>Commercial farm, sea cages, middle Norway, Farm #6</td>
<td>Winter 2003/ Fall 2004/ Spring 2006</td>
<td>AlphaJect 6-2</td>
<td>Mineral oil, emulsifiers (0.1 ml)</td>
<td>Same bacteria plus IPN virus (formalin inactivated)</td>
<td>NA</td>
</tr>
<tr>
<td>J (n = 11) 3056 g</td>
<td>Wild salmon caught in bag-net, Hovika, Namsos, Norway</td>
<td>NA/NA/Summer 2006</td>
<td>None</td>
<td>Mineral oil, emulsifiers (0.1 ml)</td>
<td>Same bacteria plus IPN virus (formalin inactivated)</td>
<td>NA</td>
</tr>
</tbody>
</table>

* Aeromonas salmonicida subsp. salmonicida, Vibrio anguillarum serotype 01/02, Vibrio salmonicida, Mortierella viscosa; IPN, infectious pancreatic necrosis; NA, not applicable.

Materials and Methods

Serum and tissue samples from salmon

Samples of salmon sera and tissue were obtained from two experimental groups of salmon (A: unvaccinated control, B: vaccinated), unvaccinated farmed fish (C), six groups of randomly sampled vaccinated fish from salmon farms (D-I), and a group of wild salmon (J) (Table I). Number of fish, weight of fish at harvesting, location, and condition of fish; time of hatching, vaccination, and sampling; type of vaccine, adjuvant, and bacterial and viral Ags in vaccines are summarized in Table I. Vaccines used include Tetra Forte Vet (Aqua Health), Alpha Ject 6-2 (Pharmaq), Pentium Forte Vet (Aqua Health), and Norvax Compact 6 Vet (Intervet Norbio AS). Each fish was treated according to the regulations for euthanasia of fish in aquaculture issued by the Norwegian Directorate of Fisheries (Forskrift om drift av fiskeriforretninger – 2005). Samples were collected and kidney, liver, peritoneal granuloma, and other tissues were sampled after euthanasia. Serum samples were then incubated and diluted in sterile PBS and the cell pellets were kept at −80°C until use. Cells were resuspended at a concentration of 5 × 10⁶/ml in PBS with PMSF, sonicated for 0.5 s twice, and centrifuged at 12,000 rpm for 30 min at 4°C. Supernatant was then incubated with sterile PBS and then resuspended in PBS (2 × 10⁶/ml), and incubated on six-well hydrophobic coating slides (Erie Scientific Company) at 22°C for 1 h, and fixed with acetone/methanol (3/1) for 2 min at −20°C. Slides were then washed with diluted sera (1/40), washed with TBS (10 mM Tris-HCl (pH 7.5), 135 mM NaCl) and incubated with mouse IgG1 mAbs to salmon IgM (1/200 culture supernatant, Cedarslane Laboratories), followed by Alexa 488-goat anti-mouse IgG1 Abs (1/200 culture supernatant, Cedarlane Laboratories), followed by Alexa 488-goat anti-mouse IgG1 Abs (1/200, Invitrogen Life Technologies) and examined by fluorescent microscopy. Titors were estimated using a titration emulation system (ImageTiter, Rhigene) (17).

ELISA

Salmon sera were tested for the reactivity with chromatins (from chicken RBC), ss- and ds-DNA (calf thymus DNA, Sigma-Aldrich), thyroglobulin, mouse IgG1 (BD Bioscience), and β2 glycoprotein-I (a gift from Dr. J. Kaburaki, Tokyo Electric Power Company Hospital, Tokyo, Japan) by ELISA (18). Each plate was coated with 2–10 μg/ml Ag, incubated with diluted salmon sera (1/100), followed by alkaline phosphatase-conjugated mouse anti-salmon IgM Abs (1/1000, Cedarslane Laboratories). ELISA using salmon blood cell extract

Cells from freshly collected salmon blood (East Coast Bio) were washed five times using sterile PBS and the cell pellets were kept at −80°C until use. Cells were resuspended at a concentration of 5 × 10⁶/ml in PBS with PMSF, sonicated for 45 s twice, and centrifuged at 12,000 rpm for 30 min at 4°C. Supernatant was then incubated with aliquots and used as salmon blood cell extract for ELISA. Cells from 10⁶ cells (contains ~100 μg proteins) were diluted in 10 ml (−10 μg proteins/ml) coating buffer (0.1M Na₂HPO₄/NaH₂PO₄ (pH 9.0)) and used to coat wells of microtiter plate (100 μl/well, Nunc, Immobilizer Amino) at 4°C for 16 h. Wells were then washed once with 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20 (TBS/ Tween), and blocked with 0.5% BSA in 0.15M NaCl, 2 mM EDTA, 50 mM Tris (pH 7.5), 0.3% NP40 (0.5% BSA, NET/NP40) for 1 h at 22°C. Supernatant was serially 1/2 diluted starting 1/100 in 0.5% BSA NET/ NP40 (1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200, 1/6400, 1/12800, 1/25600, 1/51200, 1/102400, 1/204800) and tested in duplicate. After incubation with diluted salmon sera for 2 h at 22°C, wells were washed three times with TBS/Tween 20, followed by incubation with alkaline phosphatase-conjugated mouse IgG1 anti-salmon IgM mAb (Cedarslane...
Laboratories, 1/2,000 in 0.5% BSA NET/NP40) for 1 h. Wells were washed three times, developed, and OD405 was measured.

**Total serum IgM ELISA**

Wells of microtiter plate were coated with 2 μg/ml rat mAb to mouse IgG1 (BD Biosciences) for 16 h at 4°C, followed by incubation with mouse IgG1 mAb to salmon IgM (1/500 of culture supernatant in 0.5% BSA NET/NP40, ~2 μg/ml). After washing and blocking, wells were incubated with 1/100,000 diluted salmon sera in duplicate and serially diluted standard at 4°C for 16 h. Wells were then washed three times with TBS/Tween and incubated with alkaline phosphatase conjugated mouse IgG2a mAb to salmon IgM (Cedarlane Laboratories, 1/2,000 in 0.5% BSA NET/NP40) for 2 h at 22°C, washed three times, and developed. OD405 of each sample was converted to concentration based on the standard curve.

Standards curve was created by one of the salmon serum that has high levels of IgM. The IgM concentration of the standard serum was estimated as the following. Forty microliters of protein G agarose beads (50% v/v, Sigma-Aldrich) in 20 mM Tris-HCl (pH 7.5), 0.05% NaN3, were incubated with 20 μl of mouse anti-salmon IgM mAbs (Cedarlane Laboratories) and 2 μl of the salmon serum. After washing the beads three times with 0.5 M NaCl NET/NP40 and once with NET/NP40, SDS-PAGE sample buffer was added. IgM from 0.5 μl of salmon serum was fractionated on 7.5% SDS-PAGE along with serially diluted BSA (2.5-0.167 μg/lane). After Coomassie blue staining, the staining intensity of IgM μ-chain of the serum sample was compared with those of BSA. The IgM concentration of the sample was estimated 3.4 mg/ml and used as a standard.

**Immunoprecipitation (IPP)**

The proteins recognized by salmon sera were evaluated by IPP of extracts from K562 (human erythroleukemia) cells that were metabolically radiolabeled with 35S-methionine/cysteine for 14 h (12). Cells were lysed in 0.5M NaCl, 2 mM EDTA, 50 mM Tris (pH 7.5), 0.05% NaN3, were incubated with 20 μg of mouse anti-salmon IgM Abs and 10 μl of protein G agarose beads (50% v/v, Sigma-Aldrich) in 20 mM Tris-HCl (pH 7.5), 0.05% NaN3 were incubated (1/2,000 in 0.5% BSA NET/NP40) for 16 h at 4°C for 16 h. Wells were then washed three times with TBS/Tween and incubated with alkaline phosphatase conjugated mouse IgG2a mAb to salmon IgM (Cedarlane Laboratories, 1/2,000 in 0.5% BSA NET/NP40) for 2 h at 22°C, washed three times, and developed. OD405 of each sample was converted to concentration based on the standard curve.

**Histology and immunofluorescence**

Samples from groups A, B, C, G, H, and I were processed for histology (H&E, Periodic acid-Schiff, PAS; Martius scarlet blue, MSB; and other staining) or electron microscopy (EM) (9). For IF, mid-kidney samples from fish in groups G and H were snap-frozen in liquid nitrogen and stored at −90°C. Seven micrometer slides were incubated with rabbit anti-salmon Ig serum (provided by Dr. Knut Falk, The National Veterinary Institute, Oslo, Norway) (19) diluted 1/400 followed by Alexa Fluor 594 goat anti-rabbit IgG (H + L) (1/400, Invitrogen Life Technologies).

**Statistical analysis**

Frequency and levels of autoantibodies were compared by Fisher’s exact test and Kruskal-Wallis with Dunn’s multiple comparison test, respectively, using Prism 4.0c for Macintosh (GraphPad Software, Inc., San Diego, CA). p < 0.05 was considered significant.

**Results**

Sera and tissue samples were obtained from experimental groups of salmon (A: unvaccinated control, n = 10; B: vaccinated, n = 10) kept in fish tank and groups of salmon from commercial fish farms along the Norwegian coast (C: unvaccinated control, n = 20; D-I: fish that received mineral-oil or animal/vegetable-oil based vaccines from 6 different farms, n = 19–55, total 154). In addition, sera from 11 wild salmon confirmed as wild by the absence of abdominal lesions and reading of scales (J), were included in the study (Table I).

Production of ANA (including anti-cyttoplasmic Abs) detected by indirect IF is a hallmark of human systemic autoimmune diseases such as lupus (20, 21). Thus, the presence of ANAs in salmon sera was tested using cells from salmon peripheral blood. Abs in sera from vaccinated salmon bound to nuclei and/or cytoplasm of salmon blood cells in speckled or multiple dots pattern, indicating the presence of autoantibodies to self-cellular Ags (Fig. 1, A, panels a–c) in contrast to no reactivity by sera from unvaccinated fish (Fig. 1A, panel d). Screening and titration of IF ANA were performed using commercial HEp-2 cell slide to ensure consistent quality and to take advantage of the available reference Abs to various intracellular structures or Ags. Diffuse nuclear staining, often with cytoplasmic staining, was commonly seen (Fig. 1B, panel a), but specific cytoplasmic patterns consistent with cytokeratin, vimentin, and actin (panels b, c, and d, respectively), nuclear speckled pattern (e), nucleolar staining (f), and Cajal body staining (g) were also produced by some sera. In the experimental groups, none of the unvaccinated controls (A) had ANA in a titer of >1/160, whereas 60% of the vaccinated group (B) were positive (p < 0.05, Fisher exact test) (Fig. 1C). Among farmed groups, 36–85% of vaccinated salmon from the six farms (D–I, Table I)
had positive ANA (p < 0.05-0.0001). In contrast, only 5% (1/20) of unvaccinated farmed salmon (C) and no wild salmon (0/11, J) had ANA. The titers of ANA in the vaccinated groups were also consistently higher than those in control (p < 0.01-0.0001, Kruskal-Wallis with Dunn’s multiple comparison test), suggesting that the high titers of ANA are associated with vaccination.

The sera were further screened for autoantibodies such as rheumatoid factor (RF), anti-chromatin, single stranded (ss), ds-DNA, thyroglobulin, β2 glycoprotein I, and ferritin by ELISA (18). Sera from vaccinated fish frequently contained high levels of autoantibodies to these Ags (Fig. 2, A and B). Titters of these autoantibodies were as high as 1/12,500 to 1/62,500 by ELISA (data not shown). Anti-single-stranded DNA (Fig. 2C) and β2 glycoprotein I (data not shown) appeared to be produced at low levels in some unvaccinated fish, somewhat different from the other autoantibodies tested. The reactivity of individual serum to the Ags including mouse IgG (RF) (Fig. 2A), chromatin (Fig. 2B), dsDNA, and thyroglobulin (data not shown) were very similar and the reactivity with different Ags showed a strong correlation (Fig. 2D). These characteristics are consistent with natural polyreactive autoantibodies, known to bind various dissimilar Ags at low affinity, as described in human, mice, and other mammals (22).

Autoantibodies in salmon sera by ELISA. A, IgM rheumatoid factor; B, IgM anti-chromatin Abs; C, IgM anti-ssDNA Abs. Details of groups are described in Materials and Methods and Table I. O, Unvaccinated fish; •, vaccinated fish. D, Correlation of rheumatoid factor and anti-chromatin Abs. Correlation of rheumatoid factor and anti-chromatin Abs in all sera from vaccinated salmon (n = 169) were analyzed. Spearman’s R = 0.9128, p < 0.0001.

FIGURE 2.

Sera from vaccinated salmon contained autoantibodies that react with salmon blood cells in indirect IF (Fig. 1A). To obtain data that are comparable to the IF and confirm salmon autoreactivity in a semiquantitative manner, sera from 48 salmon were serially diluted and tested by ELISA using salmon blood cell extract as Ag, and endpoint titers for each sample were determined. Titration curves for samples in experimental group (Fig. 3A, unvaccinated; B, vaccinated), those for unvaccinated wild fish (Fig. 3C, group J, n = 11), and farmed vaccinated fish (Fig. 3D, n = 17, two to four randomly selected samples/group from groups D–I) are shown. All except one vaccinated fish (100% (10/10) in experimental group A, 94% (16/17) in farmed groups D–I) had significant titers of autoantibodies to salmon blood cell extract compared with unvaccinated groups. Endpoint titers were determined from the titration curves (Fig. 4A). Vaccinated fish had significantly higher (p < 0.0001 by Mann-Whitney) titers of autoantibodies to blood cell extract vs unvaccinated fish in both experimental group (left) and nonexperimental group (wild vs farmed fish, right). Vaccination and following i.p. inflammation in fish may induce polyclonal B cell activation leading to increased serum Ig levels similar to adjuvant oil i.p. injected mice (18, 23). Thus, serum IgM levels were measured in the same set of samples to evaluate the relationship between specific autoantibody production and polyclonal B cell activation (Fig. 4B). As expected, serum IgM levels were higher in vaccinated fish vs unvaccinated fish (p < 0.0001 in experimental group, p = 0.0022 in farmed fish). Whether the increased titers of autoantibodies were merely resulted from nonspecific polyclonal B cell activation leading to increased serum Ig levels similar to adjuvant oil i.p. injected mice (18, 23). Thus, serum IgM levels were measured in the same set of samples to evaluate the relationship between specific autoantibody production and polyclonal B cell activation (Fig. 4B). As expected, serum IgM levels were higher in vaccinated fish vs unvaccinated fish (p < 0.0001 in experimental group, p = 0.0022 in farmed fish). Whether the increased titers of autoantibodies were merely resulted from nonspecific polyclonal B

FIGURE 3.
ever, the reduction of reactivity was minimal when these Abs were not to bind to target Ags when NaCl concentration in the buffer was increased from 0.15M to a more stringent 0.5 M NaCl (24). Thus, the sensitivity of autoantibodies to NaCl was evaluated by ELISA. Vaccinated fish had significantly higher (p values are by Mann-Whitney) levels of IgM vs unvaccinated fish in both experimental group (a pair on the left) and non-experimental group (wild vs farmed, right). Horizontal bar, median. C, Fold changes of total IgM levels vs Abs to salmon blood cell extract. Fold changes in total IgM levels and Abs to salmon blood cells extract in vaccinated fish compared with unvaccinated fish in experimental group (left panel) and farmed fish (right panel) are shown. Fold changes of IgM were calculated by dividing IgM levels in each sample by average IgM levels in unvaccinated group (average IgM levels in unvaccinated experimental group A (0.401 mg/ml) vs unvaccinated group B (0.337 mg/ml)). For autoantibody levels, 1/100 was used as levels in unvaccinated fish to calculate the fold changes since the median in unvaccinated vaccinated fish was <1/100 and all except one had titers of 1/100 or less. p values are by Wilcoxon matched pairs test.

To further characterize autoantibodies induced by vaccination, avidity of autoantibodies were evaluated. IgM Abs, the only known serum Ig class in salmon, were shown to be low-affinity and not to bind to target Ags when NaCl concentration in the buffer was raised from 0.15M to a more stringent 0.5 M NaCl (24). Thus, the sensitivity of autoantibodies to NaCl was evaluated by ELISA. The reactivity was significantly reduced when Abs bound in 0.15M NaCl buffer were exposed to buffer containing 0.325M NaCl, however, the reduction of reactivity was minimal when these Abs were exposed to 0.5M NaCl buffer, indicating that a significant portion of the Abs are resistant to high NaCl, consistent with high avidity of Abs (Fig. 5A). Furthermore, all tested sera from vaccinated fish (groups B, D–F, 104 sera) strongly immunoprecipitated a 20–22 kD doublet (identified as ferritin light and H chain, respectively, by mass spectroscopy/MALDI) and ~50% of sera from the vaccinated group clearly immunoprecipitated specific proteins in the presence of high (0.5M) NaCl (Fig. 5B) (12). These data collectively suggest the development of high avidity autoantibodies in vaccinated fish. Although many sera from vaccinated fish had positive ANA and immunoprecipitated unique proteins, lupus-related specific autoantibodies like those found in adjuvant-oil-injected normal mice such as anti-snRNPs, ribosomal P, or Su (argonaute protein) (12, 14, 16, 18, 25) were not detected by IPP. Although the similar results of ELISA using different Ags, are consistent with polyreactive natural autoantibodies, different staining patterns by IF ANA (Fig. 1, A and B) and IPP of unique proteins (Fig. 5B) clearly indicate that these are not simple, nonspecific reactions, but that each fish exhibits a distinct pattern of autoreactivity.

Chronic granulomatous inflammation with adhesion of internal organs was found in all salmon immunized with oil-adjuvanted vaccines (Fig. 6A), as reported previously (7, 9) and similar to changes described in mice treated ip with adjuvant oil (26). Isolated granulomas were also seen in liver (Fig. 6A). In addition, multiple thrombi in portal vein and perivascular infiltration of mononuclear cells in hepatic artery were identified (Fig. 6A, c–e) in salmon of the experimental vaccinated group (group B, Table I) of fish harvested 1 year after vaccination. Thrombi were not seen in the same group of fish examined after 2 years. Although isolated granulomas and perivascular inflammatory...
cell infiltration were commonly found in vaccinated fish from salmon farms, thrombosis as seen in the experimental group was not observed in liver from farmed salmon. Whether the difference between groups simply reflects the differences in period between vaccination and harvest (1 vs 2–3 years) or is due to other factors will be a focus of future studies. These lesions were absent in unvaccinated fish (Fig. 6Af). Because kidney involvement is frequently seen in human lupus and in adjuvantoil induced lupus (13, 16), kidneys from salmon were examined. PAS staining revealed focal proliferation of glomerular capillary in vaccinated fish (Fig. 6Ba) in contrast to its absence in unvaccinated fish (Fig. 6Bb). Granular short-linear peripheral capillary deposition of immunoglobulins was also observed in kidneys from vaccinated salmon (Fig. 6Bc) but not in unvaccinated fish (Fig. 6Bd). Transmission electron microscopy revealed irregular thickening and splitting of the glomerular basement membrane (GBM) in vaccinated fish (Fig. 6Be) in contrast to intact structure in unvaccinated fish (Fig. 6Bf). These findings are consistent with the immune-mediated glomerulonephritis in vaccinated fish.

Discussion
The induction of specific immune responses following vaccination presupposes an adaptive immune system (27). Teleosts such as salmon harbor the expressed genes of the TCR, MHC class I and II (28), and B cell Ag receptors (27). Notably, they lack the germinal center and Ig class switch, making IgM-like tetramer isotype the dominant form in both primary and secondary humoral responses (29). Therefore, innate immunity must play a crucial role in host defense. TLR (30) and the type-I IFN (I-IFN) system both exist in salmon and their roles in host defense have been investigated (31).

In addition to host defense (32), I-IFN plays a critical role in adjuvant activity as well as in the pathogenesis of human and murine lupus (21, 33). A mouse model of lupus induced by an i.p. injection of the adjuvant oil pristane is I-IFN dependent (26). Microbial constituents in vaccines may also play a role in I-IFN induction via TLR stimulation (32). In fact, up-regulation of I-IFN-inducible Mx genes in vaccinated salmon has been previously reported (34). Thus, the role of I-IFN in the vaccination-induced autoimmunity in salmon will need to be investigated in future studies.

The mechanisms for selection of particular target Ags as seen by IF (Fig. 1, A and B) and by IPP (Fig. 5B) in individual fish are not clear. Strong IPP of ferritin by virtually all vaccinated salmon sera was unexpected. Because bacterial ferritin is a major target of immune response and ferritin is conserved between species (35, 36), this may simply reflect the results of efficient immunization with bacterial Ags, inducing Abs to bacterial proteins that cross-react with mammalian ferritin. If these Abs also react with salmon ferritin in vivo, they may play a role in the tissue damage in vaccinated fish, based on animal models of immune-complex glomerulonephritis and thrombosis induced by immunization with ferritin (37, 38). Mechanisms of liver thrombosis also remain to be clarified. It is tempting to suspect that thrombosis is triggered by endothelial cell damage caused by autoantibody or immune complex, similar to what is proposed as a mechanism of thrombosis in anti-phospholipid Ab syndrome (39). Massive cytokine production induced by vaccination can also damage endothelial cells. Because adjuvant hydrocarbon oils are known to be incorporated into lipoproteins (40), these abnormal lipoproteins may play a direct or indirect role in endothelial cell damage, leading to thrombosis. The mechanism of autoimmunity in salmon may be consistent with the concept of innate autoimmunity (41). Regardless of the actual mechanisms of tissue damage, considering the occurrence of autoimmunity in fish will help us to understand the pathogenesis of the lesions reported in this study.

Despite much controversy on a possible relationship between human vaccines and induction of autoimmune diseases, little or no association has been validated by scientific studies (42). Due to concern on the side effects, IFA is not used in FDA-approved vaccines, even though a large epidemiological study on the use of IFA and autoimmune disease found no association (43). Nevertheless, induction of lupus-like autoimmune disease or arthritis by adjuvant hydrocarbon oil, including IFA, squalene, and pristane, is well described in rodents (12, 13, 44). Autoantibodies in vaccinated salmon clearly reacted with salmon Ags by indirect IF (Fig. 1A) and by ELISA (Figs. 3 and 4), in addition to their reactivity in

FIGURE 6. Pathological changes in vaccinated fish. A, Peritoneal granuloma and thrombosis in liver. a, A granuloma within the abdominal cavity. Exocrine pancreatic cells (dark color) and fat tissue are seen in the periphery, and, centrally, surrounded by inflammatory tissue, a negative imprint of an adjuvant oil droplet is seen. b, Granuloma in the liver. c, A larger vessel with peripheral degenerative changes and necrosis and multiple smaller vessels containing thrombi (white arrowheads). d, MSB staining demonstrating thrombi (intensively red) throughout the liver. e, A close-up image of a thrombus. f, MSB staining of liver of unvaccinated salmon. a–c, H&E; d–f, MSB staining. Scale bars are 200 μm except for e (30 μm). B, Kidney lesions. a and b, PAS staining. a, A glomerulus with focal endocapillary proliferation (white arrowhead) in vaccinated salmon. b, Unaffected glomerulus from unvaccinated salmon. c and d, Immunofluorescence. c, A granular-short linear peripheral capillary deposition of immunoglobulins in vaccinated salmon. Bar = 10 μm. d, Negative sample. e and f, Electron micrograph. e, Irregular thickening and splitting (arrow) in the GBM from a vaccinated salmon. Bar = 2 μm. L = lumen, p = podocyte. f, Smooth GBM from an unvaccinated salmon. Bar = 1 μm.

...to intact structure in unvaccinated fish (Fig. 6Bf). These findings are consistent with the immune-mediated glomerulonephritis in vaccinated fish.

Discussion
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assays using mammalian cells (Fig. 1B) and Ags (Fig. 2), indicating that the observation in this report is “true” autoimmunity, reacting with self Ags. Autoimmunity is often accompanied by polyclonal B cell activation leading to hypergammaglobulinemia (45) and may be induced by polyclonal B cell stimulation in certain conditions (46). Polyclonal hypergammaglobulinemia was seen in adjuvant-oil induced lupus (18, 23) and also appeared to be the case in vaccinated salmon that show average 3–4-fold increase vs unvaccinated controls (Fig. 4). However, the fold increases of Abs to salmon blood cell extract were significantly higher than those of total IgM levels in both experimental group and farmed fish (Fig. 4C). Thus, although the vaccination-induced autoantibodies have characteristic of polyreactive low affinity Abs, they are not simply resulted from polyclonal B cell activation but rather specifically induced. The role of adjuvant vs the microbial component of vaccine in autoimmune inflammatory disease described here will be examined in future studies.

Several apparent differences between human and salmon vaccination may help explain why autoimmune inflammation is so prominent in salmon. A dose of vaccine in salmon, 0.1 ml vaccine to a 40 g fish, is equivalent of 200 ml vaccine in human with 80 kg body weight. Site of vaccination, i.p. in salmon vs i.m. in human, is another major difference. The i.p. vaccination in salmon induces chronic granulomatous inflammation similar to the peritoneal granuloma of rodents with adjuvant-oil induced lupus (26). Lymphoid neogenesis in affected tissues of various human autoimmune diseases, such as synovium in rheumatoid arthritis, thyroid gland in chronic thyroiditis, and salivary gland in Sjögren’s syndrome, has been well described (47). A role of lymphoid neogenesis in rodents with adjuvant oil-induced autoimmunity has also been suggested (26). Whether granulomatous inflammation in vaccinated fish exhibits structure consistent with lymphoid neogenesis and has a role in autoimmunity, is currently investigated.

The primary considerations of successful vaccine for aquaculture are cost effectiveness and safety (48). Pathological changes caused by vaccine have hitherto been thought acceptable from an animal welfare point of view, considering the consequences of high mortality and disease when fish are left unprotected (1, 8). Our results, however, show that the situation is more complicated than previously assumed, as oil adjuvant-based vaccination provokes the development of systemic autoimmunity. Despite significant improvement in the control of infection by vaccination, ~10% of stocked fish still die during the production period (2). With these new considerations, it is critical to address the role of autoimmunity in production loss and diseases of previously unknown etiology in the future.

Studies of the negative aspects of salmonid aquaculture have so far focused on environmental contaminants (3) and unfavorable ecological effects such as environmental pollution (49), use of large amount of fish as feed, and genetic effects of escapees (50). Concerns regarding vaccine side effects were limited to nonspecific inflammation (9). The present study, however, illustrates the importance of previously unrecognized, and thus far not evaluated or considered, side effects of ordinary commercial vaccination. Vaccination-induced autoimmunity in farmed Atlantic salmon will have significant impacts on future vaccine development and salmon farming strategy, in addition to on animal welfare. Several alternative vaccine strategies are currently under development, including DNA vaccine and use of specific TLR ligand as an adjuvant (51). Evaluation for autoimmunity should be included as an important category when vaccine safety is tested in future. With these considerations in mind, the aquaculture industry has every reason to devote significant resources to the development of alternative vaccination strategies (52) and prophylaxis to ensure healthy fish and thus an optimal food source for global consumers.

In summary, autoimmunity similar to the mouse model of adjuvant oil-induced lupus, is common in vaccinated farmed Atlantic salmon. This may have a significant impact on production loss, disease of previously unknown etiology, and future strategies of vaccines and salmon farming.

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Disclosures

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