Concanavalin-A-induced Open Neural Tube Defects in Chick Embryos


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Abstract
Open neural tube defects developed in 12 of 122 alive chick embryos treated with exogenous lectin (concanavalin-A) at stages between 10 or 14 as defined by Hamburger and Hamilton. Embryos treated at stage 10, the time of anterior neuropore closure, developed exencephaly or extensive neural openings from the level of rhombencephalon to the thoracic spinal cord, while embryos treated at stages between 11 and 14, at posterior neuropore closure, developed only small myeloschisis in the thoracolumbar region. The failure of neural tube closure at a critical time is a major cause of neural tube defects.

Key words: neural tube defect, concanavalin-A, closure defect, chick embryo

Introduction
The cause of both cranial and spinal bifidum open neural tube defects is still unclear. Most investigators agree that neural tube defects such as myelomeningocele and anencephaly result from failure of initial closure rather than from reopening of the closed neural tube. On the other hand, the dysraphic state should be regarded as a postneurulation disorder.

Closure of the neuropores has been investigated, but the mechanism of the adhesion and/or fusion of the neuropore is still unclear. Extracellular material at the crests of the folds may be important in neural tube fusion and adhesion in chick, rodent, and amphibian development. Complex carbohydrates, e.g., glycosaminoglycans, approximating neuroepithelia on the cell surface may occur in many of the biological processes related to neurulation, including cellular adhesiveness, intercellular recognition, and morphological differentiation. Histochemical methods have identified an extracellular complex carbohydrate material which may bridge the gap between the neural folds. Therefore, cell surface carbohydrates are important in fusion and adhesion during neurulation.

Previous studies from our laboratory on vitamin-A-induced neural tube defects in mice suggested that the delay or failure of apposition of neural folds may be the major cause of neural tube defects. Concanavalin-A (Con-A), a plant agglutinin, is useful for the study of cell surfaces, because Con-A affects normal neurulation by binding to the cell surface. Treatment of chick embryos with Con-A in vitro often results in a reversal of neural tube closure. The present study investigated whether Con-A creates neural tube defects in chick embryos and the relationship between the incidence and treatment timing.

Materials and Methods
The White Leghorn chick embryos were chosen as the model for this experiment, because the development stage is relatively easy to evaluate at the time of treatment. Fresh fertilized eggs from a local supplier...
were stored at 10°C until used. Eggs were incubated at 38–38.5°C. Treatment was given on incubation day 2 or 3, when neural tube closure occurs in the chick. A window was made in the egg shell approximately 7 mm by 10 mm using a variable speed electrical drill equipped with a convex sanding stone. After windowing, 1% neutral red in saline was applied just above the embryo for staging (personal communication from G.C. Schoenwolf, Department of Anatomy, University of Utah). Embryos were staged according to the system of Hamburger and Hamilton. The embryos were from stages 8 to 15. The stages of embryos did vary very much in this period from our previous study (not published).

178 embryos were treated with Con-A and 40 embryos were treated with the same amount of 0.9% saline as controls (Tables 1 and 2). Con-A solution (10 μl) diluted with phosphate buffer saline (50 ng/μl) was applied to the vitelline membrane. The air space was filled with 0.9% saline, the window was covered with vinyl electrical tape, and the egg was rotated 180 degrees to turn the window down. The eggs were reincubated at same temperature as described above. Embryos were harvested on incubation days 4 to 5, which correspond to Hamburger and Hamilton stages 20 to 24, and evaluated for anomalies.

Histological specimens were prepared as follows. Harvested abnormal embryos with open neural tube defects and selected normal control embryos were immersed into freshly prepared 4% paraformaldehyde solution (in 0.1% cacodylic acid buffer, pH 7.2). Embryos were embedded in araldite according to routine protocols. Serial 1 μm sections were cut with glass knives and placed on ethanol-cleaned glass slides. Sections were stained with Richardson’s stain.

## Results

122 of 178 Con-A-treated embryos (approximately 69%) survived, which is not statistically different from the saline controls (26 of 40 alive; Chi-square test, p = 0.665) (Tables 1 and 2). Twelve Con-A-treated embryos developed neural tube defects. One embryo had exencephaly (Fig. 1) and two had extensive openings from the rhombencephalon to the level of the thoracic spine. These three embryos were treated with Con-A at stage 10, the time of anterior neuropore closure. Nine embryos had small openings of the neural tube from middle to lower spinal levels. These embryos were treated at stages 11 to 14 which corresponds to posterior neuropore closure. Four embryos had tail defects. These embryos were treated at stages 8, 9, 10, and 13. Nine Con-A-treated embryos had other anomalies, such as short wingbuds or divided wingbuds (Fig. 2).

### Table 1 Types of defect and timing of treatment with Con-A

<table>
<thead>
<tr>
<th>Embryo stages*</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryos</td>
<td>6</td>
<td>23</td>
<td>28</td>
<td>35</td>
<td>42</td>
<td>29</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>Dead embryos</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>16</td>
<td>13</td>
<td>11</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Neural tube defects</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Tail defects</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*According to the system of Hamburger and Hamilton. #Two embryos had extensive openings in the neural tube, and one had exencephaly.

### Table 2 Timing of treatment with saline and survival in control embryos

<table>
<thead>
<tr>
<th>Embryo stages*</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryos</td>
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<td>2</td>
<td>7</td>
<td>10</td>
<td>10</td>
<td>7</td>
<td>0</td>
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</tr>
<tr>
<td>Dead embryos</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

*According to the system of Hamburger and Hamilton.

Fig. 1 Photograph showing the embryo with exencephaly at the mesencephalon and the opening of the neural tube (arrowheads).

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was no relationship between the timing of treatment and defects not related to the neural tube. No embryo with neural tube defects was found in the control embryos.

Representative embryos showing the morphology and stage specificity of neural tube defects are described below. Embryo 1 was treated at stage 10. This embryo had a wide and extensive opening from the rhombencephalic level to the mid-thoracic level at harvest. Histological examination of the rostral rhombencephalon, the rostral part of the extensive defect, and the middle thoracic and caudal parts of the defect showed an open neural tube defect without covering of surface ectoderm (Fig. 3A, C). However, the caudal part of the rhombencephalon with the neural tube defect was covered by surface ectoderm (Fig. 3B). There was no abnormality at the caudal to limb bud under the dissecting microscope, but open neural tube with a covering of apparently normal surface ectoderm was found (Fig. 3D). The discontinuity of surface ectoderm and neuroectoderm was found at the level where the surface ectoderm did not cover the open neural tube (Fig. 3C).

Embryo 2 was treated at stage 12. This embryo

Fig. 3 Embryo 1. Photomicrographs, (A) showing the defect at the level of the rhombencephalon of the embryo with extensive opening (arrows) (×100), (B) just caudal to (A) showing the membrane covering the defect of neural tissue (arrow) (×100), (C) at the thoracic level showing an open neural tube defect and discontinuity of the surface ectoderm and neuroectoderm (arrowheads) (×200), and (D) caudal to the limb bud showing no abnormality, but the surface ectoderm had covered the open neural tube (asterisk) (×400).
had a small opening of the spinal neural tube with a smooth edge (Fig. 4A). The discontinuity between the surface and neuroectoderm was found at the level of the defect (Fig. 4B).

Embryo 3 was treated at stage 13. This embryo also had a rough edged defect (Fig. 5A). A similar discontinuity of surface ectoderm and neuroectoderm was observed. However, an abnormal neural tube was also found at the level slightly caudal to the open defect. Moreover, the mass of the neuroectodermal cells were arranged in a disorganized fashion (arrow) (×400).

Discussion

This experiment indicated that neural tube defects in chick may result from failure of the neural tube to close, i.e., primary neural tube defects, rather than
reopening of the closed neural tube, i.e., secondary neural tube defects. Embryos with exencephaly or extensive open neural tube defects were observed when treated with Con-A at stage 10 that corresponds to anterior neuropore closure. Embryos with small openings at the spinal level were treated with Con-A between stages 11 to 14 that correspond to posterior neuropore closure. Therefore, the timing of treatment and the type of neural tube defect are related: early Con-A treatment results in exencephaly or extensive neural tube opening, and late treatment results in small defects at more caudal spine levels. The morphological development of the embryos with extensive neural tube openings in this experiment (for example Embryo 1) resembles the monkey embryo described by Padget,21> except for the absence of ventricular coagulum in the neuroschistic bleb. He suggested “neuroschisis” rather than “non-closure” as the cause of the neural tube defect. However, his morphological data seems to us inadequate to prove that the abnormalities were due to reopening of the closed neural tube. His hypothesis is based upon the theories of Gardner,6,7) regarding the possible “hydrocephalomyelia” of the neural tube. However, recent studies have showed that early stage normal embryos have neither hydrocephalus nor a functional choroid plexus.20 Our other experiments of Con-A-treated chick embryos with wide defects of the abdominal wall (data not published) and this study suggest that Con-A may interrupt the normal organization of both neural and surface ectoderm, primarily when Con-A was applied during periods of cell migration and/or recognition. Con-A binds to mannos or glucose glycoconjugates on the cell surface, and may alter cell adhesion and/or intercellular recognition processes. Therefore, Con-A may alter cell activity resulting in abnormal neurulation.

Some forms of spina bifida may be related to abnormal development of the neural tube, separation of superficial ectoderm, or migration of the mesoderm toward the cleavage space between superficial ectoderm and neuroectoderm.15) Our study showed that the patterns of abnormal development of the neural tube in both closed and opened neuraxis levels vary. Simple opening with or without rough edges that indicates the effect of Con-A may extend into the mesoderm cells directly and/or indirectly, causing opening of the neural tube with or without skin ectoderm covering, opening of the neural tube with disorganized neuroectoderm cells rather than overgrowth of neuroectoderm, and open neural tube with intervening mesenchyme cells and covered skin ectoderm. The disjunction between the surface ectoderm and neural ectoderm was a common feature in this study, which may be result from mesenchymal accessing to the central portion of the neuroectoderm, very similar to clinical lipomyelomeningocele. Therefore, various types of open and/or closed neural tube defect may be due to the same cause acting at different stages of embryo development. Previous experiments in our laboratory showed that secondary neural tube defect may be a cause of abnormal neurulation.15) The present study did not show the toxic effect on neural cells, such as massive cell death in the neural tissue, that was observed in previous study. The present study also showed clearly that neural tube defects may be caused by multiple and complex rather than single factors. Our data indicates that the events occurring at the time of neural tube closure (both fusion and adhesion) are crucial in developing neural tube defects.

Fig. 6 Embryo 4. Photomicrograph showing another type of neural tube defect with no obvious external defect, but an abnormality of the neural tube with closed surface ectoderm and intervening mesenchyme under the surface ectoderm and neural tube defect (arrow) (x 400).

References

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Commentary

The authors report a single experimental animal model of the dysraphic state induced by canavalin-A in the chick embryo. The experimental methodology used in this study is almost identical to those reported by Lee et al. in the 1970s but use of the chick embryo as the investigated species is different. Therefore, the major interest in this report lies in the following points:

1. Do a different species treated with the same teratogen develop different CNS malformation(s)?
2. If so, is it due to different embryopathogenesis?  
3. If the same CNS malformation(s) develop(s), did the authors expand their investigation to controversial areas?

The authors found that CNS dysraphism may develop in a variety of the involved levels from the rhombencephalon to the caudal region of the spinal cord with very delicate time differences in the terato-
gen induction as the critical factor. The early phase of these dysraphic states was beautifully delineated in this study. The major criticism of the authors' concept suggested in the discussion may be to support a single theory of embryopathogenesis for dysraphism and try to explain all observations. The authors did not mention or discuss in detail reports in the literature that the chick embryo as the experimental model of dysraphism may have different pathogenesis and critical periods in the development of dysraphism, which depends upon the species and teratogen. The major hypotheses include "simple non-closure" and "overgrowth and reopening." Both theories, although not compatible, are proved in the individual experimental model of chick embryo using different species and teratogens. We should recognize these data and analyze clinical examples or other experimental trials accepting the variety of mechanisms in the development of neural tube defects.

Regarding the evidence of "overgrowth" of the neuronal tissue, the follow-up period in this report is too short at postincubation days 4 to 5 to form a conclusion. As the authors observed, a phenomenon of developmental arrest was the major finding but at the same time some neuroectoderm cells started to proliferate in a disorganized fashion. This should be observed in all neuronal developmental processes after cell proliferation and neuronal differentiation up to axonal maturation. The findings demonstrated in Figs. 5 and 6 are very important. These are the early changes of the cytoarchitectures in spinal dysraphism. I would recommend that the authors continue to investigate the direction of the neuronal maturation process in this experimental model.

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