Chemically stimulated differentiation of post-nodal pieces of chick blastoderms

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Earlier studies on the role of sulfhydryl (–SH) groups in morphogenesis have indicated that in chick and amphibian embryos, an –SH-containing substance can act as an inducing stimulus in the action of the primary organizer. Thus, blocking –SH groups by chloroacetophenone (CAP) has specific developmental effects and the inducing capacity of the Hensen’s node is affected (Lakshmi, 1962a, b). Reversal of the effects of –SH block by cysteine indicated that whatever is blocked by the –SH inhibitor is restored by cysteine (Mulherkar, Rao & Joshi, 1965; Mulherkar, Rao, Joshi & Joshi, 1966). It appears, however, that not all –SH-containing substances can reverse the effect of –SH block by CAP. Thus the teratogenic effects of CAP could not be ameliorated by a subsequent treatment with cysteamine, a decarboxylation product of cysteine, while CAP and cysteamine mixed in equimolar proportions had no effect on development (Rao, 1969). Inhibition of developing neural tissue and of inducing capacity of the organizer may be due to block of –SH groups of proteins or of the –SH in free cysteine and/or glutathione in the inducing tissue. Since the effects of –SH block can be reversed effectively by cysteine and not by cysteamine, it is likely that the action of cysteine in reversing the effects of –SH block is not the restoration of –SH groups. On the other hand, it is quite likely that an inducing stimulus can be given by substances of low molecular weight, probably cysteine or reduced glutathione. Waheed & Mulherkar (1967) have shown that post-nodal pieces (PNPs) treated with cysteine and reduced glutathione, when used as organizer grafts, can induce neural tissue and that the grafts are neuralized or differentiate into somites. Thus there is good evidence that –SH-containing substances like cysteine and glutathione have the ability to stimulate differentiation of axial structures. In the present investigation an attempt has been made to study the inductive effects of cysteine and glutathione on post-nodal pieces of the chick embryo.

MATERIAL AND METHODS

Fresh fertilized eggs of White Leghorn hens were obtained from a local poultry farm and incubated to get embryos of stage 4 of the Hamburger &

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Hamilton (1951) scale. The blastoderms were explanted and most of the opaque area was trimmed. The pellucid area was cut transversely 0.5 mm from the node (Fig. 1B). The vitelline membrane of the same egg was mounted around a glass ring and the node piece (NP) or the post-nodal piece (PNP) was placed flat on it. The ring was placed in a watch-glass which in turn was kept in a Petri dish with a ring of moist cotton in it (Fig. 1A). The contents of a whole unfertilized egg were mixed with 50 ml Ringer and after shaking thoroughly the homogenate was centrifuged at 2000 rev/min for 30 min. The supernatant thus obtained was mixed with an equal volume of Pannett–Compton solution (PC). About 1 ml of the egg-extract medium was added around the glass ring. A few drops of PC were added inside the ring. In experiments involving the use of chemicals, the chemicals were added to the PC and egg-extract medium by diluting stock solutions. The solutions of cysteine and reduced glutathione were freshly prepared to guard against oxidation. At the end of the chemical treatment the entire assembly consisting of the watch-glass, glass ring and vitelline membrane with the piece of blastoderm was washed thoroughly in PC and it was mounted again using egg-extract medium and PC without the chemicals. In all culturing experiments sterile conditions were maintained as a matter of routine.

At the end of the culture period the explants were observed, fixed and processed for histological examination. Serial transverse sections of the explants were stained with haematoxylin and eosin. All sections were examined and all doubtful identifications of tissues were eliminated from the results recorded.
FIGURE 2

(A) T.S. of a post-nodal piece cultured without chemical treatment. Note the absence of axial structures. $\times 133$.

(B) T.S. of a post-nodal piece treated with cysteine. $N.\, pl = \text{neural plate};\, som = \text{somatic layer};\, spl = \text{splanchnic layer}.\, \times 300$.

(C) T.S. of a post-nodal piece treated with cysteine and glutathione. $n.\, gr = \text{neural groove};\, so = \text{somites}.\, \times 300$.

(D) T.S. of a post-nodal piece treated with glutathione. Note the double neural tube. $\times 300$.

(E) T.S. of a post-nodal piece treated with cysteine. $tub = \text{nephric tubules}.\, \times 133$.

(F) T.S. of a post-nodal piece treated with cysteine, glutathione and actinomycin. Note the absence of axial structures. $\times 133$. 
RESULTS

1. Control cultures

(a) Node pieces. Since the method of culture described above has not been used by others, it was necessary to study the pattern of differentiation of the NPs. A large number of experiments of Spratt (1947, 1948) have shown that the NP develops into an entire normal embryo. Usually the posterior end grows into a 'tail' consisting of somites, notochord and neural tube. In all, 49 NPs were cultured by our method and it was found that they develop as described by Spratt.

(b) Post-nodal pieces. In all our experiments the PNP was obtained by cutting across the blastoderm of stage 4 embryos not less than 0.5 mm behind the node. The PNPs were cultured for at least 48 h and in some cases, longer. When examined in serial transverse sections the PNPs cultivated in normal culture medium did not show differentiation of any axial embryonic structures. The ectoderm remained thin and the mesoderm formed a loose network of mesenchyme or compact condensations (Fig. 2A). The latter were never found to be organized in the form of somites. Differentiation of blood islands was normal and in most of the PNPs blood could be detected.

2. Differentiation of post-nodal pieces by chemical stimulation

The effects of cysteine (2.5–10 µg/ml), reduced glutathione (10 µg/ml) and cysteine with glutathione (5 and 10 µg/ml respectively) on the differentiation of PNPs were studied. The PNPs were treated with the chemicals during the initial 6 h of culture in vitro. They were then washed and mounted in normal culture medium. After growing them in vitro for 48–66 h the PNPs were studied in serial sections.

Post-nodal pieces treated with cysteine, reduced glutathione or with these chemicals mixed together, develop a number of axial embryonic structures. The ectoderm shows various degrees of neuralization. The weakest reaction shows palisades in one or more regions. This reaction has not been considered significant in the present study. Definite neuralization is considered to have occurred when the ectoderm differentiates into clearly defined neural plates (Fig. 2B). Folding of the neural plate to form a neural groove is considered to be a stronger reaction (Fig. 2C). There are many cases of completely closed neural tubes (Fig. 2D), indicating a still stronger reaction. It is observed that a single PNP may have more than one region with various grades of neuralization. In many cases the neuralizations are so much away from the median axis of the PNP as to permit the conclusion that the observed differentiations are definitely stimulated by the chemical treatments.

Mesodermal differentiation is also observed in the treated PNPs. Somites and tubules resembling nephric tubules are clearly found in several cases (Fig. 2C, E). The mesoderm frequently shows differentiation of somatic and
splanchnic layers (Fig. 2B). Notochord-like tissue is also found in some PNPs. Huge blood islands are found in all PNPs.

The spatial relationships of the differentiated structures do not show, in most cases, any tendency to build an axial embryonic organization in the PNPs. A few cases, however, show some organization of an incomplete axis as revealed by association of the induced neural structures with somite or notochord-like differentiation (Fig. 2C).

Table 1. **Chemically stimulated differentiation of the post-nodal pieces (PNPs) of chick blastoderm**

<table>
<thead>
<tr>
<th>Experimental series</th>
<th>Treatment</th>
<th>No. of PNPs available</th>
<th>Differentiation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Neuralization</td>
</tr>
<tr>
<td>II PC</td>
<td>Nil (controls)</td>
<td>35</td>
<td>2.8</td>
</tr>
<tr>
<td>II PE</td>
<td>Cysteine (2.5–10 μg/ml)</td>
<td>40</td>
<td>62.5</td>
</tr>
<tr>
<td>III PE</td>
<td>Glutathione (10 μg/ml)</td>
<td>27</td>
<td>59.2</td>
</tr>
<tr>
<td>IV PE</td>
<td>Cysteine (5 μg/ml) + glutathione (10 μg/ml)</td>
<td>24</td>
<td>71.1</td>
</tr>
<tr>
<td>V PE</td>
<td>Cysteine + glutathione (as in IV PE) + actinomycin D (0.1 μg/ml)</td>
<td>34</td>
<td>Nil</td>
</tr>
</tbody>
</table>

* See text.

When the PNPs are treated with cysteine, glutathione and actinomycin D (5, 10 and 0.1 μg per ml respectively) for 6 h, differentiation of all axial structures is inhibited (Fig. 2F). The concentration of actinomycin used has been found to be non-toxic, but sufficient to inhibit differentiation of axial structures in whole embryos treated during the formation of the head process (Rao & Chauhan, unpublished observations).

The results of the various experiments performed have been summarized in Table 1. It is to be noted that a PNP is scored only once even if it shows a particular kind of differentiation in more than one region.

**DISCUSSION**

Several workers have used the PNP of the chick embryo to study chemically stimulated differentiation (see, for instance, Butros, 1960; Sherbet & Mulherkar, 1963). The level of the transverse cut of the blastoderm to ensure a reliably neutral tissue in which to study the stimulated differentiation is, however, a matter of some discussion. In the present study 35 PNPs have been studied as untreated controls. Of these, only one shows a small neural groove. From this it is concluded that the level of transverse cut used in the present study provides a reliably neutral PNP in which chemically stimulated differentiation could be studied.
Earlier studies have revealed the importance of –SH-containing substances in the action of the primary organizer. It has been shown that the inductive stimulation occurs during the formation of the head process (see Mulherkar, Rao & Joshi, 1965). Thus the stimulation of a PNP for a short time, 6h, may be expected to simulate the action of the primary organizer. The results reported here show a significantly high percentage of differentiation of axial structures in PNPs treated with cysteine and reduced glutathione. The two chemicals together seem to be more effective in causing neuralization of ectoderm and differentiation of somites. However, this conclusion may be deferred until one has a larger number of PNPs treated with a wider range of concentrations of the chemicals.

Though there is no evidence that cysteine or reduced glutathione acts as the inducing stimulus in the action of the primary organizer, the stimulation of differentiation in PNPs by these substances is quite comparable to the inductive stimulus. Finding multiple neural structures and somites in the PNPs indicates a true chemical stimulation and not a mere potentiation of already-existing organization in the PNPs. It is not possible to say at present whether the chemicals primarily stimulate the mesoderm or the ectoderm. However, finding neuralizations not spatially associated with mesodermal somites or notochord in the treated PNPs suggests that ectoderm may be directly stimulated by the chemicals. A method for culturing ectodermal pieces is now being perfected and the use of such isolated ectodermal pieces to study chemically stimulated differentiation is expected to answer the question.

A number of studies have indicated that the process of induction involves fresh synthesis of RNA (Denis, 1964; Brahma, 1966; Tiedemann, Born & Becker, 1965; Rao, 1967). Treatment for a short duration with actinomycin D has shown that neuralization of ectoderm is completely suppressed if chick embryos are exposed to the chemical during the formation of the head process. After the head process is formed, a similar treatment fails to inhibit the formation of neural tissue. These results (Rao & Chauhan, unpublished observations) lend further support to the conclusion drawn from the earlier studies. It seems quite likely that the tissue responds immediately to inductive stimulation by a fresh synthesis of RNA. From the results reported here, it is clear that if RNA synthesis is inhibited during the process of chemical stimulation, the PNPs fail to differentiate any axial structures.

From the present investigation it is clear that substances of low molecular weight can act as inducers. Studies to explore the possibility of substances like cysteine and glutathione acting as inducing stimuli in the action of the primary organizer may therefore be expected to be fruitful.
SUMMARY

1. Post-nodal pieces of the chick blastoderm when cultured in vitro do not normally differentiate axial structures such as neural tissue, somites and notochord. When treated with cysteine or reduced glutathione or both together, for 6 h, the post-nodal pieces differentiate neural tissue, somites, notochord and nephric tubules.

2. The chemically stimulated differentiation is completely inhibited by treatment with actinomycin D.

3. It is suggested that -SH-containing substances of low molecular weight like cysteine and reduced glutathione may act as a primary organizer, inducing competent ectoderm to differentiate into neural tissue.

RÉSUMÉ

Stimulation chimique de la différenciation de fragments de blastoderme postérieurs au noeud de Hensen

1. Des fragments de blastoderme de poulet, prélevés en-dessous du noeud de Hensen et cultivés in vitro, ne différencient pas de structures axiales telles que du tissu nerveux, des somites et de la notochorde. Après traitement par la cystéine ou le glutathion réduit ou encore par les deux substances à la fois, pendant 6 h, ces fragments différencient du tissu nerveux, des somites, de la notochorde et des tubules néphritiques.

2. La différenciation, stimulée par voie chimique, est complètement inhibée par un traitement à l’actinomycine D.

3. On suggère que des substances de faible poids moléculaire contenant le groupement -SH, telles que la cystéine et le glutathion réduit, peuvent agir en tant qu’organisateur primaire: induire l’ectoderme compétent à se différencier en tissu neural.

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REFERENCES


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