Histopathologic basis of the teratogenic effects of H-1 virus on hamster embryos

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WITH THREE PLATES

Rat virus (RV), an extremely small DNA virus with a diameter of 15 mμ (Dalton, Kilham & Zeigel, 1963), has exhibited a marked ability to proliferate in placental and fetal tissues following intravenous inoculations of pregnant hamsters, the maternal health remaining unaffected. Effects on the fetus, however, have varied with the strains of RV employed. In initial experiments, a strain which had been carried in forty-two passages through suckling hamsters was without apparent effect on hamster fetuses, although present in high titers in both placental and fetal tissues (Ferm & Kilham, 1963). These results encouraged similar studies on other strains of RV, attempting to find some which would proliferate in a similar manner and also induce teratogenic effects. These studies resulted in the finding of the teratogenicity of the H-1 strain of RV (Ferm & Kilham, 1964a). The purpose of the present report is to describe the histopathologic changes, including the numerous intranuclear inclusions, which accompanied fetal infections with H-1 virus and the spectrum of congenital malformations directly induced by viral action. The histopathologic processes found in these embryonic tissues may help to explain the morphogenetic basis of certain congenital malformations.

MATERIALS AND METHODS

The H-1 virus used in these experiments was kindly supplied by Dr. H. W. Toolan and has been carried through an unknown number of hamster passages. The stock preparations were from virus grown in tissue cultures prepared from rat embryos of approximately 15 days gestational age and maintained in medium 199 containing penicillin and streptomycin. Titrations of the H-1 virus from these cultures and later from tissue suspensions were carried out in rat embryo tissue culture using the appearance of cytopathic effect (CPE) and agglutinins for guinea pig or human ‘O’ erythrocytes as end points (Kilham & Ferm, 1964).

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Female golden hamsters (Cricetus auratus) were left with the male overnight if in estrus and the day following mating was considered to be the first day of gestation. The impregnated females were placed in individual cages and maintained on Purina lab chow.

Under Nembutal anesthesia, pregnant hamsters were injected with 1.5 ml. of tissue culture virus directly into the lingual vein (Anderson, 1963). Various dilutions of the tissue culture virus were used (Table 1), but the volume of injected fluid was kept constant at 1.5 ml. The maternal animals were watched daily for signs of illness or weight loss.

At the time of sacrifice on either the 10th or 13th days of gestation (normal gestation of 16 days), the animals were killed with ether and sterile specimens of uterus, placenta and fetus were obtained for viral titration. Fetal resorption sites were examined and the status of the embryos was recorded. Maternal tissues and most fetal tissues were fixed in Bouin's fixative, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

Specimens of uterus, placenta and fetuses were ground as 10 per cent. suspensions in medium 199 containing penicillin and streptomycin, then frozen at −40°C until the time of virus titration. Methods of titration for H-1 virus in these tissues have been described elsewhere (Kilham & Ferm, 1964). In summary, ten-fold dilutions were made of the original tissue suspensions and added to tubes of rat embryo tissue cultures. Hemagglutinins appeared in the tissue culture fluids in the presence of virus. The end-points of virus titrations (Table 2) were therefore taken as the highest dilutions of the tissue suspensions which were able to cause an appearance of hemagglutinins.

A second group of pregnant hamsters, serving as controls, was injected in an identical manner with another strain (HHP) of rat virus. Tissues from these animals and their fetuses were collected for similar studies. In addition, pregnant

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**PLATE 1**

**FIG. A.** Littermates showing range in fetal size and types of malformations. Animal at left is grossly normal 13-day-old hamster fetus. Next fetus shows hypoplasia of mandible. Next two fetuses are microcephalic and demonstrate hepatic enlargement. Mother received H-1 virus ($10^{-3}$) on day 6. Cardiac circulation and body movements were observed in all of these fetuses at the time of sacrifice. × 3.

**FIG. B.** Thirteen-day hamster fetus with hypoplasia of maxilla and facial asymmetry. H-1 virus ($10^{-1}$) on day 6. × 4.

**FIG. C.** Eleven-day hamster fetus with enlarged head and transverse supraorbital clefts. H-1 virus (undiluted) on day 7. × 4.

**FIG. D.** Thirteen-day-old hamster fetus with microcephaly and retarded growth. H-1 virus ($10^{-1}$) on day 6. × 4.

**FIG. E.** Thirteen-day hamster fetus with ectopic heart and abdominal masses. H-1 virus ($10^{-1}$) on day 6. × 4.

**FIG. F.** Cross section of abdominal area of littermate to fetus in Fig. E. Note apparently normal anterior abdominal wall in mid-line and large hernial masses consisting of embryonic liver. × 10.
FIG. G. Section through vertebral body and notochord (center) of 13-day hamster fetus showing typical intranuclear inclusion bodies in cartilage and notochordal cells. H & E × 350.

FIG. H. Section through kidney (lower portion) and mesenchyme surrounding kidney. Intranuclear inclusions are found in mesenchyme but not in renal parenchymal cells. Normal mitotic figures present in fetal kidney tissue. H & E × 350.

FIG. I. Section through atrio-ventricular region of 13-day fetal hamster heart. Intranuclear inclusion bodies in cells of endocardial cushions. No necrosis noted. H & E × 350.
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rats (Charles River Laboratories), were injected intravenously with 1-5 ml. of undiluted H-1 tissue culture virus on the 8th (two rats) and 15th (two rats) days of gestation. Fetuses, placentas and maternal tissues were collected on the 13th and 20th days of gestation respectively, and studied for gross and microscopic changes.

RESULTS

The H-1 virus has a marked embryocidal effect as summarized in Table 1. This effect appears to diminish as the quantity of actual viral particles injected decreases (Table 1).

Table 1

Effect of various dilutions of H-1 and HHP virus on embryonic mortality when injected intravenously into pregnant hamsters during the period of critical embryogenesis

<table>
<thead>
<tr>
<th>Day of gestation inoculated</th>
<th>Dilution of virus</th>
<th>No. of mothers treated</th>
<th>Living embryos</th>
<th>Dead or resorbed embryos</th>
<th>Per cent resorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-1:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Undiluted</td>
<td>13</td>
<td>16</td>
<td>149</td>
<td>90</td>
</tr>
<tr>
<td>6</td>
<td>10^-1</td>
<td>8</td>
<td>54</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>6</td>
<td>10^-2</td>
<td>11</td>
<td>98</td>
<td>27</td>
<td>21</td>
</tr>
<tr>
<td>6</td>
<td>10^-3</td>
<td>5</td>
<td>51</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>7</td>
<td>Undiluted</td>
<td>5</td>
<td>14</td>
<td>46</td>
<td>76</td>
</tr>
<tr>
<td>7</td>
<td>10^-1</td>
<td>6</td>
<td>22</td>
<td>48</td>
<td>68</td>
</tr>
<tr>
<td>7</td>
<td>10^-2</td>
<td>5</td>
<td>36</td>
<td>28</td>
<td>44</td>
</tr>
<tr>
<td>8</td>
<td>Undiluted</td>
<td>5</td>
<td>12</td>
<td>48</td>
<td>80</td>
</tr>
<tr>
<td>HHP (controls):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Undiluted</td>
<td>2</td>
<td>24</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>10^-1</td>
<td>5</td>
<td>47</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>7</td>
<td>Undiluted</td>
<td>2</td>
<td>25</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>10^-1</td>
<td>2</td>
<td>24</td>
<td>2</td>
<td>8</td>
</tr>
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</table>

Most important, however, is the finding of developmental malformations in some of the fetuses surviving to the time of sacrifice. Representative malformations are shown in Plate 1, Figs. A-F. In addition to the malformations depicted, exencephaly, spina bifida, umbilical hernias, facial defects and cardiac enlargements were found. These malformations do not appear to conform to any obvious pattern. However, consistent histopathology was noted in all of the fetuses studied. These changes consisted of large numbers of intranuclear inclusions especially in mesodermally derived tissues such as certain cartilages (Plate 2, Fig. G), smooth muscle, heart (Plate 2, Fig. I), notochord, meninges of nervous system and especially in the area of the mesodermal somites and mesenchyme of the limb buds. Similar inclusions were noted in the germinative layers of the central nervous system in many affected embryos. These intranuclear inclusions were noted to be adjacent to cells undergoing normal mitotic division (Plate 2, Fig.
On the other hand, inclusions were occasionally found intimately associated with areas of acute necrosis (Plate 3, Figs. K, L). Areas of necrosis were frequently noted in the developing lung tissue (Plate 3, Fig. K), but otherwise they appeared to be randomly located in the loose mesenchymal tissues (Plate 3, Fig. L).

Table 2 summarizes the data concerning the recovery of H-1 virus from uterine, placental and fetal tissues following intravenous injection of the mother. While it is apparent that the placenta of the hamster is permeable to the H-1 virus, the titers do not appear to remain elevated for longer than 3 days after injection when they begin to decline, even though intranuclear inclusions can be found 5 to 7 days later.

Table 2

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Injected Day 10</th>
<th>Injected Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sacrificed Day 13</td>
<td>Sacrificed Day 11</td>
</tr>
<tr>
<td>Uterus</td>
<td>$10^{-3}$*</td>
<td>—</td>
</tr>
<tr>
<td>Placenta</td>
<td>$10^{-3}$</td>
<td>$10^{-6}$</td>
</tr>
<tr>
<td>Fetus</td>
<td>—</td>
<td>$10^{-2}$</td>
</tr>
</tbody>
</table>

* See text for method and calculation of titer.

Sections of placentas of the malformed animals revealed no consistent evidence of placental pathology but the characteristic intranuclear inclusions could be found in trophoblast nuclei of the fetal placentas. These were not frequent and were always located in trophoblast nuclei located near the fetal surface of the labyrinthine placenta. In addition, intranuclear inclusions were quite common in the endodermal cells of the yolk sac placenta in certain specimens (Plate 3, Fig. J).

The maternal animals survived injections without any apparent ill health. Their weight gain was normal for pregnant animals and there was no evidence of lethargy, diarrhoea or vaginal bleeding.

HHP virus, used in an identical manner as the H-1 virus, had no embryocidal or teratogenic effect on the hamster (Table 1). No evidence of histopathology was found in either placental or fetal tissues. Similarly, the H-1 virus had no effect on the maternal or fetal rats after intravenous injection, all fetuses surviving to the time of sacrifice. No evidence of viral inclusions in maternal, placental, or fetal tissues was found.

DISCUSSION

Although it has been postulated that viruses act directly upon embryos to produce their teratological effects, little evidence for the mechanism has been forthcoming. Selzer (1964) has recently described the isolation of rubella virus.
FIG. J. Section of yolk sac placenta showing occasional intranuclear inclusions in endodermal cells (arrows). H & E × 350.

FIG. K. Fetal lung showing marked necrosis of bronchial elements. H & E × 140.

FIG. L. Tail bud of 13-day fetus with areas of necrosis associated with mesenchyme. H & E × 140.
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from one human embryo and placental tissues of four women who had clinical rubella 10–14 days prior to study. Inclusion bodies characteristic of the virus were found in both placental and fetal tissues. The now well-known relationship of cytomegalic inclusion disease and congenital malformations in humans is also characterized by viral inclusions in placental and fetal tissues as well as the ability to recover this virus from these tissues (Weller & Hanshaw, 1962). Thus it appears that for these two viruses a direct pathological effect, including necrosis of cells and subsequent effects on specific organs during critical stages of embryogenesis, may explain their teratogenic activity. Attenuated vaccines of hog cholera (Sautter et al., 1953) and ovine blue tongue viruses (Shultz & DeLay, 1955) in early animal pregnancies have been reported as teratogenic factors but their mechanism of action is not known. Infection of fetal tissues with the virus of equine rhinopneumonitis (EVR) causes abortion in mares which are themselves relatively unaffected by this disease (Westerfield & Dimock, 1946). The aborted fetuses show gross and microscopic lesions with widespread intranuclear viral inclusions but no relationship to developmental malformations has yet been shown for this virus in the horse. Infection of fetal tissues with bovine rhinotracheitis abortion virus has been reported (Kennedy & Richards, 1964) but viral inclusions in fetal tissues and teratogenicity have not been described. Other human and animal viruses (Blattner & Heys, 1961) have been implicated in the etiology of congenital malformations. Of these, some appear to penetrate the placenta and infect the fetus but few have been shown to cause true developmental malformations. It is, therefore, important to distinguish between fetal infection with resulting pathology (fetopathy), and true developmental defects caused by a derangement of some organogenetic process.

It is of interest to note the effect that dilution of the virus inoculum has on embryonic survival (Table 1, Day 6). This is in agreement with a general principle of teratology that the strength of the teratogenic insult is usually directly proportional to its embryocidal and teratogenic effects. On the other hand, the types of congenital malformations found in these studies are not morphologically similar to those described in other hamster experiments using different teratogens (Ferm, 1958, 1963, 1964). Thus we can assume that there is a basic difference in the primary teratogenic mechanism operating here. The histopathologic evidence of cell necrosis and viral intranuclear inclusions in these experiments suggests a direct teratogenic effect of the H-1 virus on hamster embryonic tissues. From recent similar findings in human embryopathies following rubella and cytomegalic inclusion disease it does not appear necessary to invoke an ‘auto-immune disease’ hypothesis as the causative mechanism as suggested by Ebert (1963). Toolan (1962) has not described any gross or microscopic pathology of the human fetuses or placentas from which the H-1 virus and other related viruses have reportedly been isolated.

In addition to the negative teratogenic effect of the HHP strain of rat virus as used in these experiments, and similar previous reports on the 42P strain of RV in
hamsters and rats, mumps virus (Ferm & Kilham, 1964b) and herpes simplex virus (Ferm & Low, 1965) have no teratogenic or embryocidal effect on the hamster. The placenta of the hamster is relatively impermeable to the latter two viruses. We have noted occasional malformations, not easily repeatable and not found in controls, in fetuses from pregnant hamsters injected with the 312 and Krisini strains of RV. Neither of these two strains has any embryocidal or histopathological effect.

Attempts to explain the action of other experimental teratogens have included, among others, the overgrowth of cells in response to injury (Patten, 1952), and the opposite extreme, necrosis with consequent decrease in cell mass and perhaps loss of secondary inductive mechanisms (Hicks, 1953). In these instances of H-1 virus infection of embryonic tissues it appears that quantitatively the mesenchymal tissues have been involved to a more severe degree than other tissues. The somitic or paraxial mesoderm is particularly involved but the resultant malformations are not similar to those described for the effect of vitamin A in the hamster in which necrosis of the embryonic somite appears to be the primary defect (Marin-Padilla & Ferm, 1965). The type of defect in the abdominal hernia of Plate 1, Fig. E, could be best explained on the basis of mesenchymal necrosis of the abdominal wall. Likewise, the malformations of the face as seen in Plate 1, Figs. A, B and C, and the frequent occurrence of microcephaly (Plate 1, Figs. A and D) may be the direct result of a loss of mesenchymal mass due to cellular death. The short 16-day gestation period of the hamster might not permit time sufficient to repair the damage or, in this case, to mask the causative mechanism. Thus, in other instances of virus-induced malformation, direct evidence of viral involvement may be obscured by reparative and secondary processes.

**SUMMARY**

1. Pregnant golden hamsters were injected intravenously with various dilutions of H-1 virus during critical stages of embryonic development. Undiluted virus caused a marked embryocidal effect and a wide spectrum of congenital malformations in the surviving fetuses. Dilution of the viral inoculum caused a diminution in the embryocidal and teratogenic effects.

2. The placenta of the hamster was permeable to this virus as demonstrated by actual recovery of virus from the fetal tissues and the histopathological findings of intranuclear inclusion bodies in many fetal tissues. These inclusions were found most frequently in tissues of mesodermal origin but were also noted in the central nervous system, the trophoblastic cells of the chorio-allantoic and endodermal cells of the yolk sac placentas.

3. Areas of cellular necrosis associated with viral inclusions were frequently found in random patterns most commonly in the loose mesenchymal tissues and bronchial primordia. It is concluded that this virus had a direct teratogenic effect upon the hamster embryo.
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RÉSUMÉ

Base histopathologique des effets tératogènes du virus H-1 sur les embryons de Hamster

1. Des femelles gestantes de Hamster doré ont reçu par voie intraveineuse des dilutions variées de virus H-1 au cours de stades critiques du développement embryonnaire. Le virus non dilué a provoqué une mortalité embryonnaire marquée et une gamme étendue de malformations congénitales chez les foetus survivants. La dilution du virus injecté a diminué la mortalité et les effets tératogènes.

2. Le placenta de Hamster est perméable au virus comme le montrent la récupération de celui-ci à partir des tissus foetaux et la découverte histopathologique d'inclusions virales intranucléaires dans de nombreux tissus embryonnaires. Ces inclusions se trouvent le plus fréquemment dans des tissus d'origine mésodermique mais aussi dans le système nerveux central, les cellules trophoblastiques de l'allanto-chorion et les cellules endodermiques du sac vitellin, dans les régions placentaires.

3. Des zones de nécrose cellulaire associée à des inclusions virales se trouvent fréquemment, réparties au hasard, dans les tissus mésenchymateux lâches et les ébauches bronchiques en particulier. On conclut que ce virus a une action directement tératogène sur l'embryon de Hamster.

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