Congenital Heart Disease and Other Malformations Produced by Influenza A Virus and Allantoic Fluid in the Chick Embryo

By Maria V. de la Cruz, M.D., Carlos Campillo-Sainz, M.D., Simón Muñoz-Armas, M.D., Asunción Pascual-Chávez, and Rafaela Zamudio-Bandera

Although a number of explanations have been suggested, the etiology of congenital heart disease is still unknown. The teratogenic agents used in the experiments of other investigators are seldom those encountered in clinical practice. Epidemiological studies have revealed the role of viral infections in the production of human cardiac malformations; this led us to investigate the effect of influenza A virus on the production of congenital heart disease in the chick embryo.

Influenza A virus was chosen for our experiments because: a) its effect on the development of the heart has not been studied, even though epidemiological and experimental data have shown it to be capable of producing congenital malformations; b) it is the cause of frequent infections in human beings, including pregnant women; and c) it is easy to grow in the chick embryo.

Methods

The eggs of healthy young white Leghorn hens were used throughout the experiments. All hens were fed a balanced diet. All had been immunized against Newcastle disease and fowl-pox. The anti-Newcastle vaccine was a dead virus preparation.

INCUBATION OF CONTROL GROUP

A control group of 200 eggs was incubated at 37.65°C with a constant humidity of 86% to 87%. The eggs were rolled over once every two hours. After 18 days incubation the air vents of the incubator were opened, keeping both temperature and humidity constant.

VIRUS STRAIN

Strain PR-8 of influenza A virus was used maintaining it through successive passages in the allantoic cavities of 10-day-old chick embryos. Embryos were inoculated into the allantoic cavity with 0.2 ml of infected allantoic fluid diluted in buffered saline (NaCl 0.85%, pH 7.2). They were incubated at 35°C for 48 hours, then their allantoic fluid was harvested aseptically and used as a stock suspension of virus. The infectivity of the stock suspension was determined by inoculating groups of six 10-day-old embryos with 0.2 ml of tenfold dilutions of the suspension. After 48 hours incubation at 35°C, followed by freezing of the embryos for one hour, the allantoic fluid was harvested. The ability of the fluid to agglutinate chick erythrocytes was then determined and the ID₃₀ doses were calculated by the method of Reed and Muench.

INOCULATION OF EMBRYOS

Four hundred and twenty-five eggs, divided into four groups, were simultaneously incubated under optimal conditions. The first group, 105 eggs, was inoculated with influenza virus suspended in allantoic fluid and diluted in buffered saline (pH 7.2) to contain approximately 10⁷ ID₃₀ per 0.05 ml. A second group of 105 eggs was inoculated with the same strain of virus inactivated by heating in a 56°C water bath for 10 minutes. The heat-inactivated virus lacked hemagglutinating ability when tested in the same way as the stock virus suspension, which showed that it had lost its infectivity. The 107 eggs of the third group were inoculated with sterile allantoic fluid harvested after 12 days incubation. A fourth group of 105 eggs was inoculated with the buffered saline used as a diluent.

All eggs were inoculated 17 hours after the beginning of incubation. A volume of 0.05 ml was injected immediately beneath the blastoderm with introducing a 24-gauge, 1.5-inch needle through a puncture over the air chamber. Eggs were transilluminated for the inoculation. The inocula for the second and third groups of eggs were diluted in the same amount of buffered saline as was necessary to adjust the titer of live virus for the first group.


TABLE 1

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Fertile eggs</th>
<th>Dead embryos</th>
<th>Per cent mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimal conditions</td>
<td>179</td>
<td>50</td>
<td>27.9</td>
</tr>
<tr>
<td>Inoculation with active virus</td>
<td>87</td>
<td>86</td>
<td>98.85</td>
</tr>
<tr>
<td>Inoculation with heat-inactivated virus</td>
<td>101</td>
<td>88</td>
<td>87.13</td>
</tr>
<tr>
<td>Inoculation with allantoic fluid</td>
<td>87</td>
<td>61</td>
<td>70.12</td>
</tr>
<tr>
<td>Inoculation with buffered saline</td>
<td>70</td>
<td>56</td>
<td>80.00</td>
</tr>
</tbody>
</table>

*Except for the factor which was modified and which appears in the table, incubation conditions were optimal in every case.


OBSESSION AND STUDY
OF EMBRYOS

Incubated eggs were observed once every two days, each time harvesting only dead embryos. Embryo ages were calculated according to classification of Hamburger and Hamilton. Those with a morphological age under stage 22 were block-stained in hematoxylin. The external features of the embryos beyond stage 22 were observed and then hearts were microdissected studying atrial and ventricular cavities, valves, great vessels, and the interventricular septum.

Results

CONTROL GROUP

The control group of embryos incubated under optimal conditions and not inoculated served as a means to investigate whether the white Leghorn hens whose eggs were used carried any genetic factor capable of inducing congenital heart disease. In this group 179 of the 200 eggs were fertile. Fifty of these died during incubation (table 1), with a normal mortality distribution (fig. 1).

The frequency of congenital heart disease in the dead embryos was 8%, and that of malformations in other structures and systems was 4% (table 2). The first figure rules out the existence of a genetic factor producing congenital heart disease.

GROUP INOCULATED WITH ACTIVE VIRUS

Among the 105 eggs inoculated with active influenza virus, 87 were fertile, and 86 of them died (table 1). The mortality curve was quantitatively and qualitatively abnormal because of the appearance of a great peak...
FIGURE 2
Chick embryos after five and one-half to six days of incubation. A: embryo inoculated 17 hours after the beginning of incubation with active influenza A virus, stained with hematoxylin. Notice the greatly retarded development, the gross alterations of the nervous system, and the microcephaly. The arrows point to the abnormal torsion of the longitudinal axis of the body (1) to the flattening of the mesencephalon (2), and to the absence of auditory vesicles (3). B: normal control embryo.

shortly after the beginning of incubation (fig. 1). The difference between the mortality in this group and that in the control group was highly significant ($P < 0.00002$).

The frequency of heart disease in this group was 10.4%, a statistically insignificant difference from the control group. On the other hand, malformations in other structures

TABLE 2
Most Frequent Congenital Heart Diseases and Other Malformations in Chick Embryo Incubated Under Optimal Conditions and Under Diverse Experimental Conditions*

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>No. of embryos</th>
<th>Ventricular septal defect</th>
<th>Valve disease</th>
<th>Other heart disease</th>
<th>Total</th>
<th>Malformation of other structures and systems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimal conditions</td>
<td>50</td>
<td>—</td>
<td>—</td>
<td>4 (8%)</td>
<td>9 (10.4%)</td>
<td>2 (4.0%)</td>
</tr>
<tr>
<td>Inoculation with virus</td>
<td>86</td>
<td>—</td>
<td>—</td>
<td>13 (14.8%)</td>
<td>32 (38.3%)</td>
<td>13 (14.77%)</td>
</tr>
<tr>
<td>Inoculation with heat-inact.</td>
<td>88</td>
<td>9 (10.9%)</td>
<td>13 (14.8%)</td>
<td>30 (35.3%)</td>
<td>16 (18.65%)</td>
<td></td>
</tr>
<tr>
<td>Inoculation with allantoic fluid</td>
<td>61</td>
<td>13 (21.3%)</td>
<td>12 (19.6%)</td>
<td>16 (26.3%)</td>
<td>16 (26.3%)</td>
<td></td>
</tr>
<tr>
<td>Inoculation with saline</td>
<td>56</td>
<td>—</td>
<td>—</td>
<td>2 (3.6%)</td>
<td>3 (3.6%)</td>
<td>16 (28.85%)</td>
</tr>
</tbody>
</table>

*Except for the factor which was modified and which appears in the table, incubation conditions were optimal in every case.
†Embryos inoculated with active virus died before the stage of development in which these malformations can be detected.
and systems were found in 34.88% of the embryos, a highly significant figure ($P < 0.0002$) in comparison to the control group (table 2). Fourteen of the 86 dead embryos (16.28%) showed alterations similar to those observed by Hamburger and Habel and by Williamson et al. after inoculation with influenza A virus. These alterations included abnormal torsion of the body axis, microcrania, microcephaly, and other deformities of the nervous system, and malformations of the eyes and ears (fig. 2). Sixteen embryos (18.6%) showed multiple malformations, total loss of their normal morphology and many vesicular formations in the blastoderm (fig. 3).

As a complement to the experiments with active virus, its multiplication in 17-hour chick embryos was studied. Groups of 12 embryos were incubated under optimal conditions and inoculated as previously described. After intervals of 15 minutes, 1, 5, and 25 hours, the embryos were harvested, washed three times in buffered saline, weighed, ground in a mortar, and suspended in nine volumes of buffered saline. The tissue emulsions were tested for infectivity titers by the tests previously described for the stock virus suspension. No infectivity was found at 15 minutes; but a titer of 1.5 log appeared at 1 hour and increased to 3.5 log and 4.5 log at 5 and 25 hours, respectively (fig. 4).

GROUP INOCULATED WITH HEAT-INACTIVATED VIRUS

Of the 108 eggs inoculated with heat-inactivated virus 101 were fertile; 88 of these died (table 1). The mortality distribution during incubation is not unlike that found under optimal conditions (fig. 1). The mortality figure, on the other hand, was significantly different ($P < 0.0002$).

The frequency of heart disease was 36.3%, significant to the level of $P < 0.0006$. The types of heart disease found were: interventricular septal defect, 10.2%; valvular disease, 14.8%; other malformations, 11.3%

**FIGURE 3**

Chick embryos after four days of incubation, stained with hematoxylin. A: embryo inoculated 17 hours after the beginning of incubation with active influenza A virus. Notice the total loss of normal configuration and the presence of many vesicles. B: normal control embryo.
FIGURE 4

Multiplication of influenza A virus (strain PR-8) in chick embryos inoculated after 17 hours of incubation.

(table 2). The statistical significance of the finding of interventricular septal defect and of valvular disease is obvious, since neither of these lesions was found in the control group. Valvular disease was predominantly stenosis of the pulmonary artery (11 cases); in two cases thickening of the mitral valve was observed. It should be especially noted that in two cases pulmonary stenosis and interventricular septal defect were associated and that the anatomy of the heart in these cases was similar to that found in patients with Fallot's tetralogy.

Malformations of other structures and systems were found in 14.77% of the embryos in this group, which is significant to the level of \( P < 0.04 \) when compared with the control group. In seven cases (7.95%) the abnormalities were like those described by the above-mentioned authors,\(^5\) \(^1^1\) and in six cases (6.82%) they were the alterations in body shape and the blastodermic vesicles already described.

GROUP INOCULATED WITH ALLANTOIC FLUID

Among 107 eggs inoculated with allantoic fluid, 87 were fertile and 61 died during incubation (table 1). The mortality distribution was more or less normal (fig. 1), but the mortality figure was significantly higher than normal (\( P < 0.0002 \)).

Heart disease was present in 49.2% of the embryos; the difference between this ratio and the control value is significant to \( P < 0.0002 \). Interventricular septal defect was found in 21.3% of the embryos; valvular disease in 19.6%; and other malformations in 8.3% (table 2). The significance of the interventricular septal defects and of the valvular abnormalities is, once again, obvious. Thickening of the mitral valve was seen in seven cases and pulmonary stenosis in five. Two of the latter also had interventricular septal defects and the Fallot-like morphology (fig. 5).

Malformations of other structures were present in 13.11% of the embryos. (\( P < 0.07 \) compared to the controls.) Seven (11.47%) had gross alterations in body shape and in cephalic vesicles, as well as blastodermic vesicles. Only one embryo (1.64%) showed the alterations described by Hamburger et al.\(^8\) and by Williamson et al.\(^1^1\)

GROUP INOCULATED WITH BUFFERED SALINE

Of this group of 105 eggs, 70 were fertile and 56 of these died (table 1). Mortality distribution was essentially the same as in the group inoculated with inactivated virus (fig. 1). The mortality figure was significantly higher than in the group incubated under optimal conditions (\( P < 0.0002 \)). Interventricular septal defects and valvular disease were not found in this group. Other cardiac abnormalities were found in 3.6% of the embryos, a figure even lower than the 8.0% found in the control group. Malformations of other organs, however, were found in 28.6%, a significantly higher frequency (\( P < 0.0004 \)) than under optimal conditions (table 2).

Discussion

The results of incubation under optimal conditions prove that the strain of hens used as a source of eggs does not have the genetically transmitted congenital heart disease described in other strains.\(^1^4\)

Inoculation with active virus produced alterations incompatible with survival, which
led to a very high mortality rate during the first few days of incubation. This high early mortality, occurring before development of the septa that divide the heart cavities and of partition of the truncus conus to originate the aorta and the pulmonary artery have hardly begun, determined the absence of congenital heart disease in this group.

The alterations observed by Hamburger and Habel,9 and by Williamson et al.11 in almost the entire lot of embryos inoculated with influenza A virus appeared in only a small percentage (16.28%) of the embryos studied in these experiments. Instead, such gross malformations as loss of body shape and vesicle formation were observed in 18.6% of the embryos. These malformations were not described by the authors quoted. This disagreement in results may be attributed to the fact that in this study inoculations were performed at an earlier stage of development (17 hours of incubation) than in the other authors' experiments (48 hours of incubation).

The appearance of lethal malformations was to be expected, considering Wilson's statement16 that teratogenic agents acting on the embryo early in development, when differentiation begins, can produce more malformations—and more severe ones—than the same agents acting later. The beginning of differentiation is a period of maximum susceptibility for every individual organ and system.

The different route of inoculation may also account for some of the differences between the results of these experiments and those of Hamburger et al.9 and Williamson et al.11 These authors inoculated over the blastoderm, whereas in our experiments the needle was placed under it.

There is enough evidence to attribute the high early mortality and the gross malformations to the multiplication of the virus in susceptible embryonic cells. Such multiplication was adequately demonstrated.

FIGURE 5
Twenty-one-day embryo, inoculated with chick allantoic fluid after 17 hours of incubation. A: general appearance of a chick with congenital heart disease. B: inside view of the microdissected heart, showing right ventricular infundibulum and pulmonary artery. Notice the interventricular septal defect (1) and the gross thickening of the pulmonary cusps (2).
Cardiac malformations observed after inoculation with heat-inactivated virus and with allantoic fluid were similar. In both cases interventricular septal defects, valvular disease, or both, were observed. The pathogenesis of these malformations is not clear. The infective action of the virus cannot be blamed; neither can the mediation of an immunological process, for the embryo is an immunologically immature being. The results of the buffered saline experiment rule out the possibility that inoculation trauma could be the cause. In fact, the injection of buffered saline caused a significant number of deaths and led to malformations outside the heart, but did not increase or modify the heart lesions found in the control group. Furthermore, unlike the embryos inoculated with active virus, the group that received buffered saline survived long enough so that heart lesions, had there been any, would have been detected.

Finally, a direct toxic action of the allantoic fluid on the developing heart could be postulated. This is reasonable inasmuch as allantoic fluid contains catabolic products of the chick embryo.

Other investigators do not refer to the teratogenic action of allantoic fluid on the heart, possibly because they have not carried our detailed studies of this organ.

Summary
The effect of both active and heat-inactivated influenza A virus, chick allantoic fluid, and of buffered saline solution on the mortality and appearance of congenital malformations in the white Leghorn chick embryo, especially in the heart, was studied. Active influenza A virus produced a high mortality rate during the initial stages of development, when formation of the cardiac septa had barely begun and the primordia of the valves were yet to appear. Consequently, no congenital heart disease was found in the group inoculated with active virus, though profound alterations of the nervous system, sensorial organs and general body shape were observed. The effects of allantoic fluid with and without heat-inactivated virus were the same: high incidence of congenital valvular disease (pulmonary stenosis, thickening of the mitral valve), and interventricular septal defect. Inoculation of buffered saline solution produced an increased mortality rate and structural malformations outside the heart, but had no effect on the development of the heart. Possible mechanisms of action of these teratogenic agents are discussed.

References
11. Williamson, A. P., Simonsen, L., and Blattner, R. J.: Specific organ defects in early chick


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