The development of hepatogenic potency in the endoderm of quail embryos

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SUMMARY

Hepatogenic potency of the endoderm is detectable in the anterior half of the endoderm of quail embryos older than 2-somite stage when endodermal fragments are cultured with or without heterologous chick mesenchymes, in the coelomic cavity of 3-day chick embryos. On the other hand, the posterior half of the endoderm never has hepatogenic potency. The hepatogenic potency of the endoderm is gradually stabilised with increasing age. However, expression of hepatogenesis can be affected when the endoderm is associated with inductively active digestive tract mesenchymes.

Mesenchyme taken from the presumptive cardiac region (‘cardiac’ mesenchyme) of chick embryos is necessary for the uncommitted anterior endoderm to acquire hepatogenic potency, and this effect is specific for the ‘cardiac’ mesenchyme. The ‘cardiac’ mesenchyme, however, fails to induce hepatic epithelium in the allantoic endoderm, which can differentiate heterotypically when cultured in combination with digestive tract mesenchymes. The evidence presented in this study suggests that the effect of ‘cardiac’ mesenchyme on the acquisition of hepatogenic potency in the endoderm is limited.

INTRODUCTION

Embryonic development of the liver involves a complex series of cellular interactions. In the chick embryo, the primordium of the liver appears around the 20-somite stage as an endodermal evagination of the floor of the anterior intestinal portal. The hepatic endoderm proliferates, and together with the surrounding mesenchymal tissue including the endothelium, finally differentiates into the hepatic structure composed of hepatic cords and sinusoids (Kingsbury, Alexanderson & Kornstein, 1956; Romanoff, 1960). We have previously described the normal morphogenesis of the liver of the chick embryo with special reference to the developmental interrelation of liver elements: the hepatic endoderm, the hepatic mesenchyme and the endothelium (Fukuda, 1976). Interactions of these components during the morphogenesis of the liver have been experimentally analysed (Le Douarin, 1964; Houssaint & Le Douarin, 1971; Sherer, 1975).

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Le Douarin (1964, 1975) showed that the endoderm becomes capable of differentiating into hepatic epithelium when associated with the hepatic mesenchyme ('determination' of hepatic endoderm) at the 4- to 5-somite stage far earlier than the initial hepatic evagination, and that the ‘determination’ of the hepatic endoderm depends on the presence of the pre-cardiac mesenchyme. The importance of the cardiac mesenchyme in hepatic differentiation has been indicated by Hunt (1931), Willier & Rawles (1931) and Rawles (1936).

Recent experiments with the chick embryo (Sumiya, 1976; Sumiya & Mizuno, 1976; Mizuno & Sumiya, 1977) have established that anterior fragments of the endoderm older than the 7-somite stage can differentiate into hepatic epithelium in vitro in the absence of mesenchymal cells.

This paper deals with the developmental processes of the hepatic endoderm before hepatic primordium formation in quail embryos. The developmental interrelation of the liver elements before the initial hepatic evagination is described, and the first appearance of endoderm possessing hepatogenic potency is clarified. The degree of hepatogenic potency in endoderm from various developmental stages, when cultured in association with heterologous mesenchymes, is defined. Some mesenchymes from the digestive tract have been demonstrated to have inductive ability (Sigot, 1971; Mizuno & Yasugi, 1973). Finally, the specificity and effect of the ‘cardiac’ mesenchyme on the acquisition of hepatogenic potency in the endoderm is demonstrated: the ‘cardiac’ mesenchyme is associated with the anterior endoderm or the allantoic endoderm, which has been proved to differentiate heterotypically under the influence of inductively active digestive-tract mesenchymes (Mizuno & Yasugi, 1973; Yasugi & Mizuno, 1974; Yasugi, 1976a, b; Gumpel-Pinot, Yasugi & Mizuno, 1978).

MATERIALS AND METHODS

Embryos

Japanese quail (Coturnix coturnix japonica) and White Leghorn (Gallus gallus domesticus) embryos were used. The stages were identified by the appearance of blastoderms or by the number of paired somites for younger embryos, and by days of incubation for older embryos.

To exclude cellular contamination of either of the tissue components, the difference of nuclear structure between chick and quail cells was used as a biological cell marker (Le Douarin, 1969). Endodermal tissues were obtained from quail embryos and mesenchymal tissues from chick embryos.

Isolation of endodermal fragments

Quail embryos from definitive streak to 21-somite stage were cut into two pieces (the anterior and posterior pieces) at various antero-posterior levels according to the developmental stages: for pre-somite stages, the level was posterior to the posterior end of Hensen’s node; for 1- to 4-somite stage,
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posterior to the last somite; for older than 5-somite stage, between the 4th and the 10th somite. These anterior and posterior pieces were treated in 0.03% collagenase (Worthington, CLSPA) in Tyrode's solution for 80 min at 37 °C, and anterior and posterior endodermal fragments were isolated. The areas of secure adhesion between the endodermal layer and overlying tissues, the area under the anterior half of the primitive streak and the head process and the apex of the foregut, were excluded from the preparation of endodermal fragments. Allantoic endoderm was obtained from the allantois of 3-day-old quail embryos. These endodermal fragments were washed thoroughly in three changes of serum-supplemented Tyrode's solution to eliminate the enzyme, and finally in fresh Tyrode's solution.

Preparation of mesenchymes

The mesenchymes of several definite parts of the digestive tract (oesophagus, proventriculus, gizzard and small intestine), were obtained from 5- to 15-day chick embryos by collagenase treatment.

‘Cardiac’ mesenchyme was obtained from the presumptive cardiac and the cardiac region (Rudnick, 1938; Rawles, 1943; Le Dourain, 1964) of head fold to 11-somite-stage chick embryos with the aid of collagenase. Non-cardiac mesenchyme was obtained from the area posterior to the 4th somite for 4- to 6-somite-stage embryos and posterior to the 5th to 7th somite for 7- to 11-somite-stage embryos.

In vitro association of tissue fragments

The endodermal fragments or allantoic endoderm of quail embryos were cultured in interspecific combination with various heterologous (non-hepatic), ‘cardiac’, or non-cardiac mesenchymes for 1 day in vitro according to the modified method of Wolff & Haffen (1952): the culture medium consisted of seven parts of 1% Difco Bacto-Agar in Gey’s solution, three parts of foetal calf serum (Flow Laboratories Ltd.), three parts of medium 199 containing Earle’s balanced salt solution (Morgan, Morton & Parker, 1950) and one part of Tyrode’s solution containing potassium penicillin G.

In vivo cultivation of endodermal fragments and recombinants

Isolated anterior and posterior endodermal fragments were transplanted directly into the coelomic cavity of 3-day chick embryos and cultured for 6-8 days.

Interspecific quail-chick recombinants of various tissue fragments cultured in vitro were transplanted into the coelomic cavity or grafted onto the chorio-allantoic membrane, and were harvested after 6-8 days.

Histological methods

The explants were fixed in Bouin’s fluid, embedded in paraffin, and sectioned at 5 µm. The sections were stained with Carazzi’s haematoxylin and eosin. To detect glycogen, PAS-staining was carried out after fixation in Gendre’s fluid.
Table 1. Sequence of development of liver elements

<table>
<thead>
<tr>
<th>Stages</th>
<th>Splanchnic mesenchyme</th>
<th>Embryonic endothelium</th>
<th>Hepatic mesenchyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Definitive streak</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Head process</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Head fold</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1–2 somite</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3–4 somite</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5–6 somite</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>7–8 somite</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>9–10 somite</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>11–13 somite</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>14–16 somite</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>17–19 somite</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>20–22 somite</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>23–25 somite</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>26–28 somite</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>29–32 somite</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Criteria for identifying the differentiation of hepatic epithelium

The formation of bile canaliculi characteristically bordered by several hepatic cells and of hepatic cords composed of two rows of hepatocytes identifies the hepatic epithelium. Glycogen storage in cytoplasm of hepatic cells and the presence of intrahepatic bile ducts composed of cuboidal epithelial cells with homogeneous cytoplasm are also standards for the identification of hepatic epithelium.

RESULTS

Development of three major tissue components related to the differentiation of the liver

The early development of the hepatic endoderm, the hepatic mesenchyme and the embryonic endothelium of quail and chick embryos was studied with special reference to their temporal and spatial interrelations. The results with the quail and chick embryos were basically similar and are summarized in Table 1 and Fig. 1.

Around the 20-somite stage the endoderm of the margin of the anterior intestinal portal evaginates and forms the hepatic primordium. At this time, the hepatic endoderm is in close contact with the endothelium of the omphalomesenteric vein and splanchnic mesenchyme.

Splanchnic mesenchyme develops at the latest by the 4-somite stage, and contacts with the endoderm of the anterior intestinal portal. The splanchnic mesenchyme develops into the cardiac mesenchyme, endothelium and a loose mesenchymal tissue.
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<table>
<thead>
<tr>
<th>Stages</th>
<th>Hepatic endoderm</th>
</tr>
</thead>
<tbody>
<tr>
<td>14–16 somite</td>
<td></td>
</tr>
<tr>
<td>17–19 somite</td>
<td></td>
</tr>
<tr>
<td>20–22 somite</td>
<td></td>
</tr>
<tr>
<td>23–25 somite</td>
<td></td>
</tr>
<tr>
<td>26–32 somite</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Schematic drawings of the formation and branching of hepatic primordial endoderm.

The loose mesenchymal tissue develops around the ductus venosus somewhat after the onset of hepatic-bud evagination. The hepatic primordial endoderm and the endothelial cells proliferate and invade the loose mesenchymal tissue, which is identical to the hepatic mesenchyme, and form hepatic cords and sinusoids.

The endothelium of cardiac and anterior intestinal portal region first appears at the 5- to 6-somite stage, much earlier than the beginning of the hepatic-bud evagination.

Anterior and posterior endodermal fragments cultured alone in the coelomic cavity

Anterior and posterior endodermal fragments isolated from various stages of quail embryos were transplanted into the coelomic cavity of 3-day chick embryos (Fig. 2). After 6–8 days' cultivation, about two-thirds of explants could be found to attach loosely to various mesenchymes of the host, adjacent to the coelomic cavity, or to float in the abdominal cavity forming cyst-like structures. Histological sections showed that the endodermal epithelium consisted of quail cells and the mesenchyme, chick cells. As shown in Table 2, the hepatogenic potency of the anterior endoderm can first be demonstrated at the 5-somite stage in these experimental conditions (Fig. 5), and the frequency of hepatic epithelium formation becomes higher as development proceeds. After the onset of hepatic morphogenesis (as shown by * in Table 2) the incidence becomes about 84%. The hepatic epithelium never develops from the posterior endodermal fragments regardless of stage. Besides the hepatic epithelium,
Fig. 2. Schematic representation showing the isolation of anterior and posterior endodermal fragments from definitive streak to 21-somite-stage quail embryos. These endodermal fragments alone are transplanted into the coelomic cavity of 3-day chick embryo. AIP, anterior intestinal portal.

Table 2. Areas and stages of the quail endoderm from which hepatic epithelium differentiated, when the endoderm was cultured in the coelomic cavity of chick embryos

<table>
<thead>
<tr>
<th>Stages</th>
<th>Anterior endoderm</th>
<th>Posterior endoderm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Definitive streak</td>
<td>0/3 (0)</td>
<td></td>
</tr>
<tr>
<td>Head process</td>
<td>0/9 (0)</td>
<td></td>
</tr>
<tr>
<td>Head fold</td>
<td>0/7 (0)</td>
<td>0/16 (0)</td>
</tr>
<tr>
<td>1–2 somite</td>
<td>0/9 (0)</td>
<td></td>
</tr>
<tr>
<td>3–4 somite</td>
<td>0/8 (0)</td>
<td></td>
</tr>
<tr>
<td>5–6 somite</td>
<td>5/22 (23)</td>
<td></td>
</tr>
<tr>
<td>7–8 somite</td>
<td>4/16 (25)</td>
<td></td>
</tr>
<tr>
<td>9–10 somite</td>
<td>7/18 (38)</td>
<td></td>
</tr>
<tr>
<td>11–12 somite</td>
<td>3/9 (33)</td>
<td>0/20 (0)</td>
</tr>
<tr>
<td>13–14 somite</td>
<td>4/7 (57)</td>
<td></td>
</tr>
<tr>
<td>15–21 somite</td>
<td>3/6 (50)</td>
<td></td>
</tr>
<tr>
<td>21–35 somite</td>
<td>26/31 * (84)</td>
<td></td>
</tr>
</tbody>
</table>

No. in parentheses designates the percentage of differentiation of the hepatic epithelium.
* Hepatic primordial endoderm was cultured.

It can be concluded from these results that the endoderm possesses hepaticogenic potency at the latest by the 5-somite stage in quail embryos, and that this potency is restricted to the anterior half of the endoderm, and becomes gradually stable as development proceeds.

Combination of anterior endoderm and heterologous mesenchyme

To define the stability of the differentiation capacity of the hepatic endoderm, anterior endodermal fragments derived from definitive streak to 19-somite-
stage quail embryos were associated with heterologous mesenchymes of the digestive tract of chick embryos. Two series of experiments were prepared (Fig. 3): series A, the anterior endoderm was combined with the mesenchymes of 8- to 15-day digestive tract; series B, the anterior endoderm was combined with mesenchymes of 5-day digestive tract.

In series A, we found that oesophageal, proventricular, and small intestinal mesenchymes of 8- to 14-day embryos permitted differentiation of hepatic
epithelium from the anterior endoderm of embryos older than 2-somite stage (10 cases out of 46) (Fig. 6 and Table 3).

In series B, hepatic epithelium differentiated less often (12 cases out of 125). The differentiation of hepatic epithelium was limited to endoderm older than the 4-somite stage (Table 3).

The results of these two series of experiments indicate that the anterior endoderm possesses hepatogenic potency at the earliest from the 2-somite stage or at the latest from the 4-somite stage, and that this potency is expressed even in the presence of heterologous mesenchymes, although the expression of hepatogenesis by the anterior endoderm can be affected, especially when associated with inductively active mesenchymes of 5-day digestive tract (series B).

Specificity and effect of 'cardiac' mesenchyme in the development of the liver

The specificity and effect of mesenchyme taken from the presumptive cardiac region ('cardiac' mesenchyme) in the acquisition of the differentiation potency of the hepatic endoderm in quail embryos were investigated. The experimental procedure is shown in Fig. 4. The anterior endodermal fragments from definitive streak to 1-somite-stage embryos were associated with 'cardiac' or non-cardiac mesenchyme. The recombinants were cultured in the coelomic cavity or on the chorio-allantoic membrane. To test the inducing effect of the 'cardiac' mesenchyme, allantoic endoderm of 3-day quail embryos was also combined with these mesenchymes and cultured in the coelomic cavity.

Hepatic epithelial cells with heavy glycogen storage in their cytoplasm, and forming hepatic cords and bile canaliculi often differentiate from the anterior endoderm associated with the 'cardiac' mesenchyme. (Fig. 7). Most of these explants were accompanied by rhythmically pulsating cardiac tissue. In contrast, the anterior endodermal fragments cultured with non-cardiac mesenchyme seldom differentiated into hepatic epithelium and mostly remained undifferentiated (Table 4). The allantoic endoderm associated with the 'cardiac' or non-cardiac mesenchyme, never differentiated into hepatic epithelium even when pulsating cardiac tissue differentiated, and remained undifferentiated (Fig. 8).

These results strongly suggest specific dependence on 'cardiac' mesenchyme of the differentiation of hepatic epithelium in quail embryos as has been suggested in chick embryos. However, 'cardiac' mesenchyme fails to induce hepatic epithelium in the allantoic endoderm, which possesses considerable ability to undergo heterotypic differentiation. Other types of epithelia such as intestine-like epithelium often differentiate in the allantoic endoderm associated with 'cardiac' as well as non-cardiac mesenchymes.

Discussion

The hepatogenic potency of endoderm has been investigated extensively in the chick embryo. The endoderm of the presumptive hepatic area taken from embryos older than 4-somite stage can differentiate into hepatic epithelium only
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Fig. 4. Diagram showing the mode of association of quail anterior or allantoic endoderm with chick 'cardiac' or non-cardiac mesenchyme.

Table 4. Differentiation of the anterior endoderm of definitive streak to 1-somite stage or allantoic endoderm under the influence of 'cardiac' or non-cardiac mesenchyme

<table>
<thead>
<tr>
<th>Origin of the endoderm</th>
<th>'Cardiac' mesenchyme</th>
<th>Non-cardiac mesenchyme</th>
<th>Culture condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior</td>
<td>9/10</td>
<td>2/13</td>
<td>Coelomic cavity</td>
</tr>
<tr>
<td>Anterior</td>
<td>20/27</td>
<td>0/36</td>
<td>Chorio-allantoic</td>
</tr>
<tr>
<td>Allantoic</td>
<td>0/17</td>
<td>0/7</td>
<td>Coelomic cavity</td>
</tr>
</tbody>
</table>

under the influence of hepatic mesenchyme (Le Douarin, 1964). However, foregut endoderm older than the 7-somite stage can differentiate into hepatic epithelium when it is associated with lateral plate mesenchyme (Le Douarin & Bussonnet, 1966; Le Douarin, Bussonnet & Chaumont, 1968).

The present investigation revealed that the hepatogenic potency of the anterior endoderm of the quail embryo appeared between the 2- and 5-somite stages, when the endoderm was cultured in the host chick, with or without various heterologous mesenchymes. Though these stages vary a little according to the experimental conditions (Tables 2, 3), the first appearance of hepatogenic potency precedes the appearance of hepatic primordia and hepatic mesenchyme. These stages almost coincide with the stages when the endoderm becomes capable of differentiating to hepatic epithelium under the influence of hepatic mesenchyme.
It has been reported that the hepatic primordial endoderm can never express its hepatogenic potency, if introduced into the coelomic cavity of a 3-day host (Le Douarin, 1964, 1975). However, in the present study of more than 200 endodermal fragments transplanted into the coelomic cavity before development of hepatic primordia, two-thirds survived and differentiated into hepatic epithelium as well as many kinds of digestive tract epithelia. As the explants were loosely attached to the mesenchymes adjacent to the coelomic cavity, or were floating in the abdominal cavity, they seemed to be under minimal influence of the mesenchymes and to express their own differentiation potency, that is, for self-differentiation.

Sumiya (1976), Sumiya & Mizuno (1976) and Mizuno & Sumiya (1977) demonstrated that endodermal fragments taken from embryos older than the 7-somite stage can differentiate into hepatic epithelial cells when foregut endodermal fragments are cultured in vitro enveloped in a fragment of the vitelline membrane, but in the absence of any type of mesenchyme. These results, together with those obtained in the present study, indicate that the foregut endoderm at these developmental stages has been committed towards hepatic development.

It must be noted that the time of appearance of self-differentiation potency in the endoderm for the hepatic epithelium, coincides with that of the first differentiation of embryonic endothelium in the anterior intestinal portal region. The endothelium of this region is generally considered as the most important in hepatic morphogenesis (Kingsbury, Alexanderson & Kornstein, 1956; Karrer, 1961; Le Douarin, 1964; Sherer, 1975; Fukuda, 1976).

Data from the present investigation show that the expression of the potency to differentiate into hepatic endoderm is often prevented by inductively active heterologous mesenchymes: the differentiation of hepatic epithelium from endodermal fragments was strongly prevented by association with 5-day digestive tract mesenchymes. The regionally specific inductive ability of these mesenchymes has been demonstrated (Sigot, 1971; Mizuno & Yasugi, 1973).

Figures 5-8

Fig. 5. Quail anterior endoderm of 6-somite stage cultured in the coelomic cavity of a 3-day chick embryo for 8 days. Differentiation of hepatic epithelium forming hepatic cords and bile canaliculi.
Fig. 6. Quail anterior endoderm of 4-somite stage cultured in combination with small intestinal mesenchyme of a 8-day chick embryo for 8 days in the coelomic cavity. Differentiation of hepatic cords and bile canaliculi.
Fig. 7. Quail anterior endoderm at head-fold stage cultured in combination with chick 'cardiac' mesenchyme of 6-somite stage. Hepatic tissue with hepatic cords and bile canaliculi develops well after 8 days' cultivation in the coelomic cavity.
Fig. 8. Quail allantoic endoderm (3-day) cultured combined with chick 'cardiac' mesenchyme of 4-somite stage. The endoderm remains as undifferentiated cellular masses or sheets.
In the present study, other types of epithelia such as oesophageal, proventricular, gizzard, intestinal, were frequently observed to differentiate in the endoderm associated with the mesenchymes of these organs.

The differentiation potency of hepatic primordial endoderm in heterologous combination (Le Douarin, 1964; Houssaint & Le Douarin, 1971; Sherer, 1975) or in coelomic culture (Fukuda & Mizuno, 1978) has been investigated. According to our work, the hepatic primordial endoderm differentiates into hepatic epithelium and further into the epithelia of intra- and extrahepatic bile ducts and gall bladder in coelomic cultures. These facts, together with the results obtained in the present study, indicate that the hepatogenic potency of the hepatic endoderm is stabilized at the stage when the hepatic primordium forms.

The importance of cardiac mesenchyme in hepatic differentiation has been indicated previously (Hunt, 1931; Willier & Rawles, 1931; Rawles, 1936). According to these authors, liver differentiation is always accompanied by differentiation of cardiac tissue. Le Douarin (1964, 1975) reported that the presence of pre-cardiac mesoderm is necessary for 'determination' of hepatic endoderm. The present study also demonstrates the necessity and specificity of the 'cardiac' mesenchyme for the acquisition of hepatogenic potency in the uncommitted endoderm, by the results obtained from experimental combinations between endoderm and 'cardiac' or non-cardiac mesenchyme (Table 4). Though the 'cardiac' mesenchyme used in the present study was taken from the presumptive cardiac region or the cardiac region, it is conceivable that it contained mesenchymal cells which may participate in the formation of organs other than the heart. What kinds of cell population in mesenchyme taken from the presumptive cardiac region are really responsible for the acquisition of the hepatogenic potency, and whether there is a causal relation between the evolution of hepatic endoderm and the cardiac differentiation are unresolved.

Allantoic endoderm, which is known to have considerable ability for heterotypic differentiation (Mizuno & Yasugi, 1973; Yasugi & Mizuno, 1974; Yasugi, 1976a, b; Gumpel-Pinot et al. 1978), did not acquire hepatogenic potency when exposed to 'cardiac' mesenchyme. Our experimental data have also shown that the 'cardiac' mesenchyme does not affect the posterior part or area opaca of definitive streak to head-fold-stage blastoderms (Fukuda, unpublished). These results indicate that the inducing ability of the 'cardiac' mesenchyme is limited. The distribution of endoderm responsive to stimulation by 'cardiac' mesenchyme will be described in a separate communication.

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S. FUKUDA


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