Culture of mouse embryos during neurulation

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SUMMARY
A comparison between static versus rotator culture systems and a variety of media (rat serum, new born calf serum, DMEM and Waymouth's) was made in an attempt to promote in vitro growth of mouse embryos from the beginning of neurulation (headfold stage) to the closure of the neural tube and formation of the limb buds (48 h). The results demonstrate that good development can be achieved for 48 h using a rotator system and that 80% of embryos cultured on rotators show growth and differentiation similar to that obtained for the same time period in vivo. Static cultures are less successful and embryos grown in this system show lower protein content and somite numbers than those maintained on rotators. Undiluted rat serum is superior to all other media tested and supports better growth and development as monitored by total protein and developmental abnormalities.

INTRODUCTION
Two studies have been made recently of the development of post-implantation mouse embryos in culture: Sadler (1979) has shown that embryos explanted at early somite stages can be maintained in culture throughout much of the period of organogenesis, and Tam & Snow (1980) have shown that primitive-streak-stage embryos can be grown to early somite stages. However, these investigators achieved little success in maintaining embryos beyond this early somite stage. In both studies rat serum provided a good nutrient medium and Tam & Snow also report favourable results with mouse serum and serum diluted with Dulbecco's modified Eagle's medium. Interestingly, Tam & Snow's success was obtained using a static culture technique, whereas Sadler's study employed a rotator system.

Results from these studies raise the obvious question of whether or not mouse embryos can be cultured continuously throughout the period of neurulation, i.e., from primitive streak to complete closure of the neural tube. Rat embryos fulfill this requirement excellently (New, Coppola & Cockroft, 1976), but there would be several advantages if a similar success could be achieved with mice: Since embryos show maximum sensitivity to teratogenic insult during early organogenesis, systems that can support embryonic growth at this time are valuable. Use of mice for these studies is desirable since they are cheaper

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to purchase and maintain than rats, but respond in a similar fashion to terato-
gen. A greater advantage of mice is the wide selection of genetic mutants
available including those which demonstrate neural tube abnormalities (Kalter,
1980). Comparing morphogenetic events in the mutants with normal develop-
ment offers an excellent opportunity to understand basic developmental
processes. Use of in vitro techniques to study these mutants, especially if they
can be maintained in culture throughout formation of the organ system to be
studied, provides the investigator with an opportunity to observe and manipu-
late developmental events at initial morphogenetic stages. Culturing throughout
entire stages of organ formation also provides the opportunity to observe all
phenomena related to this process including the end result, i.e. the final pattern
of normal or abnormal morphogenesis.

Thus, development of a successful system for culturing pre-somitic mouse
embryos throughout the process of neurulation was undertaken. Included in
this attempt was an evaluation of static versus rotator culture systems and the
possibility of using diluted serum as a culture medium. Use of a static system
had met with success in Tam & Snow's study and if capable of maintaining
embryos throughout the period in question would have the advantage of being
simpler and less expensive than rotator techniques. Also the possibility of
employing even partially diluted rat serum would offer a great advantage since
serum is both costly and time consuming to obtain. In all studies particular
attention has been given to the quality of embryonic development obtained and
the frequency of malformations.

**MATERIALS AND METHODS**

*Embryos*

Embryos were obtained from random bred ICR mice. These embryos were
explanted on day 8½ of gestation (plug day = day 1) and prepared for culture
as described previously (New, 1971; Sadler, 1979). (It should be noted that this
day of gestation corresponds approximately to Tam & Snow's day 7½ since they
record their days as post coitum with the day of mating as day 0, whereas we
count this as day 1. Also the embryonic stages are similar at this time, i.e. late
primitive streak–early headfold, Fig. 1.) The parietal yolk sac was removed,
but the visceral yolk sac left intact. The ectoplacental cone was removed from
some embryos, but left intact in others (see next section). Only late-primitive-
streak–early-headfold-stage embryos with visceral yolk-sac dimensions of
0·6–0·8 × 1·1–1·4 mm were selected for culture. These embryos showed initial
elevation of cranial neural folds, but no spinal neural fold elevation or somite
formation. All embryos were cultured 48 h.
Culture systems

(1) Rotator with intermittent gassing: Stoppered glass flasks (10 ml) were attached to a disc rotating at 30 rev./min. (Sadler, 1979). Each flask contained 1 ml medium and 1 embryo. The ectoplacental cone was left intact in all embryos and flasks were gassed with 5% O₂/5% CO₂/90% N₂ at the beginning of culture and at 12 h and 24 h, followed by gassing with 20% O₂/5% CO₂/75% N₂ at 36 h.

(2) Rotator with continuous gassing: Unsealed glass-bottles (10 ml) were plugged into a hollow drum rotating at 30 rev./min. (New & Cockroft, 1979). Inlet and outlet tubes from the drum allowed continuous gassing of the cultures throughout incubation. Each bottle contained 2 ml medium and two embryos. The ectoplacental cone was removed from half the embryos (for comparison with embryos in dishes), while in the others it was left intact. Cultures were gassed with 5% O₂/5% CO₂/90% N₂ during the first 36 h and 20% O₂/5% CO₂/75% N₂ for the remainder of the culture period.

(3) Falcon plastic Petri dishes. The method was similar to that of Tam & Snow (1980). The dishes were 35 mm diameter, each containing 2 ml medium and two embryos, or 3 ml medium and three embryos. The ectoplacental cone was removed from each embryo to prevent attachment to the floor of the dish. Dishes were incubated in sealed humidified containers gassed with 20% O₂, 5% CO₂, 75% N₂.

Culture media

Rat serum was prepared by immediate-centrifugation (Steele, 1972) of blood from Sprague-Dawley retired breeders, and was heat-inactivated at 56 °C for 30 min. It was stored frozen at −20 °C for up to one month before use.

Newborn calf serum (NCS) (found by Tam & Snow, 1980, to give better results than foetal calf serum) was obtained from Flow Laboratories (Dublin, Virginia) and heat-inactivated.

Dulbecco’s modified Eagle’s medium (DMEM), with glutamine, but without pyruvate, was freshly prepared by the tissue-culture laboratory facility at the University of Virginia Diabetes and Research Training Center. For some cultures, extra glutamine at a final (additional) concentration of 2 mM, and sodium pyruvate at a final concentration of 0.1 mM, were added (DMEM +) as recommended by Tam & Snow (1980).

Waymouth’s MB751 medium was obtained from Flow Laboratories (Dublin, Virginia).

In all cultures, rat serum was used either undiluted or as 1:1 mixtures with DMEM, DMEM + or Waymouth’s medium. The newborn calf serum was used in a 1:2 mixture with DMEM +. Streptomycin (50 µg/ml) was added to all media.
Table 1. Comparison of growth of embryos maintained in vivo and under different culture conditions from late primitive streak-early headfold to completion of neural tube closure (48 h)

<table>
<thead>
<tr>
<th>Culture system and medium</th>
<th>No. of embryos (mean ± SD)</th>
<th>Protein (µg) (mean ± SD)</th>
<th>Somites (mean ± SD)</th>
<th>Blood circ.*</th>
<th>Turning complete*</th>
<th>Turning folds closed*</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo</td>
<td>19</td>
<td>116 ± 23</td>
<td>23 ± 0-9</td>
<td>19 (100)</td>
<td>19 (100)</td>
<td>18 (95)</td>
</tr>
<tr>
<td>Rotator/intermittent gassing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat serum</td>
<td>16</td>
<td>135 ± 32</td>
<td>23±0±1·1</td>
<td>16 (100)</td>
<td>15 (94)</td>
<td>12 (75)</td>
</tr>
<tr>
<td>1:1 Rat serum: DMEM</td>
<td>12</td>
<td>65 ± 31†</td>
<td>13±0±0·9</td>
<td>10 (83)</td>
<td>1 (10)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1:1 Rat serum: DMEM+</td>
<td>7</td>
<td>36 ± 11†</td>
<td>10±1±0·9</td>
<td>5 (71)</td>
<td>1 (14)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1:1 Rat serum: Waymouth's</td>
<td>10</td>
<td>33 ± 15†</td>
<td>11±0±0·8</td>
<td>5 (50)</td>
<td>1 (10)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Rotator/continuous gassing</td>
<td></td>
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<td></td>
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<tr>
<td>Rat serum</td>
<td>18</td>
<td>115 ± 22</td>
<td>23±8±1·0</td>
<td>16 (89)</td>
<td>16 (89)</td>
<td>15 (83)</td>
</tr>
<tr>
<td>1:1 Rat serum: DMEM</td>
<td>6</td>
<td>116 ± 32</td>
<td>22±8±0·9</td>
<td>5 (83)</td>
<td>5 (83)</td>
<td>2 (33)</td>
</tr>
<tr>
<td>1:1 Rat serum: DMEM+</td>
<td>8</td>
<td>79 ± 22†</td>
<td>17±6±0·8</td>
<td>6 (75)</td>
<td>4 (50)</td>
<td>1 (12)</td>
</tr>
<tr>
<td>1:2 NCS: DMEM+</td>
<td>6</td>
<td>no growth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Petri dishes (static cultures)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat serum</td>
<td>10</td>
<td>69 ± 26†</td>
<td>19±3±0·8</td>
<td>5 (50)</td>
<td>5 (50)</td>
<td>5 (50)</td>
</tr>
</tbody>
</table>

* Percentages in brackets.
† P ≤ 0.001 compared to in vivo controls or rotator systems using rat serum.
‡ P ≤ 0.001 compared to in vivo controls or rotator systems using rat serum.
P ≤ 0.05 compared to rotator continuous gassing, 1:1 rat serum: DMEM Student’s t-test employed for statistical analyses.

Assessment of results

After 48 h, cultures were terminated and embryos were examined for defects, particularly inhibition of neural tube closure. Somites were counted, blood circulation and turning of the embryos to the foetal position were noted, and protein determinations (of embryos without membranes) were made using the method of Lowry, Rosebrough, Farr & Randall (1951).

Fig. 1. Headfold-stage embryo as prepared for culture. Visceral yolk sac (YS); ectoplacental cone (EC); headfolds (H). × 400.

Fig. 2. 10½-day embryo maintained in vivo demonstrating development similar to that achieved by maintaining 8½-day embryos for 48 h in culture. × 45.

Fig. 3. Embryo cultured 48 h in undiluted rat serum using a rotator system. Turning is complete and normal growth and development have occurred including neural tube closure and somite formation. × 45.

Fig. 4. Embryo cultured 48 h in a 1:1 mixture of rat serum and DMEM on a rotator with constant gassing. Although significant growth has occurred, turning is incomplete and headfolds have failed to close in a pattern characteristic of exencephaly. × 45.

Fig. 5. Embryo cultured 48 h in a 1:1 dilution of rat serum and Waymouth’s medium. Little or no growth has occurred and no normal morphology remains. × 75.

Fig. 6. Embryo cultured 48 h in rat serum in static culture showing no rotation, poor growth and open neural folds. × 75.
RESULTS

Results from various combinations of culture systems and medium are summarized in Table 1. Best results were obtained with rotators and whole rat serum and both types of rotator gave similar results. Approximately 90% of the embryos completed turning, developed a rapid uninterrupted heart beat and circulation (embryonic and visceral yolk sac), and attained a size, protein content, and stage of organogenesis similar to those of comparable embryos in vivo (Figs. 2 and 3). In approximately 80% of the embryos the neural tube closed normally, but in the remainder closure of cranial folds was incomplete (exencephaly). No difference was noted between embryos cultured with or without the ectoplacental cone.

The rat serum/DMEM mixture gave different results in the two rotator systems: In the rotator with intermittent gassing, growth and development were poor, whereas in the rotator with continuous gassing, embryonic growth (total protein) was equal to that achieved by embryos in whole serum. However, four out of six embryos showed failure of cranial neural fold closure (Fig. 4). Interestingly, serum diluted (1:1) with DMEM+ was even less successful, and produced lower protein values and somite numbers than DMEM. Cultures with serum and Waymouth's medium also produced unsatisfactory growth and development and mixtures of NCS and DMEM+ were embryolethal (Fig. 5).

Growth in rat serum in Petri dishes was less satisfactory. Although a few embryos developed well, both average protein content and somite number were significantly lower than in the rotators ($P \leq 0.001$) and half the embryos were abnormal (Fig. 6).

DISCUSSION

The results indicate that good development of headfold-stage mouse embryos can be obtained for 48 h in undiluted rat serum in a rotator system. Forty-eight h of normal growth and development is sufficient for the entire process of neural tube closure to occur (Geelen & Langman, 1977) and represents an improvement from Tam & Snow's results in which growth and development of embryos was retarded in the second 24 h, i.e. beyond the early somite stages. Approximately 80% of the embryos show growth and differentiation similar to that in vivo. The commonest defect, occurring in the remaining 20% of the embryos, is failure of closure of the cranial neural folds (exencephaly). In this respect, the culture system is not yet as successful for mouse embryos as for rat embryos (New et al. 1976). The reasons for differences between rats and mice are not clear, although failure of closure of the neural tube is a common response of embryos to inadequate culture conditions (Cockroft, 1979), and to teratogens (Sadler, Langman & Burk, 1980). It is, therefore, possible that mouse embryos are affected adversely by heterologous (rat) serum. However, in this respect
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Tam & Snow (1980) found no improvement in younger mouse embryos grown in mouse serum compared with those in rat serum.

Rotators supported better development than Petri dishes, perhaps because they provided better oxygenation. Although rotators were gassed with only 5 % $O_2$ for the first 36 h (because it had been shown previously that rat embryos exposed to higher oxygen levels at headfold stage become exencephalic) (Morriss & New, 1979) followed by gassing with 20 % $O_2$ for the remainder of the culture period (12 h), oxygen in this system would be conveyed rapidly to the embryos by the flowing medium. In contrast, in the static medium of the Petri dishes oxygen reaches the embryos only slowly by diffusion even though the oxygen concentration is maintained at 20 % throughout the culture period (48 h) in accordance with the procedure of Tam & Snow (1980) (even when the embryos float, most of the tissue is below the surface of the medium). The benefits of flowing medium also increase with age and size of the embryos (New, 1978), but rat embryos as young as early primitive streak stage grow better in flowing than in static medium (Buckley, Steele & New, 1978).

Tam & Snow (1980) tested several mixtures of serum with different chemically defined media and found that the best was 50 % serum (rat or mouse) with 50 % DMEM supplemented with 2 mM glutamine and 0.1 mM pyruvate. In our cultures, this mixture gave variable results. It supported better growth of the embryos in the rotator with continuous gassing than in the rotator with intermittent gassing, possibly because in the former the pH is likely to have fluctuated less. It was also better without the addition of glutamine and pyruvate. This may have been because our DMEM was freshly prepared and any addition of extra glutamine produced an abnormally high concentration of this amino acid, whereas Tam & Snow added the extra glutamine to commercially supplied DMEM which had probably lost some of the original glutamine by deterioration. The pyruvate is unlikely to have been harmful at the concentration used, although Cockroft (1979) has shown that at higher concentrations it can be deleterious to headfold rat embryos.

Even our best cultures with rat serum/DMEM did not yield as many embryos with closed neural folds as those grown in undiluted rat serum. Furthermore, results with rat serum/Waymouth and NCS/DMEM+ demonstrated the inadequacy of these media to support normal growth. Of the media tested therefore, undiluted rat serum appears to be the most satisfactory for growing mouse embryos during this period of organogenesis.

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