Investigation of Conditions Optimum for Spontaneous Rosette Formation by Lymphocytes of Yellowtail, *Seriola quinqueradiata*

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**Abstract**

Various factors involved in spontaneous rosette formation with erythrocytes by peripheral blood lymphocytes of yellowtail, *Seriola quinqueradiata* were investigated. The percentage of the rosette forming lymphocytes was higher with sheep red blood cells (SRBCs) than either with rabbit red blood cells or bovine red blood cells. Binding of SRBCs to lymphocytes was enhanced after treating SRBCs with 2-aminoethylisothiouronium bromide and trypsin but not with neuraminidase. There were no significant differences among the percentage of rosette forming lymphocytes incubated at 4, 25 and 37 ºC within 3 h of incubation. After 3 h, the percentage began to decrease and rosette could be detected only at 4 ºC after 24 h. Higher value of rosette formation was obtained when the lymphocytes were incubated in L-15 medium non-supplemented with fetal calf serum.

Spontaneous rosette formation with sheep red blood cells (SRBCs) has been considered as one of the most important feature of T cells in mammalian peripheral blood lymphocytes. Later it was known that the spontaneous rosette formation also occurred in fish lymphocytes. However the percentage of the rosette forming lymphocytes in fish was lower comparing with those of mammals. Therefore it seems that the percentage of the rosette formation is directly dependent upon the experimental conditions.

In this study, the conditions optimum for the spontaneous rosette formation by the lymphocytes of yellowtail, *Seriola quinqueradiata* which is one of the most important commercial marine fish of the fishery industry of Japan were investigated.

**Materials and Methods**

**Fish**

Yellowtail weighing about 50 g cultured in Uranouchi Bay, Kochi Prefecture were used.

**Lymphocyte Separation**

Peripheral blood was drawn from the heart with an heparinized syringe and was diluted up to four-fold with phosphate buffered saline (PBS, pH 7.2). Separation of lymphocyte from the blood was carried out according to the methods previously described. The separated lymphocytes were washed 3 times and resuspended in L-15 medium (modified, Flow Laboratory) supplemented with 5 % fetal calf serum (FCS). The lymphocyte concentration was adjusted to approx-
approximately $5 \times 10^6$ cells/ml using L-15 medium.

**Rosette Formation with Red Blood Cells (RBCs)**

Red blood cells from sheep, rabbit (RRBCs) and bovine (BRBCs) were used to assay rosette formation. The RBCs were washed 3 times and the concentrations were adjusted to approximately $3 \times 10^8$ cells/ml in L-15 medium. Hundred $\mu l$ of the lymphocyte suspension was added to each of the sedimintered RBCs and mixed in a round-bottomed sampling tube. Hundred $\mu l$ of FCS was added to the above mixtures. Following centrifugation at $50 \times g$ for 5 min, the mixtures were incubated for 12 h at 4°C. The mixtures were then gently resuspended and fixation-stain solution (0.005 % brilliant cresyl blue, 0.25 % glutaraldehyde in PBS) was added to each of them. Rosettes consisting of a minimum of two red blood cells bound to a lymphocyte molecule were counted on a hemocytometer. A total of 200 lymphocytes were counted in triplicate and the percent of the rosette forming lymphocytes was calculated.

**Effect of Various Treatments of SRBCs on the Rosette Formation**

Equivalent amounts of 4 % 2-aminoethylisothiouronium bromide (AET) and SRBCs ($3.0 \times 10^8$ cells/ml) in PBS were mixed and incubated for 20 min at 37°C, then washed 4 times with PBS$^{8)}$. In a second experiment, equivalent mixture of 2 mg/ml trypsin and SRBCs ($3.0 \times 10^8$ cells/ml) in PBS were incubated for 1 h at 37°C. After incubation, 2 mg/ml soybean-trypsin inhibitor was added to the mixture and then incubated for 10 min at room temperature. At the end of the incubation, SRBCs were washed 3 times. Neuraminidase was diluted to 2 U/ml in PBS (pH 6.4) and two tenth milliliter of this suspension was added to 1 ml of SRBCs ($3.0 \times 10^8$ cells/ml). The mixture was incubated for 1 h at 37°C, then washed 3 times$^9)$. Neuraminidase, bovine red blood cells.

**Results**

Results of rosette formation with RBCs are shown in Fig. 1. SRBCs, RRBCs and BRBCs showed different degrees of rosette formation. The percentage of rosette forming yellowtail lymphocytes was higher with SRBCs than either with RRBCs or BRBCs.

![Fig. 1](image)

**Rosette formation (%)**

<table>
<thead>
<tr>
<th></th>
<th>SRBCs</th>
<th>RRBCs</th>
<th>BRBCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The effect of various treatments of SRBCs on the rosette formation is shown in Fig. 2. Rosette formation with SRBCs was enhanced after treating SRBCs with AET and trypsin, but not with neuraminidase.

![Fig. 2](image)

**Rosette formation (%)**

<table>
<thead>
<tr>
<th></th>
<th>AET</th>
<th>Trypsin</th>
<th>Neuraminidase</th>
<th>Non-treatment</th>
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<tbody>
<tr>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
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<tr>
<td>3.0</td>
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</table>

**Effect of Incubation Medium and FCS on the Rosette Formation**

The effect of incubation medium on the rosette formation with SRBCs was investigated using L-15 medium supplemented with and without equivalent ratio of FCS, PBS supplemented with and without FCS and sole FCS.

The effect of various treatments of SRBCs on the rosette formation is shown in Fig. 2. Rosette formation with SRBCs were enhanced after treating SRBCs with AET and trypsin, but not with neuraminidase.

**Effect of Incubation Period and Temperature on the Rosette Formation**

The effect of incubation period and temperature on the rosette formation with SRBCs were investigated at 4, 25 and 37°C from 1 to 24 h of incubation.

The effect of incubation period and temperature on the rosette formation is shown in Fig. 3. There were no significant differences in rosette formation incu-
Rosette formation of yellowtail lymphocytes

bated at 4, 25 or 37 °C within 3 h of incubation though the percentage of rosette forming lymphocytes at 37 °C were somewhat lower than at 4 or 25 °C. After 3 h, the percentage decreased at either temperatures and the formation occurred only at 4 °C after 24 h.

Fig. 3. Effect of temperature and incubation period on rosette formation of yellowtail lymphocytes with SRBCs. ○, 4 °C; △, 25 °C; □, 37 °C.

Results of the effect of incubation medium and FCS on the rosette formation is shown in Table 1. The percentage of the rosette formation was higher in L-15 medium non-supplemented with FCS compared to all other media.

Table 1. Effect of incubation medium and fetal calf serum on rosette formation of yellowtail lymphocytes

<table>
<thead>
<tr>
<th>Medium</th>
<th>Fetal calf serum (%)</th>
<th>Rosette formation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-15</td>
<td>50</td>
<td>0.9</td>
</tr>
<tr>
<td>L-15</td>
<td>0</td>
<td>1.7</td>
</tr>
<tr>
<td>PBS</td>
<td>50</td>
<td>0.2</td>
</tr>
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<td>PBS</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>-</td>
<td>100</td>
<td>0.5</td>
</tr>
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</table>

Discussion

The population of rosette forming lymphocytes in yellowtail was as small as other species of fish in comparison to various kinds of animals. The finding still compels more to clarify the detail conditions for the rosette formation in order to investigate rosette forming lymphocytes of yellowtail.

Binding of the peripheral blood lymphocytes of yellowtail was specific to SRBCs among SRBCs, RRBCs and BRBCs. As the result was consistent with that reported for mammalian rosette formation, it seems that yellowtail lymphocytes have SRBC receptors similar to those of mammalian lymphocytes.

In mammal, rosette formation was known to be enhanced by using SRBCs treated with AET and neuraminidase, but not with trypsin. The result of enhanced rosette formation of AET treated SRBCs with yellowtail lymphocyte was consistent with that reported for mammalian lymphocytes. On the other hand, the results of trypsin and neuraminidase treatment differ from those reported for mammalian. Earlier investigators demonstrated that the number of rosette forming lymphocytes of human was reduced because of the degradation of glycoprotein sequences, α-1, α-2 and α-3 on the erythrocyte cell membrane due to the trypsin treatment, conversely degradation of sialic acid due to the neuraminidase treatment enhanced rosette formation. The result may shows that yellowtail and human lymphocytes recognize different glycoprotein sequences on erythrocytes cell membrane.

Incubation temperatures 4, 25 or 37 °C has little effect on rosette formation. Such independence is similar to those reported for mammals and carp.

In human, it has been reported that rosette formation is favored by the presence of FCS. Nevertheless, the percentage of rosette forming lymphocytes of yellowtail was higher when incubated with SRBCs in L-15 medium without FCS compared to all other conditions.

From these results, it can be concluded that the conditions optimum for the rosette formation are 3 h of incubation of lymphocytes at 4 or 25 °C with AET treated SRBCs in L-15 medium non-supplemented with FCS.

References

ブリ末梢血リンパ球の自然ロゼット形成の好適条件

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ブリ末梢血リンパ球の自然ロゼット形成の好適条件を調べるために、赤血球の種類、赤血球膜の処理法、リンパ球と赤血球との反応時間、温度および培地によるロゼット形成率の差異について検討した。その結果、ロゼット形成率は、赤血球の種類ではヒツジ赤血球（SRBC）が、赤血球の膜処理では2-aminoethylisothiouroniumhydrobromide（AET）あるいはトリプシン処理が、反応時間では3時間が、反応温度では4℃あるいは25℃が、反応培地では牛胎児血清を含まないL-15培地が、それぞれ高かった。これらのことから、自然ロゼットを好適な条件で形成させるためにはSRBCのAET処理したものを使い、4℃において3時間、牛胎児血清を含まないL-15培地中で反応させるのがよいと思われる。