Erythrocytic Inclusion Body Syndrome: Resistance to Reinfection

Nobuaki Okamoto¹, Kiyotaka Takahashi², Akira Kumagai³, Masashi Maita¹, John S. Rohovec⁴ and Yayoi Ikeda¹

¹Department of Aquatic Biosciences, Tokyo University of Fisheries, Konan 4, Minato-ku, Tokyo 108, Japan
²Miyagi Prefectural Freshwater Experimental Station, Yamato, Taiwa-cho
Kurokawa-gun, Miyagi 981–36, Japan
³Kesennuma Miyagi Prefectural Fisheries Experimental Station, Hashikami, Kesennuma, Miyagi 988–01, Japan
⁴Laboratory for Fish Disease Research, Department of Microbiology, Oregon State University, Corvallis, Oregon 97331–3804, USA

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Acquired resistance to erythrocytic inclusion body syndrome (EIBS) was investigated in coho salmon (Oncorhynchus kisutch). Fish which were experimentally infected with EIBS and maintained at 15°C for 242 days were challenged by EIBS virus and kept at 8°C. The fish survived the challenge, though they showed a slight decrease in Ht values which recovered presently. This result indicates that acquired resistance to EIBS continues at least as 242 days. Follow-up examination of coho salmon which survived EIBS in freshwater farm ponds revealed that they maintained acquired resistance after being transferred to marine farms.

Erythrocytic inclusion body syndrome (EIBS) is a serious viral disease of salmonid fish. The signs of EIBS are severe anemia and the erythrocytes have characteristic inclusion bodies which contain enveloped viral particles with a diameter of approximately 77 nm. Natural and artificially-induced infections show that most salmonid fish are susceptible to EIBS (Lunder et al., 1990; Rodger et al., 1991; Leek, 1987; Piacentini et al., 1989; Takahashi et al., 1992a, b; Okamoto et al., 1992a). In Japan, EIBS is a major contributor to mortality of coho salmon reared in saltwater net-pen culture (Takahashi et al., 1992a) and in freshwater ponds (Okamoto et al., 1992b) and is responsible for great economic losses to the aquaculturists.

Progression of EIBS has been studied and the stages of the disease were determined from the hematocrit (Ht) values and the morphology of the erythrocytes (Takahashi et al., 1992b). Onset of EIBS was correlated with water temperature declining to below 10°C and mortality from the disease ceased when the temperature surpassed 13°C (Takahashi et al., 1992a). Fish that recovered from the disease were much less susceptible to reinfection (Piacentini et al., 1989). Here we report the use of artificially-induced infections to demonstrate resistance of coho salmon (Oncorhynchus kisutch), to reinfection with EIBS.

Materials and Methods

Artificially Induced Infection

To study resistance of coho salmon to reinfection with EIBS, we artificially infected groups of healthy coho salmon (mean body weight, 6 g) that were maintained in fish pathogen-free well water at 8°C. Duplicate groups of 30 control or experimental fish were held in 60-l tanks. Blood for the artificial infections was taken from moribund fish with EIBS or from healthy animals for controls. Blood was disrupted by sonication for 2 min at 20W (20 kHz). The resulting material was clarified by low speed centrifugation, the supernatant diluted 1:100 in Hanks' balanced salt solution and passed...
through a 0.45 µm filter. This homogenate (0.25 ml/g body weight) was injected intraperitoneally into fish anesthetized with 10% benzocaine (ethyl aminobenzoate). Fish were fed commercial dry pellets daily.

**Sampling**

Disease progression was monitored by lethally sampling of five fish on selected days. The caudal peduncle was severed and blood was collected in heparinized capillary tubes for hematocrit determination. In addition, blood smears from each fish were made, allowed to air dry, then fixed in absolute methanol for 5 min and stained with Giemsa, pinacyanol chloride or acridine orange (Yasutake, 1987; Piacentini and Rohovec, 1989). Thirty, 1,000 microscopic fields of each stained blood smear were examined and the number of cytoplasmic inclusion bodies was recorded.

**Duration of Resistance to Reinfection**

Coho salmon were artificially infected with EIBS and maintained at 8°C for 28 days during which time the fish showed signs of EIBS. Subsequently, the affected fish were maintained at 15°C. At 71, 150 or 242 days after initial infection, the fish were reinjected with EIBS and transferred to 8°C. Disease progression was monitored by lethally sampling on day 20, 30 or 40 after reinjection. Fish that were previously injected with the blood homogenates from healthy fish were injected with EIBS and were treated as positive controls.

**Procedure to Induce Resistance**

To induce resistance to reinfection with EIBS, 6 groups of fish were set and tested. After initial infection with EIBS, the fish were maintained at 15°C until day 242 after initial infection. On day 242 after initial infection, the fish were reinjected with EIBS and transferred to 8°C. Disease progression was monitored by lethally sampling on day 20, 30 or 40 after reinjection. Fish injected with the blood homogenates from healthy fish and maintained at 8°C for 14 days were injected with EIBS day 242 after initial infection and were treated as positive controls. Fish that were injected with EIBS and maintained for 28 days at 8°C were injected with the blood homogenates from healthy fish day 242 after initial infection and were negative controls.

**Resistance to EIBS in Seawater Acquired from Natural Exposure in Freshwater**

Fish that survived a natural infection of EIBS in freshwater in June 1991 were transferred to saltwater net-pens 6 months later (in December). At the same time, coho salmon which had been previously unexposed to the EIBS virus were placed in adjacent net-pens and treated similarly to those which had recovered from EIBS in freshwater. Groups were monitored daily and the numbers of dead fish recorded and sampling was done to determine the cause of death.

**Results and Discussion**

Hematocrit values of the fish that recovered from EIBS and were reinjected with EIBS days 71, 150, or 242 after initial infection decreased little (the lowest Ht values, 20-25%) and inclusion bodies were never seen in the erythrocytes. Typical disease did occur in the control fish (the

![Fig. 1. Successive changes of the Ht values and the incidence of fish with inclusion bodies in erythrocytes. The fish that were injected with EIBS, maintained at 8°C for 28 days and subsequently transferred to 15°C were reinjected with EIBS on days 71, 150 or 242 after initial infection and maintained at 8°C for 40 days after reinjections.]

- ●: Injected with EIBS for initial infection.
- ○: Injected with the blood homogenates from healthy fish for initial infection (positive control).
lowest Ht values, 2–6%, incidence of inclusion bodies, 60–100%) (Fig. 1). These results indicated that fish that recovered from EIBS were resistant to reinfection with EIBS and the resistance was acquired at least by day 71 or less after initial infection and remained until day 242.

Hematocrit values of the fish that were maintained at 8°C for 0, 7, 14 or 28 days after initial infection with EIBS and subsequently transferred to 15°C 242 days after initial infection decreased slightly when the fish were reinjected and maintained at 8°C (the lowest Ht values, 20–23%). Inclusion bodies in the erythrocytes of the fish were never seen (Fig. 2). The positive control fish had Ht values of 2% and inclusion bodies were seen in all the fish. Hematocrit values of the negative controls did not decrease and inclusion bodies in the erythrocytes were not seen.

The fish that were transferred to 15°C day 0 after initial infection were resistant to reinfection with EIBS. This indicated that it is not necessary for fish to have severe disease to obtain the resistance to reinfection. Even when the fish that obtained the resistance were reinjected with EIBS and maintained at 8°C (the temperature most suitable for virus propagation), the Ht values of the fish only slightly declined and recovered quickly. This rapid response may be attributed to humoral immunity which the fish acquired by recovering from EIBS (Piacentini et al., 1989).

When fish infected with EIBS were maintained at 15°C, it was determined that the fish gained resistance to reinfection at least by day 71 or less after initial infection and it was maintained until day 242. Piacentini et al. (1989) showed coho salmon that recovered from EIBS had resistance to reinfection day 50 after initial infection. The shortest period necessary to acquire resistance and its subsequent duration should be clarified when fish are maintained at 15°C. Also, lower temperature than 15°C should be tested because the immune response of salmonid fish is decreased as the temperature decreases (Avtalion, 1981; Groberg et al., 1983).

Similar to the controlled laboratory experiments, we determined that coho salmon which had survived a natural epizootic of EIBS during their freshwater stage were resistant to reinfection after being transferred to saltwater net-pens (Table 1, Fig. 3). Previously unexposed fish had high (60%) incidence of erythrocytic inclusions and reduced hematocrits. There was no incidence of viral inclusions in fish which had survived EIBS in freshwater and the hematocrits

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Fig. 2. Successive changes of the Ht values and the incidence of fish with inclusion bodies in erythrocytes. The fish that were maintained at 8°C for 0, 7, 14, 28 days after initial infection with EIBS and subsequently transferred to 15°C until day 242 after initial infection. Reinjection with EIBS was carried out on day 242 after initial infection and the fish were maintained at 8°C for 40 days after reinjections.

- : Injected with the blood homogenates from healthy fish for initial injection and maintained at 8°C for 14 days and then transferred to 15°C. Reinjected with EIBS as a positive control.
- : Injected with EIBS for initial injection and the fish were maintained at 8°C for 0, 7, 14 or 28 days, respectively, and transferred to 15°C. Reinjected with EIBS.
- : Injected with EIBS for initial injection and the fish were maintained at 8°C for 28 days and transferred to 15°C. Reinjected with the blood homogenates from healthy fish as a negative control.
Table 1. Ht values and incidence of fish with erythrocytic inclusions in animals which recovered from EIBS in freshwater compared to previously uninfected fish during mass mortalities in seawater on March 24, 1992

<table>
<thead>
<tr>
<th>Fish</th>
<th>Ht values (°H)⁴¹</th>
<th>Incidence of fish with erythrocytic inclusions (°H)⁴²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survivors of EIBS</td>
<td>35.0 ± 3.2</td>
<td>0</td>
</tr>
<tr>
<td>Previously uninfected</td>
<td>23.3 ± 7.5</td>
<td>60</td>
</tr>
</tbody>
</table>

⁴¹ Five fish were examined. Mean ± SD.
⁴² Thirty fields of view of each smear were examined under light microscope at 1,000 × magnification.

Fig. 3. Mortality attributable to EIBS in sea-cultured coho salmon in 1992. Survivors (●) of EIBS in freshwater and previously uninfected fish (○) were segregated in adjacent net-pens. Initial numbers of fish in net-pens were 7503 (●) and 8772 (○).

were normal. These field observations corroborated results gained in the laboratory and further indicate that a long lasting resistance to EIBS can be acquired from previous experience with the disease.

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References


