Reconstitutive ability of axial tissue in early rat embryos after operations and culture in vitro

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SUMMARY

Longitudinal incisions have been made in the axis of 10-day-old rat embryos (post-neurula stage with 5-10 pairs of somites) at mid-trunk levels, dividing the axis into right and left halves. The embryos have then been cultured in vitro by New’s method and their ability to reconstitute tissues in each half has been studied.

After 20 h culture at 37 °C, there was no longer any external sign of the division of the axis. Histological studies showed that in nearly all cases, however, the neural tube was duplicated in the operated region. The two neural tubes lay in close contact in the midline, and ventral to them the gut was single. Apart from four cases in which the gut roof was slightly broadened and forked, all other tissues were normal.

The reconstitutive ability of the neural and gut tissue has been compared with that of amphibian and avian embryos, as observed in ‘twinning’ experiments by other workers.

The apparent delay in axial rotation in the operated rat embryos, as compared with controls, is attributed to the inability of the two separated halves of the somite series to co-ordinate their contractions. Further details of the rotation process in operated embryos will be the subject of a future study.

INTRODUCTION

All vertebrate embryos that have been studied experimentally have been found to have remarkable powers of reconstitution after injury. Even if as much as half of the embryonic tissue is removed, at the gastrula or neurula stage, it is often possible for the remaining half to reconstitute what is lacking and form a whole embryo. This was pointed out some time ago by Ruud & Spemann (1922) in work on two newt species, Triturus taeniatus and T. alpestris. Further details of the process by which each half-embryo adapts to form a whole, after sagittal divisions at blastula and gastrula stages, were described later by Dalog & Huang (1948) and Brice (1958). Bickerstaffe (1968) obtained less complete reconstitution, particularly of axial mesoderm and the nervous system, in halved embryos of the frog Xenopus laevis, and concluded that in this species the ability to replace lost tissue is severely limited, from the gastrula stage onwards. So it seems that there is considerable variation in restitutive ability in embryos of different species, even within the Amphibia.

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Among higher vertebrates, chick and duck embryos are the types which have been much investigated experimentally, and it has been shown that transections of the blastoderm at primitive streak (gastrula) or earlier stages result in twinning or even in multiple embryos. Thus Lutz (1949) was able to produce multiple embryos by several transections of a duck blastoderm, and more recently Rogulska (1968) obtained twin embryos, in various orientations, by a series of bisections of duck blastoderms before the appearance of the embryonic axis. At late primitive streak stages in the chick, Abercrombie & Bellairs (1954) obtained duplications of the anterior embryonic axis when movements at the node were impeded by grafts of tissue from more posterior regions. The invaginating axial tissue had become divided into two halves, and each half had been able to form a somewhat incomplete embryonic axis. This implies, as must also be true of the cases described in amphibians, that the depleted cells are versatile enough to be able to proliferate more than usual and to differentiate into a wider range of tissues than they would have normally in their own half of the embryo. This versatility of the embryonic cells becomes more restricted as development proceeds, however (cf. Spemann, 1903); for instance, division of the roof-plate of the neural tube after the end of neurulation in the chick (Fowler, 1953) results in spina bifida in a large majority of cases. Neither half of the divided neural tube is able to grow a median wall or to modify in shape to form a whole intact spinal cord: the two halves are also unable to re-fuse in the midline and form one normal tube.

Relatively little is known about the abilities of mammalian early embryos at primitive streak or neurula stages to replace tissues that are damaged or lost. The possibilities of operating on mammal embryos at these stages are very limited, as they are accessible only if grown in vitro, and do not survive long under these conditions. The technique of New (1966) enables experiments of limited duration to be performed successfully, however, on rat embryos of stages from neurulation until all the main organ systems have formed. Since the embryo must be cultured within its yolk sac and amnion, which play important nutritive and protective roles in vitro (Payne & Deuchar, 1972), only very simple operations which do not damage the membranes excessively can be carried out. For instance, it is possible to transect the axis completely at levels between cervical and lumbar regions, where the bent axis protrudes near the surface of the membranes. Experiments of this kind (Deuchar, 1969,1971) have shown that the posterior portion of the severed axis is able to continue differentiating, and in most cases eventually heals to the anterior portion. However, there is no evidence of additional growth or modified differentiation in any of the tissues in these cases. Recently Jolly (1974) has amputated the tail region of slightly older rat embryos to see if these show evidence of tail regeneration, but during the time that the embryos survived in vitro only an epithelial growth over the wounded stump of the tail was seen, and no regeneration occurred.

The experiments to be reported here were aimed to discover what reconstitu-
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Fig. 1. Diagram to illustrate operative procedure. Embryonic vesicle is resting on the surface of the watch-glass culture dish, immersed in serum. One prong of a pair of watchmaker's forceps is inserted as shown (arrow). t, Trophoblast; y, yolk sac; am, amnion; al, allantois; hd, head of embryo; ht, heart rudiment.

tive abilities exist in lateral halves of the embryonic axis of the rat at post-neurula stages. It was found possible to make a midline longitudinal cut dividing the embryonic axis into right and left halves, in the mid-trunk region where it protrudes ventrally (cf. Fig. 1). It was anticipated either that the two halves of the axis would remain separate, and perhaps each reconstitute its medial portion, resulting in a double axis at this point, or that the halves would heal together and form a normal single axis. As will be seen below, however, in by far the majority of cases neither of these things happened. The two half-axes did not remain separate, but did heal in the midline: nevertheless, duplications of these healed tissues did occur.

MATERIAL AND METHODS

Rat embryos of 10 days' gestation (day 0 = the morning after mating) were dissected out of the uterus and placed in watch-glass culture dishes containing serum, as described by New (1966); 0.5 ml serum was used in each culture dish. Reichert's membrane and the outer layer of yolk sac adhering to it were partially
Table 1. Extent of axial rotation in operated and control embryos

Figures are numbers of embryos in each category.

<table>
<thead>
<tr>
<th></th>
<th>Fully turned</th>
<th>3/4 turned</th>
<th>1/2 turned</th>
<th>1/4 turned</th>
<th>Not turned</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Operated…</td>
<td>9</td>
<td>7</td>
<td>12</td>
<td>18</td>
<td>7</td>
<td>53</td>
</tr>
<tr>
<td>Control…</td>
<td>24</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>50</td>
</tr>
</tbody>
</table>

Fig. 2. Diagrams to show the stages of rotation of the embryonic axis that are referred to in Table 1. Position of mid-dorsal surface is indicated by hatched line. A, Not turned; B, 3/4 turned; C, 1/2 turned; D, 1/4 turned; E, fully turned.

reflected towards the trophoblast cone, rendering the embryonic axis visible (cf. Fig. 1). The embryo was at the post-neurula stage, with brain and heart rudiments clear and from 5–10 pairs of somites present.

To make a longitudinal split in the embryonic axis, one prong of a pair of watchmaker's forceps was inserted in the midline in the cranial arm of the bend in the axis (Fig. 1), posterior to the heart, and it was then pressed right through all the midline tissues so that it severed both arms of the bent axis. The two lateral axis halves so formed were then spread well apart by grasping each with a separate pair of forceps. Any embryos in which the division was incomplete or in the wrong plane were discarded from the results.

Some damage to the embryonic membranes was inevitable in the above operations. Control embryos, in which only the membranes near to the bend in
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Table 2. Histological observations on operated and control embryos

<table>
<thead>
<tr>
<th>Total embryos examined</th>
<th>Neural tube duplicated</th>
<th>Neural tube irregular shape</th>
<th>Neural tube normal</th>
<th>Gut roof slightly forked</th>
<th>Gut roof normal</th>
<th>Dead cells in mid-trunk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Operated...</td>
<td>53</td>
<td>37</td>
<td>10</td>
<td>6</td>
<td>49</td>
<td>14</td>
</tr>
<tr>
<td>Control ...</td>
<td>50</td>
<td>0</td>
<td>1</td>
<td>49</td>
<td>50</td>
<td>9</td>
</tr>
</tbody>
</table>

The axis had been damaged, were therefore used for comparison with the experimental series. Approximately equal numbers of control and operated embryos were cultured in each experiment. They were incubated for 20 h at 37 °C, after gassing the culture chambers with a mixture of 95% O₂/5% CO₂ as described by New (1966).

After the culture period, embryos were examined externally, then divested of their membranes and fixed in Bouin’s fluid. They were stained in toto with 1% aqueous eosin to make them readily visible in later procedures, then dehydrated in alcohols, cleared in methyl benzoate and embedded in paraplast. Sections 5 μm thick were stained with Weigert’s haematoxylin and counterstained with eosin.

RESULTS

The general health of both operated and control embryos at the end of the culture period was good: though the membranes had often partly collapsed, they had healed and the embryo had undergone further growth and differentiation. The head and pharynx region were well formed, the heart was beating rapidly and blood, often noticeably red with haemoglobin, was circulating in embryo and yolk sac. The hind end of the embryo showed less growth but was intact and healthy. In the operated embryos, the split in the mid-trunk region was no longer visible externally and the axis appeared normal. The only significant difference in external appearance between experimental and control embryos was that axial rotation was further advanced in the controls. Twenty-four of the 50 control embryos had undergone complete rotation so that the dorsal surface lay externally and was convex (cf. Fig. 2E), while only 9 of the 53 operated embryos showed this full rotation. Table 1 gives further details of the numbers of embryos in experimental and control groups which had undergone different degrees of axial rotation (cf. also Figs. 2A–D).

All of the 53 experimental and 50 control embryos were examined histologically. Table 2 summarizes the observations. Special attention was paid to the neural and other midline axial tissues, in the mid-trunk region. These were normal in all except one of the controls: this one control embryo showed small irregularities in the neural tube wall, extending for about 20 μm in the region posterior to the heart (cf. Figs. 3, 4). By contrast, 47 of the 53 operated embryos...
showed irregularities or duplications in the neutral tube, extending for about 100 μm. Thirty-seven of these showed a completely doubled neural tube (cf. Figs. 5, 6), the two tubes being either fused or in close contact with one another at their medial walls. The only other evidence of duplication was that four embryos showed forking of the gut roof (cf. Fig. 7) immediately below their doubled neural tube. In the six embryos of the experimental series which showed no doubling of the neural tube, the original split had evidently healed without trace, and no sign of abnormality could be seen, nor could the site of operation be identified. There were also ten embryos in which the neural tube showed evidence of damage, i.e. had not healed completely, and its floor was discontinuous, or its walls were malformed slightly (cf. Fig. 8). There was no other sign of abnormality in the operated embryos, except for some dead cells in the region of the mid-trunk; however, dead cells also appeared in this region in some of the controls (cf. Table 2).

**DISCUSSION**

The two most remarkable features of the present results are that in nearly all cases the neural tube has doubled, but the gut tissue, which also lies in the midline, has not. The absence of any doubling in the gut (except in a very few cases – see Table 2 – where slight forking had occurred as shown in Fig. 7) could not have been due to incompleteness of the midline incision. This incision was necessarily started from the ventral surface, which lies outermost in the 10-day-old rat embryo, so the gut tissue was necessarily cut through first. If the cut were at all incomplete, it would have failed to divide the innermost, dorsal layers of axial tissues. But in fact care was taken (see Material and Methods) to separate the two halves of the axis completely, by teasing them apart with forceps after the cut had been made. We can therefore conclude that the normal appearance of the gut in 49 out of 53 operated embryos was due to its having healed very rapidly and completely. In the four cases where it was abnormally broad or

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**FIGURES 3-8**

Photomicrographs of transverse sections in the mid-axial region of embryos, stained with H. & E. n.t., Neural tube; ntch., notochord; g., gut roof.

Fig. 3. Normal neural tube and gut roof in a control embryo. × 270.

Fig. 4. Slightly abnormal neural tube in a control embryo. Note vertical tag of tissue in lumen of tube. × 690.

Fig. 5. Duplication of the neural tube in an operated embryo. The medial walls of the two tubes are completely fused in this case, and the gut roof is normal. × 270.

Fig. 6. Duplicated neural tube in another operated embryo. In this case the medial walls of the two tubes are separate. Note also the broadening of the gut roof, which lies in close contact with the neural tubes here. × 270.

Fig. 7. Forking of the gut roof, below a partially duplicated neural tube. × 270.

Fig. 8. Slight abnormality of the neural tube in an operated embryo. In this case the tube is only slightly forked ventrally. × 270.
slightly forked (cf. Figs. 6, 7), the two floor-plates of the doubled neural tube were slightly more separate than in other embryos. Had the neural tubes been more widely separated, one might have expected the gut to be duplicated, as occurred in the twin axes obtained in chick embryos by Abercrombie & Bellairs (1954). Their results showed that in regions where the two axes and their neural tubes were some distance apart, there were two separate foregut tubes, but at levels where the two axes converged, only a single gut, with a rather broader roof than normal, was seen. No experimental evidence is available, either from bird or mammal embryos, as to whether there is interaction between the neural tube and the gut roof at this stage of development: but the general absence of duplications in the gut of these rat embryos, despite duplications of the neural tube, suggest that the two structures develop fairly independently in the rat.

There was considerable variation in the morphology of the duplicated neural tubes, so that it is difficult to draw more than a few general conclusions from them. It is clear, however, that in all the operated embryos the severed halves of the neural tissue had been able to reapproach each other and to make contact in the midline. In all but six cases they had not apparently been able to fuse, however, but instead the medial cells of each had formed a new medial wall, in contact with that of the other side. The combined thickness of these two medial walls (which were often so closely in contact as to appear single) was equivalent only to one normal lateral wall of the tube, or was even thinner than this (cf. Figs. 3, 5, 6). It is well known from classic experiments on amphibians (Holtfreter, 1934) and also from studies on birds (Watterson, Goodheart & Lindberg, 1955) that the embryonic neural tube forms thickened walls on those faces that lie adjacent to somite tissue. There was never any extra somite tissue formed medial to either half of the neural tube, in the present experiments: so this may account for the thinness of the medial wall of the duplicated tubes.

Another generalization that may be made about the neural tissue in these cases of duplication, is that its cross-sectional area was greater than that of the normal, single neural tube in controls (cf. Fig. 3 with Figs. 5–7). It was not, however, equivalent to twice the area of a control neural tube: i.e. each half had not reconstituted a complete medial half of the normal size. In both these features the neural tissue of the rat embryo appears to have greater propensities for reconstituting a normal embryo than is the case in amphibians or birds, as far as present evidence goes. In post-neurula stage chick embryos, Fowler (1953) found that longitudinal splitting of only the roof-plate of the neural tube resulted in spina bifida in a large majority of cases. The neural tube halves neither formed double tubes nor re-joined in the midline successfully. In embryos of the frog *Xenopus laevis*, Bickerstaffe (1968) found after halving experiments at much earlier stages (gastrulae and neurulae) that there was little or no reconstitution of neural tissue on the medial side of each half. Only a very thin medial wall was formed in the neural tube, and in the brain region there were a very few cases in which a small eye or otic vesicle formed on the medial side of the cut. It seems,
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then, that one may conclude, within the limits of the present experimental procedure, that neural tissue of the rat embryo is at least as good, if not better at reconstituting itself after injury, when compared with equivalent tissue of other vertebrate embryos.

The observation that axial rotation did not proceed as far in the operated embryos as in the controls (Table 1) is of interest in connexion with studies still in progress (cf. also Deuchar, 1971) on the mechanism of this axial rotation in rat embryos. It was concluded from transections of the embryonic axis that the movements of rotation are probably initiated in the cervical region, possibly aided by the pulsations and twisting of the heart tube. It has also been observed in recent cinematographic studies (Deuchar & Parker, 1975) that there is a very rapid reversal of curvature in 10½-day-old embryos when these are released from their membranes, owing apparently to a contraction of the ventral borders of the somites in the mid-trunk region. For this movement, or the more gradual twisting that occurs when the embryo’s membranes are intact, to take place normally, the somites on each side of the mid-trunk must work in good co-ordination. This co-ordination would be difficult, if not impossible, in an embryo whose mid-trunk region is divided into lateral halves. The sequence of rotation movements in these embryos with longitudinally split axes will be the subject of a further study.

I am very much indebted to Mrs F. Parker for her invaluable and skilled technical assistance in this work. I am also grateful to Miss A. Featherstone for help with the illustrations, and to Mrs P. Budd for typing the manuscript.

REFERENCES


(Received 18 July 1974)