Hyperbilirubinemia of Coho Salmon Oncorhynchus kisutch Infected with Erythrocytic Inclusion Body Syndrome (EIBS) Virus

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Anemia caused by hemolysis occurred in coho salmon Oncorhynchus kisutch infected with the erythrocytic inclusion body syndrome (EIBS) virus. Bilirubin levels were significantly higher in the plasma of diseased fish than in healthy fish. Hyperbilirubinemia occurred in fish infected with EIBS virus. Erythrocytic superoxide dismutase activities were induced in the diseased fish. The liver bilirubin level of diseased fish was much higher than that of control fish, while the total bile acid level in the plasma of diseased fish was much higher than that in the control. The plasma astaxanthin level in the diseased fish was lower than that in the control, whereas plasma vitamin A levels of both were similar. These results suggest that excretion of bile acid decreased in the diseased fish. The accumulation of bilirubin in the liver and the disfunctional bile acid excretion indicate that secretory disorder of bilirubin occurred in the diseased fish and was one of the causes of hyperbilirubinemia observed in coho salmon infected with EIBS virus.

Key words: hyperbilirubinemia, EIBS, superoxide dismutase, bile acids, astaxanthin, vitamin A

Materials and Methods

Materials

Four coho salmon infected with EIBS virus were obtained from fish farms at Kesennuma in Miyagi Prefecture on April 23, 1991 and four control fish were obtained from another farm confirmed to be free of this disease on May 22, 1991. The fish were anesthetized with MS-222 and blood was collected with a heparinized syringe. An aliquot of the sample was used for hematochemical analyses and the remainder was centrifuged at 500 x g for 10 min. Bilirubin IXα, total bile acid, astaxanthin, and vitamin A levels were analyzed in the plasma. Because of the limited plasma volume obtained, total bile acid, astaxanthin and total vitamin A were analyzed in pooled plasma samples. The erythrocyte fraction was washed with physiological saline 2 times and diluted with about 4 volumes of water to lyse the erythrocytes. One milliliter of ethanol and 0.6 ml of chloroform were added to 4 ml of the hemolysate to remove the hemoglobin. The water-ethanol layer (100 μl) was aspirated, then diluted with about 0.7 ml of water, and assayed for superoxide dismutase.

Hematological Analyses

Hematocrit values were analyzed by the method of Ikeda et al. Blood smears were allowed to air dry, then were fixed in methanol for 5 min and stained with Giemsa, pinacyanol chloride or acridine orange. Thirty microscopic fields in each stained blood smear were examined at 1000 x and the numbers of cytoplasmic inclusion bodies and the frequency of immature erythrocytes were recorded. Five stages, including substages, of disease progression were defined on the basis of Takahashi et al. The results are summarized in Table 1.

Plasma and Liver Bilirubin IXα Levels

Plasma and liver bilirubin IXα levels were measured using HPLC as described by Sakai and Tabata. A plasma sample was mixed with the same volume of methanol, centrifuged for 1 min at 5,000 x g, and 50 μl of the supernatant was analyzed by HPLC. Bilirubin IXα was extracted from the liver with chloroform-methanol (2:1). The extract obtained was...
evaporated to dryness in vacuo. The residue was dissolved with methanol and analyzed by HPLC. Because of the limited liver volume obtained, a pooled sample was used for the extraction. The analytical conditions were as follows. Column: Waters µ-Bondapak C18 column (0.39 × 30 cm). Flow rate: 1 ml/min. Detection wavelength: 436 nm. Column temperature: 40°C. The mobile phase consisted of methanol (solvent A) and 5% acetic acid (solvent B). At zero time the proportion of solvent A/B was brought to 60% (v/v). During the first 20 min of the run, the solvent A/B ratio was increased to 90% (v/v) and maintained for the next 15 min. The limit of detection of bilirubin IXα was > 0.5 μg/ml plasma.

Superoxide Dismutase (SOD) activities in the erythrocytes were assayed by the nitrite method of Ōyanagi.27,28) The results were expressed as nitrite unit (NU) mg hemoglobin. Hemoglobin levels were measured by the cyan–methemoglobin method described by Kawatsu.28

Analysis of Plasma Total Bile Acids
Total bile acid contents in the plasma were determined by the enzymatic method of Mashige et al.24) Bile acids were oxidized by 3-hydroxy bile acid oxidase. The formed indoxyl was oxidized by diaphorase in the presence of nitroblue tetrazolium blue and the resulting formazan was determined spectrophotometrically.

Analysis of Plasma Astaxanthin and Vitamin A Contents
Astaxanthin was extracted with n-hexane and 50 μl of the extract was analyzed by HPLC as described by Yamada et al.24) The analytical conditions were as follows. Column: Gasukuro Kogyo Unisil Pack-FB-100B (6 × 100 mm). Flow rate: 2 ml/min. Detection wavelength: 340 nm. The mobile phase consisted of methanol (solvent A) and water (solvent B). At zero time the ratio of solvent A/B was 95% (v/v). During the first 20 min of the run, the proportion of solvent A was increased to 100% (v/v). Vitamin A was extracted with n-hexane, 50 μl of which was analyzed by the HPLC method of Hayashi et al.25) Analytical conditions were as follows. Column: Gasukuro Kogyo Unisil Pack-FB-100B (6 × 100 mm). Flow rate: 2 ml/min. Detection wavelength: 340 nm. The mobile phase consisted of methanol (solvent A) and water (solvent B). At zero time the ratio of solvent A/B was 90% (v/v). During the first 15 min of the run, the proportion of solvent A was increased to 100% (v/v).

Statistical Analyses
The significance of differences between means was determined using Student’s t test.

Results and Discussion
Judging from the microscopic observations of stained blood smears, stages in the progression of disease of diseased fish were classified as IIa—IIIa and compared with those of control fish which were not infected (Table 1). As shown in Table 2, the hematocrit values of fish infected with EIBS virus were significantly lower than those of control fish. Anemia, the characteristic sign of EIBS disease, occurred in the fish infected with EIBS virus, and may have been caused by lysis of infected erythrocytes. Bilirubin IXα levels in the plasma of the diseased fish were significantly higher than those of the control fish. Hyperbilirubinemia occurred in the fish infected with EIBS virus. Erythrocytic SOD activities of disease fish were significantly higher than those of the control fish (Table 2). EIBS virus infection appears to induce the enzyme activities. SOD in mammals and fish is induced in tissues that are oxidatively stressed.27,28) In addition, poly-unsaturated fatty acid levels in the erythrocytes of fish infected with EIBS were much lower than those of healthy controls (Okamoto, unpublished result). In vivo lipid peroxidation may progress in erythrocytes of coho salmon infected with the virus, then SOD may be induced to suppress it. The liver bilirubin level of disease fish was much higher than that of the control. Bilirubin was accumulated in the liver of disease fish. The total bile acid level in the plasma of disease fish was much higher than that in control fish, and the plasma bilirubin level in the diseased fish was much lower than that in control fish. On the contrary, the plasma vitamin A level in the diseased fish was similar to that in control fish, and the plasma astaxanthin level in the diseased fish was much lower than that in control fish.

Table 1. Stages of disease progression in jaundiced coho salmon

<table>
<thead>
<tr>
<th>Group</th>
<th>Specimens</th>
<th>Incidence of erythrocytes with inclusion bodies</th>
<th>EIBS Stage*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jaundiced fish (J)</td>
<td>1</td>
<td>+</td>
<td>Iib</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
<td>Iib</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>++</td>
<td>IIa</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>+</td>
<td>Iib</td>
</tr>
<tr>
<td>Healthy fish (H)</td>
<td>1</td>
<td>–</td>
<td>Non-infected</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>–</td>
<td>Non-infected</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>–</td>
<td>Non-infected</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>–</td>
<td>Non-infected</td>
</tr>
</tbody>
</table>

* The stages were defined based upon the criteria of Takahashi et al.13) Stage IIa: inclusions observed in immature erythrocytes were larger than those in mature erythrocytes. Stage IIb: inclusions were observed in both immature and mature erythrocytes. Stage IIIa: lysed erythrocytes were observed in blood smears. The prevalence of inclusions in mature erythrocytes increased rapidly and the incidence of immature erythrocytes decreased rapidly.

Table 2. Body weight, hematocrit values, plasma, and liver bilirubin contents, erythrocytic SOD activities, plasma total bile acid contents, plasma astaxanthin, and plasma total vitamin A contents of coho salmon infected with EIBS virus

<table>
<thead>
<tr>
<th>Group (Date)</th>
<th>Specimen number</th>
<th>Jaundiced (4/23)</th>
<th>Healthy (5/22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.W.</td>
<td>(g)</td>
<td>450 ± 34</td>
<td>1260 ± 80</td>
</tr>
<tr>
<td>Ht</td>
<td>(%)</td>
<td>8.1 ± 2.8**</td>
<td>31.0 ± 0.7</td>
</tr>
<tr>
<td>P.B.</td>
<td>(μg/ml)</td>
<td>5.9 ± 1.2**</td>
<td>n.d.</td>
</tr>
<tr>
<td>L.B.</td>
<td>(μg/g)</td>
<td>68.8</td>
<td>n.d.</td>
</tr>
<tr>
<td>E.SOD*1</td>
<td>(μN/μg Hb)</td>
<td>14.6 ± 2.6**</td>
<td>4.6 ± 0.5</td>
</tr>
<tr>
<td>P.BA.</td>
<td>(μg/ml)</td>
<td>15.35</td>
<td>6.62</td>
</tr>
<tr>
<td>P.A.</td>
<td>(μg/ml)</td>
<td>0.02</td>
<td>1.71</td>
</tr>
<tr>
<td>P.TV.</td>
<td>(μl/g)</td>
<td>0.45</td>
<td>0.39</td>
</tr>
</tbody>
</table>

**1: Mean ± Standard Error.
**2: Significantly different from Healthy at p<0.05.

hemolysis induces the overproduction of not bilirubin IX\(_\alpha\) but biliverdin IX\(_\alpha\) and that hemolysis alone may not cause jaundice.\(^{18,32}\) Because bilirubin IX\(_\alpha\) and its conjugates but biliverdin IX\(_\beta\) and that hemolysis alone may not cause hyperbilirubinemia observed in coho salmon with the EIBS virus may be caused not only by hemolysis but also by a secretory disorder of bile pigments.

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References