An interaction between dorsal and ventral regions of the marginal zone in early amphibian embryos

By J. M. W. SLACK1 AND D. FORMAN1

From the Imperial Cancer Research Fund, Mill Hill Laboratory, London

SUMMARY

When small explants from early gastrulae of *Xenopus laevis* are allowed to develop in a buffered salt solution there is a considerable difference between the patterns of differentiation obtained from different dorsoventral levels of the marginal zone. These patterns of differentiation correspond to the fates of the different regions in the course of normal development. They are not altered if several explants of the same type are fused before culture.

If a ventral marginal zone explant from *Xenopus* is cultured in contact with a piece of dorsal marginal zone from the axolotl, it forms structures more dorsal in character than it would in isolation or in normal development. This behaviour is shown only feebly with other regions of the axolotl gastrula. A piece of dorsal marginal zone from *Xenopus* is not affected in its development by culture in contact with an explant of ventral marginal zone from the axolotl. The dorsalization of ventral marginal zone explants is shown only if there is a large area of direct contact with the dorsal explant and if the pieces remain in contact for a period of 48 h or more.

It is proposed that this *in vitro* interaction is the same as the dorsoventral component of action of the ‘organizer’ graft discovered by Spemann and Mangold.

INTRODUCTION

In 1924 Spemann and Mangold published a paper the results of which dominated experimental embryology for the following thirty years (Spemann & Mangold, 1924). They showed, using early gastrulae of the newt, that implantation of the dorsal lip into the ventral marginal zone could cause the formation of a secondary embryo in which the central mesodermal structures (mainly the notochord) were derived from the graft, and the lateral mesodermal structures (mainly somites and kidney), and also the secondary nervous system, were derived from the host. This experiment demonstrated that the fate map of presumptive territories in the mesodermal rudiment could be altered in response to a signal from the dorsal region, and this area was called by Spemann the ‘organization centre’ or ‘organizer’.

Most subsequent research on early amphibian development has concentrated either on the initial formation of the mesodermal rudiment in the equatorial region of the blastula (‘mesodermal induction’ see Chuang, 1939; Yamada,
1958; Nieuwkoop, 1969, 1973; Nakamura, Takasaki & Ishihara, 1971; Geithe et al. 1975), or on the induction of the nervous system by the action of the archenteron roof on the overlying ectoderm (‘neural induction’ see Mangold, 1928; Spemann 1938; Nieuwkoop et al. 1952a, b). In our opinion the essence of the organizer phenomenon does not lie in either of these processes but in the development of the graft into the mesodermal structures of the dorsal midline combined with a reprogramming of host mesoderm along the dorsoventral axis. Since the mesoderm controls the arrangement of territories in the other germ layers by means of subsequent inductive interactions we believe that in normal development the regional subdivision of the mesoderm is the key step in the formation of the general vertebrate body plan.

One of the few pieces of classical embryological work devoted specifically to this problem was that of Yamada (1937, 1939, 1940), which led to the idea that the dorsoventral pattern of structures in the mesoderm is evoked from the competent tissue by a graded signal whose high point coincides with the notochord rudiment (see also Dalcq & Pasteels, 1937; Pasteels, 1951). According to this conception the result of the organizer graft should not be regarded as the induction of a complete secondary embryo by an instructive signal but as a mirror duplication of the mesodermal structures in the dorsoventral axis analogous to the duplications of limbs (e.g. Tickle, Summerbell & Wolpert, 1975; Slack, 1977) or insect eggs (e.g. Sander, 1976; Nüsslein-Volhard, 1977). Neither the primary nor the secondary embryo are actually complete since they both lack the structures which would normally lie ventral to the mirror plane, for example the ventral blood islands.

In recent years the problem of the organizer has been largely neglected by those interested in pattern formation in favour of such subjects as limb development or the imaginal discs of Drosophila. However, Cooke in a series of papers (1972, 1973, 1979) has resurrected the concept of a dorsoventral gradient to interpret the results of grafting experiments on the gastrulae of Xenopus laevis. He concludes that there is indeed a signal from the dorsal region which can evoke a mirror duplication of the mesoderm and that this reprogramming can occur without any additional cell division.

The present work was commenced with the intention of setting up a simple in vitro system to investigate this dorsoventral interaction, the long-term aim being to bridge the gap between phenomenological studies of pattern formation and modern biochemical and physiological technology. In this paper we report results of the states of determination in different regions of the marginal zone of Xenopus laevis, as assayed by the self-differentiation of explants in simple buffered salt solutions. We show that in combinations between explants taken from Xenopus and from the axolotl, ventral tissue can be induced to develop into more dorsal structures than it will form in isolation or in vivo. Several characteristics of the signal are determined, including the regional distribution of its activity and the time relations of signalling and competence.
MATERIALS AND METHODS

All the experiments were performed using explants from stage-10 gastrulae of *Xenopus laevis* (Nieuwkoop & Faber, 1967) and *Ambystoma mexicanum* (the axolotl – Bordzilovskaya & Dettlaff, 1979). The former species is an anuran with relatively small eggs (diameter 1-4 mm) and very rapid development. In intact embryos at 18 °C the general body plan is fully established one day after the early gastrula stage and reasonable cytodifferentiation of mesodermal tissues has occurred after another day. The axolotl is a urodele with larger eggs (diameter 2-2 mm) and a normal tempo of development about three times slower than that of *Xenopus*.

Spawnings were induced in *Xenopus* by injection of human chorionic gonadotrophin (‘Pregnyl’: Organon, 300 i.u. for females and 200 i.u. for males). The hormone was injected into the dorsal lymph sac and the animals were left in the dark at 20 °C. Eggs were laid about 18 hours after injection (Henriques 1964).

Axolotl embryos were obtained by artificial fertilization. The female was injected intramuscularly with 35 units of porcine follicle-stimulating hormone (Sigma) and put into 50 % Steinberg solution at 20 °C. Spawning of unfertilized eggs commenced after 20-24 h. The animal was then transferred to full-strength Steinberg solution, the eggs were collected every 15 min and fertilized with sperm taken from the vas deferens of a sacrificed male.

Operations were carried out on embryos in which the dorsal blastopore lip had just become externally visible. The embryos of both species were washed several times in sterile 1/10 strength ‘normal amphibian medium’ (NAM) and decapsulated manually with sharp forceps. NAM consists of 110 mM-NaCl, 2 mM-KCl, 1 mM-CaCl₂, 1 mM-MgSO₄, 0·5 mM-NaHCO₃, 0·1 mM EDTA, 1 mM-sodium phosphate pH 7·4, 100 u/ml penicillin, 60 u/ml streptomycin and 2 mg/l nystatin. The dissections were performed in full strength NAM using hair loops and electrolytically sharpened tungsten needles.

The operating dishes were coated with 1 % agar (Noble agar, Difco Laboratories). Explants were cultured for 3–5 days in 1·5 ml NAM in multi-well plates, the bottoms of the wells being covered with 1 % agar. In some of the earlier experiments Niu-Twitty solution was used for culture of explants but this gave less satisfactory survival and differentiation of the explants than the NAM.

Millipore collars, which were used to retain tissue combinations in a dumb-bell shape, were made by cutting ultrathin Millipore sheet into strips, burning round holes in these with a sharp red-hot needle, and selecting collars of the appropriate sizes using a dissecting microscope fitted with an eyepiece graticule. The 0·4 and 0·2 mm diameter collars referred to in the text are uniform in diameter to about 10 %. The collars were washed in distilled water overnight, dried, and sterilized under u.v. light. Transfilter experiments were carried out using the same ultrathin filter material glued to a plexiglass ring with MF-(Millipore filter) cement. The ring was supported by pieces of 100 µm thick Millipore, which allows a
space for the bottom tissue, and the top tissue was kept in place by a strip of Nucleopore filter held down with coverslip fragments.

After the culture period the explants were fixed overnight in Zenker's fixative containing 5% acetic acid. They were washed in tap water and in 35% ethanol containing a small amount of iodine. They were then embedded in 56 °C m.p. wax via n-butanol and sectioned at 10 μm. Smaller explants were block stained in Grenacher's borax carmine before embedding and were counterstained with 0·1% naphthalene black in saturated aqueous picric acid. Larger ones were usually stained in periodic acid–Schiff, followed by 0·5% naphthalene black, followed by 1% methyl green in 0·1 M-sodium acetate pH 4·8. The stained sections were mounted in Euparal and mesodermal tissues were identified according to the following criteria which are based on the appearance of the tissues in intact larvae. Notochord consists of cells with very large internal vacuoles, stellate nuclei with prominent nucleoli and a PAS-positive external sheath. Muscle cells are elongated and contain longitudinal and transverse striations and large lipid-filled vacuoles. Nuclei are elongated with prominent nucleoli. Kidney tubules are composed of a single-cell-thick epithelium with a PAS-positive luminal lining. The cells are cuboidal and the nuclei round. Erythrocytes are oval with clear cytoplasm and condensed nuclei lacking nucleoli. Undifferentiated tissue retains an embryonic appearance, the cells are large and round and their cytoplasm is packed full of yolk granules.

RESULTS

Development of isolated portions of the marginal zone of Xenopus laevis

This part of the work was undertaken to establish the self-differentiation behaviour of different parts of the marginal zone of Xenopus laevis. The marginal zone lies in the subequatorial region of the early gastrula and in anuran embryos the deep portion forms the mesoderm by an involution movement which is part of the general process of gastrulation (Keller 1975, 1976). In normal development the dorsal marginal zone becomes dorsal mesoderm and ventral marginal zone becomes ventral mesoderm, although there is a dorsal convergence of tissue such that the notochord and somites which occupy about 90° of the mesodermal mantle cross section in the neurula are drawn from the dorsal 180° of the marginal zone of the gastrula. The mesodermal structures of normal larvae run in the following sequence from dorsal to ventral: notochord, somites, kidney, lateral plate and blood islands.

The explants consisted of small wedges about 30° wide taken from the region in which the blastopore lip was about to form. They include both superficial and deep tissue (Fig. 1). Some explants were also taken from the animal and vegetal poles. In Table 1 are shown the types of mesodermal tissue formed after incubation which was usually for 4 days at 18 °C, and in Fig. 2 are shown some histological sections of differentiated isolates.
Interaction in marginal zone of amphibian embryos

Fig. 1. Positions from which *Xenopus* gastrula explants were taken. (a) View from vegetal pole. (b) View from side. D, dorsal; V, ventral; A, animal; V, vegetal; P, pole; MZ, marginal zone. (c) Fate map of deep marginal zone according to Keller (1976). Lateral view; ch, prospective notochord; hm, prospective head mesoderm; s prospective somites; lm, prospective lateral mesoderm.

Table 1. Self differentiation of isolated explants from stage-10 *Xenopus gastrulae*

<table>
<thead>
<tr>
<th>Explant</th>
<th>Total cases</th>
<th>Notochord</th>
<th>Muscle</th>
<th>Kidney</th>
<th>Mesenchyme</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMZ</td>
<td>20</td>
<td>20</td>
<td>9</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>2DMZ</td>
<td>9</td>
<td>9</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3DMZ</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>60° MZ</td>
<td>22</td>
<td>5</td>
<td>22</td>
<td>8</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>120° MZ</td>
<td>18</td>
<td>0</td>
<td>11</td>
<td>3</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>VMZ</td>
<td>22</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>2VMZ</td>
<td>11</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>3VMZ</td>
<td>10</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>VMZ(A)</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>VMZ(V)</td>
<td>11</td>
<td>All undifferentiated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP</td>
<td>10</td>
<td>All undifferentiated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP</td>
<td>8</td>
<td>8/8 epidermis, 7/8 sucker</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D, Dorsal; V, ventral; MZ, marginal zone; AP, animal pole; VP, vegetal pole. 2DMZ indicates a fusion of two DMZ explants and similarly for the other cases.
Fig. 2. Cytodifferentiation of *Xenopus* gastrula explants. (a) Dorsal marginal zone, showing notochord and undifferentiated tissue. (b) 60° marginal zone, showing striated muscle. (c) Ventral marginal zone, showing red blood cells. (d) Muscle. (e) Level-3 development showing muscle cells, a kidney tubule and loose mesenchyme. (f) Blood. Scale bars indicate 100 μm.

In the salt solution used the explants round up within an hour or two of isolation. As spheres they have a diameter of approximately 0.5 mm and thus a volume somewhat less than 1/20 of the whole embryo. In all types of explant there is extensive cell division over the culture period although no attempt has been made to quantify this in the present study because of the difficulty of counting cells in the final structures. There is of course no net growth of the isolates because the medium is not nutritive; as in the intact embryo before the
establishment of the blood circulation, the cells live on their own internal yolk reserves. The rate of cytodifferentiation in the explants is somewhat slower than that of intact control embryos; for example the number of yolk granules persisting in striated muscle cells after three days of incubation is about the same as that in the trunk somites of embryos after two days of incubation. Once the proper culture conditions had been found the number of explants which died or failed to differentiate was extremely small and any such cases were excluded from the study.

The explanted dorsal lips invariably formed a clump of notochord, often with a block of striated muscle in close association with it. In addition there were usually prolific ectodermal derivatives: neural vesicles and blocks of neural-crest-type cartilage. Epidermis was not usually found. When two or three dorsal isolates were fused together and cultured as an aggregate there was no difference in the type of differentiation shown.

Explants from 60° around the marginal zone always formed a large block of striated muscle surrounded by an epidermal covering. Frequently the muscle was accompanied by notochord, neural structures, kidney tubules and loose mesenchyme.

Ventral explants usually yielded a concentric arrangement of epidermis, loose mesenchyme, mesothelium and erythrocytes. When two or three ventral explants were fused together before culture, this pattern of differentiation was maintained. When the ventral explants were divided into animal and vegetal portions as shown by the dashed line in Fig. 1b, the animal portion differentiated in the same way and the vegetal portion did not differentiate at all. A failure to differentiate was also found for isolates from the vegetal pole. Since nearly all the marginal zone isolates contained an undifferentiated region it is probable that they all extended beyond the marginal zone proper into the endoderm. The epidermal coverings which were formed by the lateral and ventral marginal zone explants are probably derived from the superficial epithelium which covers the whole external surface of anuran embryos.

Explants from 120° around the marginal zone tended to develop either the ventral pattern or a mixture of muscle, tubules and mesenchyme. There is never any notochord and usually less muscle than is found in the 60° isolates. A few explants were also taken from the animal pole. These formed convoluted masses of epidermis containing one or more suckers.

It is clear from these results that at the commencement of gastrulation there is considerable difference in the self-differentiation behaviour of explants from the different dorsoventral levels and that this behaviour is not altered by the size of the cultured pieces over a three-fold range. These results serve as controls for the experiments described in the next section in which the differentiation of the ventral explants is modified by combination with axolotl tissues. For the purpose of presentation the observed patterns of differentiation will be classified into four groups which, by comparison with
the isolates, can be regarded as running from an extreme dorsal to an extreme ventral type:

<table>
<thead>
<tr>
<th>Level</th>
<th>Type of differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Notochord alone</td>
</tr>
<tr>
<td></td>
<td>Notochord and muscle</td>
</tr>
<tr>
<td>2</td>
<td>Large muscle mass alone</td>
</tr>
<tr>
<td></td>
<td>Large muscle mass with tubules</td>
</tr>
<tr>
<td></td>
<td>Large muscle mass with mesenchyme</td>
</tr>
<tr>
<td>3</td>
<td>Mixed muscle and tubules</td>
</tr>
<tr>
<td></td>
<td>Large mass of tubules</td>
</tr>
<tr>
<td></td>
<td>Mixed muscle and blood (rare)</td>
</tr>
<tr>
<td>4</td>
<td>Mesenchyme, mesothelium, blood</td>
</tr>
<tr>
<td></td>
<td>Mesothelium, blood</td>
</tr>
<tr>
<td></td>
<td>Blood alone</td>
</tr>
<tr>
<td></td>
<td>Mesenchyme, mesothelium (rare)</td>
</tr>
</tbody>
</table>

Development of combinations between *Xenopus* and *axolotl* explants

Interspecies combinations were used for these experiments because of the need for unambiguous identification of the cells derived from the two components. Axolotl cells are larger than those of *Xenopus* and the nuclei stain much more intensely with basic dyes. In addition, axolotl explants do not usually differentiate over the culture period which was used and are therefore particularly prominent because of their content of large yolk granules. Even single cells can be identified as belonging to one or the other species with complete confidence.

The experimental design is shown in Fig. 3. Explants were cut out from various regions of the stage-10 axolotl gastrula. They were allowed to round up, which takes 3–4 h, and were fused with *Xenopus* ventral isolates dissected out 1–2 h previously by lightly pressing the two components together. After about 30 mins contact they adhere quite tightly and can be transferred to another dish by pipette. The axolotl tissue partially surrounds the *Xenopus* tissue after 1–2 days of culture. This may be a little more pronounced for the dorsal than for the ventral tissue but on the whole it seemed that any regional variation in adhesive properties was less obvious than the species difference.

When the combinations were examined histologically it was usually found that the cells from the two species remained in distinct blocks, although occasionally some loose axolotl cells had penetrated the *Xenopus* region. In Table 2 are shown the results of combining ventral *Xenopus* tissue with tissue from different regions of the axolotl gastrula. In most cases the course of development of the *Xenopus* tissue is the same as that shown by the ventral *Xenopus* isolates, i.e. level 4. But when combined with the axolotl dorsal marginal zone a high proportion of cases developed the level-2 pattern (Fig. 4). In no case was any *Xenopus* notochord found.

Once this result had been obtained it became of some interest to determine the outcome of the reciprocal combination: *Xenopus* dorsal and axolotl ventral
Fig. 3. Design of the combination experiments.

Table 2. Differentiation of Xenopus ventral marginal zone tissue in combination with explants from the axolotl gastrula

<table>
<thead>
<tr>
<th>Position of origin of axolotl component</th>
<th>Total cases</th>
<th>Differentiation of Xenopus VMZ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level 2</td>
<td>Level 3</td>
</tr>
<tr>
<td>DMZ</td>
<td>23</td>
<td>18</td>
</tr>
<tr>
<td>60° MZ</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>90° MZ</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>120° MZ</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>VMZ</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>VP</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>AP</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

D, dorsal; V, ventral; MZ, marginal zone; AP, animal pole; VP, vegetal pole.

If the axolotl tissue always imposes its own regional character on the Xenopus tissue then we should expect the latter to become ventralized, whereas if dorsal tissue respecifies ventral tissue irrespective of species then the Xenopus tissue should be unaffected. The latter result was in fact obtained, level-1
Fig. 4. Cytodifferentiation of axolotl-Xenopus combinations. (a) Axolotl DMZ + Xenopus VMZ. AD, Axolotl tissue; xm, Xenopus muscle. (b) Axolotl VMZ + Xenopus VMZ. AV, axolotl tissue; xb, Xenopus blood cells. (c) Axolotl VMZ + Xenopus DMZ. AV, axolotl tissue; xn, Xenopus notochord; xm, Xenopus muscle. Scale bars indicate 100 μm.
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Table 3.

<table>
<thead>
<tr>
<th>Combination</th>
<th>Total Cases</th>
<th>Level 2</th>
<th>Level 3</th>
<th>Level 4</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aged dorsal + fresh ventral</td>
<td>8</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>75</td>
</tr>
<tr>
<td>Aged dorsal + aged ventral</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Fresh dorsal + aged ventral</td>
<td>12</td>
<td>0</td>
<td>1</td>
<td>11</td>
<td>8</td>
</tr>
</tbody>
</table>

development being maintained in 10/10 cases (Fig. 4). It was not, unfortunately, possible to tell whether the axolotl tissue had become dorsalized because there was no differentiation over the period of culture used.

Two types of experiment were carried out to investigate the time course of the interaction between the dorsal axolotl and ventral Xenopus tissue. In the first type, the combinations were assembled in the same way but one or both components had been ‘aged’ by culturing in isolation for 24 h. The results are shown in Table 3, and indicate that the ventral tissue has lost its competence by this time. This is not surprising in view of the rapid normal tempo of development of Xenopus as compared to the axolotl.

In the second experiment the combinations were physically separated after a certain period of contact. This is not easy to do on the normal fusions because much of the Xenopus tissue is buried in the middle. So the pieces were stuck together through a Millipore collar containing a hole 0.4 mm in diameter. The axolotl tissue was cut a little larger than the Xenopus piece (0.6-0.7 mm diameter) and because it is restrained by the collar the combination remains in a dumb-bell-like configuration indefinitely with a contact area between the two pieces of 0.5 mm² (Fig. 5). It was then possible to remove the Xenopus tissue in such a way that no axolotl cells were taken with it although usually some Xenopus cells remained attached to the axolotl piece.

With the arrangement a rather lower frequency of positives was obtained than without the collars (60% as against 100%) but the results indicate, rather surprisingly, that 48 h of continuous contact is required in order to obtain this effect (Table 4). There was no indication that a lesser time of contact was required for level-3 development than for level-2 development. In terms of the time scale of Xenopus development, 48 h is a long time. By 48 h after stage 10, intact embryos show a fair amount of overt cytodifferentiation as do dorsal explants. Ventral explants have usually acquired some recognisable cytodifferentiation by the third day after stage 10, and so it would seem that there is not much of a time interval between the labile state of determination and visible differentiation. In the case of the preincubation experiments, the negative outcome is perhaps due to the fact that such a long time is no longer available before the onset of visible differentiation.

Some experiments were also carried out on the type of contact required for
Fig. 5. Axolotl DMZ – *Xenopus* VMZ combination mounted on a Millipore collar. The contact area is 0.5 mm² and the photograph was taken after 20 hours of incubation. Scale bar 200 μm.

Table 4 *Differentiation of Xenopus ventral marginal zone tissue after different periods of contact with axolotl DMZ*

<table>
<thead>
<tr>
<th>Duration of contact</th>
<th>Total cases</th>
<th>Level 4</th>
<th>Level 2 or 3</th>
<th>% Positives</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td>15</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>8</td>
<td>7</td>
<td>1</td>
<td>12</td>
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<td>24</td>
<td>23</td>
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<td>13</td>
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<td>48</td>
<td>17</td>
<td>10</td>
<td>7</td>
<td>41</td>
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<tr>
<td>72</td>
<td>18</td>
<td>7</td>
<td>11</td>
<td>61</td>
</tr>
<tr>
<td>≥96</td>
<td>22</td>
<td>8</td>
<td>14</td>
<td>63</td>
</tr>
</tbody>
</table>

Successful dorsalization of the ventral piece. When the collar diameter was reduced to 0.2 mm, corresponding to a contact area of 0.13 mm², the proportion of positive cases was further reduced to two cases out of nine. When the pieces were cultured on opposite sides of a Millipore filter (25 μm thickness, nominal pore size 0.3 μm) there were no positives out of nine cases. So it seems that to obtain a high proportion of positives the tissues should be actually touching over a large area and should remain in contact for most of the time preceding overt differentiation.
DISCUSSION

The self differentiation of small explants from embryos is the best available method for revealing the state of determination of the region concerned. Amphibian tissues are particularly suitable for this type of experiment because the culture medium does not have to be nutritive; each cell contains its own nutrient reserve in the form of yolk granules. The use of very simple media makes it possible largely to avoid the difficulty, so often encountered in avian or mammalian organ culture, of influencing the course of development by the culture conditions.

Amphibian embryo explants were first cultured in buffered salt solutions by Holtfreter and the method was used extensively in the 1930s. In those days workers were careful to distinguish between the state of determination of a region, its fate in the course of normal development, and its possible potency in abnormal situations (see Holtfreter & Hamburger, 1955). These important distinctions have unfortunately not always been remembered in more recent embryological work.

The results presented here for *Xenopus laevis* are similar to those obtained by Holtfreter for the anurans *Rana fusca* and *R. esculenta* (Holtfreter 1938 b), the only significant difference being that he obtained extensive muscle differentiation from ventral as well as lateral marginal zone material. It seems probable that there are at least three or four distinct states of determination in the dorso-ventral axis, corresponding roughly to the normal *Xenopus* fate map (Keller, 1976). According to Holtfreter (1938 a) the urodele gastrula is more labile in the sense that tissue from most regions of the marginal zone will form all the mesodermal tissues when cultured in isolation. But more recent work by Japanese embryologists has shown a difference between dorsal and ventral explants commencing as early as the morula stage in the newt *Cynops (Triturus) pyrrohogaster* (Nakamura & Matsuzawa, 1967; Nakamura & Takasaki, 1970).

The present results also show that explants of the same type can be combined without the pattern of differentiation being altered. Other authors have reported that the size of the explants does make a difference (e.g. Lopashov, 1935; Holtfreter-Ban, 1965), but their experiments were not quite the same as ours. Lopashov used *Triturus vulgaris (taeniaturs)* and combined as many as ten dorsal lips. Holtfreter-Ban used *Cynops pyrrohogaster*, subdivided explants from the dorsal marginal zone, and cultured them in a nutrient medium. For our purposes, the lack of an effect of aggregation over the range studied serves as one of the controls for the changes observed in the interspecies combinations.

The axolotl–*Xenopus* combinations indicate that *Xenopus* ventral marginal zone can be dorsalized by axolotl dorsal marginal zone. The fact that a position-specific interaction can occur between two species so diverse in taxonomy and rate of development is further evidence for the universality of basic pattern-forming mechanisms in animals (Wolpert, 1971). The fact that complete dorsal-
isation (level-1 development) is never attained in the combinations is of uncertain significance. It might indicate that the notochord rudiment is in some way qualitatively distinct from the rest of the mesoderm such that they cannot be interconverted at this stage. On the other hand it might simply be that the signal does not work very effectively in the interspecies combinations. A number of homospecific dorsoventral combinations using triploid ventral tissue have been attempted but the results are not yet clear enough to enable us to make a decision on this point. It does however seem clear that the states of determination in the marginal zone are such that the ventral tissue can still be transformed to a more dorsal type at a stage when the dorsal tissue cannot be made more ventral.

The regional distribution of dorsalizing activity in the axolotl gastrula is roughly the same as the distribution of organizer activity in the newt gastrula mapped by Bautzmann (1926). It should be noted however that he used as an assay the formation of secondary embryos after insertion of the test tissue into the blastocoel of the host, and this is a method which does not clearly distinguish between dorsalization of the mesoderm and neural induction. Our results are also consistent with the report by Minganti (1949) that 20% of grafts corresponding to our 60° MZ-induced secondary mesodermal axes when grafted to the ventral marginal zone. According to the axolotl fate map prepared by Pasteels (1942), the active area would correspond approximately to the prospective notochord if only the level-2 results are counted as positives, and to the prospective notochord plus prospective somites if the level-3 results are also counted.

The results of the timing experiments were rather surprising. Because of the great speed of *Xenopus* development it was expected that an exposure of a few hours duration would be sufficient to reprogramme the tissue irreversibly. In fact 48 h are required and it seems that contact must be continuous over this period since combinations in which the ventral piece had been preincubated for 24 h showed no dorsalization. Even in the explants this period takes up most of the time before visible cytodifferentiation. It must be remembered however that in this set of experiments the contact area between the explants was restricted to 0.5 mm². The effect of the contact area is very dramatic, for if we make an estimate for the non-restrained combinations the results can be summarized as follows:

<table>
<thead>
<tr>
<th>Contact area</th>
<th>Cases</th>
<th>Level 2 + 3</th>
<th>Dorsalization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 5 mm²</td>
<td>20</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>0, 5 mm²</td>
<td>22</td>
<td>14</td>
<td>63</td>
</tr>
<tr>
<td>0, 13 mm²</td>
<td>9</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>Transfilter</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

If, for example, the limiting factor was the amount of a morphogen which had diffused across the junction then it would be expected that for a given contact time the percentage dorsalization would be proportional to the area, and for a
Interaction in marginal zone of amphibian embryos

given area the percentage dorsalization would be proportional to the contact
time. Since the area of 0-5 mm² seems to be near the minimum required to ob-
tain a significant number of positives, it is possible that the time of 48 h is a
measure of the maximum duration of competence of the responding tissue rather
than the time which would be needed for the process to occur in vivo. Some
preliminary measurements made in collaboration with Anne Warner of Univer-
sity College, London have shown that the dorsal and ventral components of the
combinations are electrically coupled to one another. The coupling varies in
individual cases from barely detectable to quite strong, but is not as strong as the
coupling within each of the two components. This shows that gap junctions can
be formed at the interspecies junction and although this cannot prove that their
presence is necessary for transmission of the signal, it does keep the possibility
open.

In summary, the results presented here show that ventral tissue can be dorsal-
ized by contact with dorsal tissue. We believe that the same signal is involved in
the dorsoventral regionalization of the mesoderm in normal development, and
in the production of double embryos as a result of the organizer graft.

In the future we intend to use the in vitro system to study the mechanism of the
interaction in more detail, particularly with respect to the cell cycle, the role of
intercellular junctions and intercellular matrix material and the effects of
alleged dorsalizing and ventralizing substances (e.g. Lehmann, 1937; Yamada,

REFERENCES

BAUTZMANN, H. (1926). Experimentelle Untersuchungen zur Abgrenzung des Organisation-
BORDZILOVSKAYA, N. P. & DETLAFF, T. A. (1979). Table of stages of normal development of
axolotl embryos and the prognostication of timing of successive developmental stages at
various temperatures. Axolotl Newsletter no. 7.
(Niere, Leber) nach ihrer Verpflanzung in Explante und verscheidene Wirtsregionen von
V. Regulation after removal of the head organizer, in normal early gastrulae, and in those
COOKE, J. (1979). Cell number in relation to primary pattern formation in the embryo of
Xenopus laevis. I. The cell cycle during new pattern formation in response to implanted
DALCQ, A. & PASTEELS, J. (1937). Une conception nouvelle des bases physiologique de la
homogeneous morphogenetic factor inducing mesoderm and endoderm derived tissue


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