Vertical Transmission to Embryo and Fetus in Maternal Infection with Rat Virus (RV)

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Abstract: The influence of maternal rat virus (RV) infection on rat embryogenesis and fetus was examined by viral reisolation, immunostaining and PCR analysis. Vertical transmission caused by the UT-1 strain of RV depended on the stage of gestation when maternal infection occurred. When females were infected at the pre-mating point, the number of fetuses was smaller than that normally obtained, possibly due to infection at the stage of the hatched blastocyst, but almost all of the fetuses obtained were free from infection and developed normally. The incidence of transplacental infection was the highest when pregnant females were infected in the middle of the gestation stage, and some of the fetuses died. In pregnant females which were infected late in the gestation stage, all fetuses developed normally. Some of them were infected transplacentally and harbored the infectious virus. Much attention should be paid to performing reliable rederivation of RV-infected rat colonies by hysterectomy and embryo transfer.

Key words: embryo, fetus, rat virus, vertical transmission

Introduction

The rodent parvovirus group includes three variants of the virus, the rat virus (RV), H-1 and the minute virus of mice (MVM). New variants, so-called "orphan parvoviruses" have recently been recognized in mice [12] and rats [20]. These viruses are believed to be common viruses in laboratory rats and mice from the results of microbiological monitoring [6, 10]. Infection is usually asymptomatic, but severe disease can occur in fetal, newborn and immunodeficient animals [3]. Fetal death and absorption in particular are known to be caused by the involvement of not only rodent parvoviruses but also human or other animal parvoviruses [17].

Vertical transmission of rodent parvoviruses in spontaneous cases was first reported by Kilham et al. [8, 9]. Further investigations on pregnant hamsters experimentally infected with H-1 have performed by Toolan [18]. She reported her findings on infection at various stages of the gestation period in pregnant hamsters, and demonstrated higher morbidity and mortality in newborns from mothers injected in the middle of the gestation period, on days 6–8. Although she suggested a pos-
sible relation between maternal antibodies or hormones and susceptibility to transplacental infection, further study for confirmation has not yet been performed.

Recently, embryo transfer techniques have been applied for rederivation of laboratory rodents harboring microbial pathogens [1, 13, 15], but careful studies on the mechanism of vertical transmission of various viruses should be conducted to establish this new method.

This study was performed to determine the influence of maternal RV infection on embryogenesis and the fetus.

**Materials and Methods**

**Virus:** The UT-1 strain of RV isolated from an asymptomatic adult rat [4] was passaged three times in rat embryonic (RE) cells and used for the following experiments.

**Animals:** Specific pathogen-free outbred Wistar-Iamamichi rats were purchased from Imamichi Institute for Animal Reproduction, Ibaraki, Japan, and housed in a negative flow isolator. They were fed commercial diets (NMF; Oriental Yeast Ltd., Tokyo) and given autoclaved tap water ad libitum. These animals were confirmed to be seronegative to common pathogens including Sendai virus, *Mycoplasma pulmonis*, rat coronavirus and RV.

**Animal experiment I:** Female rats at the age of 9–10 weeks were oronasally inoculated with 100 μl of 5 × 10^6 TCID<sub>50</sub> (50% tissue culture infectious doses) /ml of the UT-1 by dropping it into the nasal and oral cavities under ethylether anesthesia at one of three different points, 5–7 days before, and 7 and 14 days after mating. These animals were mated at the ratio of one male to two females in a polycarbonate cage (260 × 415 × 200 mm) with a bedding of autoclaved wood shavings. The mating was confirmed by microscopic observation of sperm in the vagina.

The infected pregnant rats were sacrificed by bleeding under deep anesthesia with ethylether 20 days after mating and fetal and placental specimens were collected. These specimens were offered for viral isolation and immunostaining.

**Animal experiment II:** Ten of the infected females prepared by the procedure described above were mated 5–7 days after infection, and sacrificed 3 days after mating. Developing oocytes at the morular stage were obtained by flushing the oviducts with TYH medium [19]. They were washed with the medium and used to detect of the RV antigen and viral DNA by immunostaining and polymerase chain reaction (PCR), respectively. Some maternal tissues (ovary, oviduct and uterus) and the washing medium for oocytes were also examined by PCR analysis.

All of the animal experiments including fetus sectioning were performed in a humane manner under the Regulation for Animal Experimentation of our university and Japanese law No. 105.

**Viral isolation and immunostaining:** The procedure previously described was used for viral isolation and immunostaining of the fetal specimens [21]. Briefly, RE cells were inoculated with the supernatant of 10% tissue suspension 2 hr after seeding in 24-well tissue culture plates (Falcon, NJ, USA). After 60 min of absorption at 37°C, the cells were cultured with Eagle's minimum essential medium (MEM) supplemented with 5% fetal calf serum and antibiotics for 5 days. Viral growth was confirmed by detection of hemadsorption on the infected cell monolayers. Negativity in isolation was determined on the secondary passaged cells. The sections of the fetal specimen fixed with buffered formaldehyde solution were stained with anti-RV guinea pig serum and peroxidase-conjugated anti-guinea pig IgG serum. The sections were visualized with a substrate solution containing 3, 3 dianinobenzidine. Some sections were also examined with anti-NS protein of MVM monoclonal antibody [23] supplied by Prof. Dr. C. Astell, of the University of British Colombia, and an ABC kit (Vectastain ABC kit; Vector Laboratories, CA, USA).

**Cell culture derived from inner-cell mass (ICM):** Cells derived from the inner-cell mass (ICM) of blastocysts were prepared from ACI/Jc rats by co-cultivation with mitomycin C treated RE cells [16]. The medium for mouse embryonic stem (ES) cells [14] was modified by elimination of 2-mercaptoethanol and supplement with 5,000 units/ml of mouse leukemia inhibitory factor (LIF; ESRO™, AMRAD Corporation, Kew Victoria, Australia) and rat insulin-like growth factor II (IGF-II; supplied by Dr. Nisley, National Institutes of Health, USA), and it was used as a growth and maintenance medium. The concentration of rat IGF-II was 100 ng/ml for the growth medium and 50 ng/ml for the maintenance medium. The RV-infected
cells were examined for viral replication by immunostaining and the hemadsorption described above.

**PCR analysis:** To detect rodent parvoviral DNA, PCR analysis which amplified the conserved sequences coding NS protein of rodent parvoviruses was conducted. The procedure and the specificity of the PCR analysis were described previously [22]. Tissue specimens were homogenized and a volume of approximately 100 mg was subjected to DNA extraction with Sepa-Gene (Sanko Junyaku Co., Ltd., Tokyo). Morula cells from the infected females and positive control cells (infected RE cells) were adjusted to a cell concentration of 5–7 cells per 100 μl, and treated in the same manner as for DNA extraction from the tissue specimens. The amplified products were photographed after electrophoresis in 2% agarose gel and ethidium-bromide staining.

**Results**

**Mortality and Morbidity:** The pregnant females infected in the middle stage of gestation (7 days after mating), the late stage (14 days) and at pre-mating point (5–7 days before mating) were sacrificed 20 days after mating, and the fetuses were collected by hysterecomy. The mortality and morbidity data for the fetuses are summarized in Table 1.

The fetuses from pregnant females infected in the late stage were normal in number, body size, movement and morphological findings, and no abnormality was found in the placenta. But low titer (averaged 3.17 and 2.38 log_{10} TCID_{50}/0.1 ml of 10% homogenate) of virus was isolated from three of twelve (25%) fetuses and two of four pooled placental specimens, respectively.

In the group of pregnant females infected in the middle stage, the most severe implications were recognized in the fetuses. The number of the fetuses was significantly small, and four of twenty-four (12.5%) fetuses were found dead and showing remarkable abnormalities such as delay in development and pantropic subcutaneous hemorrhage. The amniotic fluid was clouded with hemorrhage. Implantation signs in the uterus were observed in two of the three pregnant females and this finding indicated embryonic absorption. A high titer (averaged 7.30 and 7.55 log_{10} TCID_{50}/0.1 ml of 10% homogenate) of the virus was isolated from all of the fetuses and five of six pooled placentas, respectively.

In three of ten females infected at the pre-mating point, no fetus was observed, though sperm were detected in vaginal smear from these females. In these three cases, no implantation sign was observed in the uterus. All of the fetuses from the other seven pregnant females developed normally. The number of fetuses observed in two litters was smaller than normally obtained. No implantation sign indicating embryonic absorption was recognized in their uteri. A few viruses were recovered from only one of the placental specimens, and there were no viruses in the

### Table 1. Vertical transmission of RV in pregnant rats infected at the different gestation stages

<table>
<thead>
<tr>
<th>Group (Gestation stage of infection)</th>
<th>No. of pregnant rats</th>
<th>Mean No. of fetuses (Range)</th>
<th>No. of dead / Total No. of fetuses</th>
<th>Viral isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Late-stage (14 days after mating)</td>
<td>4</td>
<td>13.5 ± 1.9 (12–16)</td>
<td>0 / 54</td>
<td>3 / 12 (3.17)</td>
</tr>
<tr>
<td>Mid-stage (7 days after mating)</td>
<td>3</td>
<td>8.0 ± 4.6 (4–13)</td>
<td>4 / 24</td>
<td>6 / 6 (7.30)</td>
</tr>
<tr>
<td>Pre-mating stage (5–7 days before mating)</td>
<td>7</td>
<td>12.0 ± 3.1 (9–15)</td>
<td>0 / 84</td>
<td>0 / 21 (0.5)</td>
</tr>
<tr>
<td>Uninfected</td>
<td>5</td>
<td>13.6 ± 1.5 (12–15)</td>
<td>0 / 68</td>
<td>NT</td>
</tr>
</tbody>
</table>

*Two or three fetuses from each pregnant female were examined. Three placentas from each pregnant female were pooled and examined. Mean ± S.D. No. positive / No. examined (Mean infective titer of positive samples: log_{10} TCID_{50}/0.1 ml of 10% homogenate). NT: Not tested.
fetuses, and no viral DNA signal was detected in the fetuses by PCR analysis (Data not shown). These findings suggested that impediments to embryogenesis or implantation must have occurred early in gestation in these two litters.

Viral isolation from maternal organs was also carried out, and low titers of virus were detected in several organs (uterus, spleen, kidneys and blood) in the pre-mating and the middle stage groups (<0.5 TCID<sub>50</sub>/0.1 ml) and in the late stage group (<1.5 TCID<sub>50</sub>/0.1 ml). In the viral isolation from the fetal and placental specimens, the possibility of viral contamination from maternal blood must be ignored. Viral antigens in the fetal organs: Three fetuses each from the three infection groups were sectioned and several organs, liver, kidneys, heart, lungs and brain, were examined for the presence of viral antigen by immunostaining. The results are shown in Table 2. The most antigenic signal was demonstrated in the fetuses from the pregnant females infected at the middle stage of gestation, and the results corresponded well with those of viral isolation. Much viral antigen was observed in the lung and the heart from both developmentally delayed and apparently normal fetuses. A little antigen was detected in the liver, especially in hepatocytes. In one of the abnormal (developmentally delayed) fetuses, most of the hepatocytes were extremely degenerated and destroyed, though the viral antigen found was less in the liver than in the lungs and heart. No viral antigen was detected in the kidneys or the brain. On the other hand, a little antigen was observed in the fetal lungs and the heart in the late infection group, but no antigen was found in any fetal organs in the pre-mating infection group.

**Table 2.** Distribution of viral antigen in the fetal organs detected following immunoperoxidase staining

<table>
<thead>
<tr>
<th>Group*</th>
<th>Liver</th>
<th>Kidneys</th>
<th>Heart</th>
<th>Lungs</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Late-stage</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mid-stage</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Pre-mating</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* See text and Table 1. Quantity of viral antigen was graded as follows: small quantity (+), large quantity (++), and negative (−).

![Fig. 1. Photomicrograph showing paroviral NS antigens in the nucleus of ICM-derived cells infected with RV. Four days after infection. Immunoperoxidase staining. Bar=10 μm.](image)

**Discussion**

The results demonstrate that the UT-1 strain of RV causes transplacental infection to the fetus, and that
vertical transmission to the fetus or embryogenesis varies in degree according to the stage of gestation when maternal infection occurs. Abnormalities reported here included death, developmental delay, pantrropic hemorrhage and severe liver degeneration of the fetus just before parturition, and embryonic death early gestation.

Kilham et al. [8, 9] were first to report spontaneous vertical transmission of RV and preliminary findings related to the disease in infected suckling rats. Thereafter, Tooan [18] has systematically studied transplacental infection in pregnant hamster with H-1 virus and reported that the fetus was most susceptible to infection in the middle gestation stage, i.e., Days 6-8. Although it is difficult to compare the results presented here with her findings because of the difference between the gestation periods of rats and hamsters, both sets of results were essentially consistent as the highest susceptibility to infection in the middle gestation stage. We considered that viral transmission via the placenta should occur at the beginning of viremia [3-5 days after the peroral inoculation [21]], i.e., Day 10-12 in the group infected in the middle stage in this study. Since Tooan performed her experiments by subcutaneous or intraperitoneal inoculation into hamsters, viremia may be caused immediately.

Viremia in pregnant female rats infected at the pre-mating point may continue until approximately 2 weeks after infection as described previously [21]. Jacoby et al. [7] also reported that viral antigen was found in the myometrium and in muscles of the lower genital tract and the endometrial epithelium in the second and third weeks of infection. Almost all the embryos from the pre-mating infection group showed no sign of infection in our study, even though the embryos might have been exposed to the virus after implantation because of maternal viremia. One of the reasons for the highest susceptibility to transplacental infection in the middle gestation stage may be a temporary reduction in immuno-competence in pregnant females to maintain their pregnancy and not to reject the embryos.

The distribution of viral antigen in the infected fetuses did not correspond with the severity of the lesions. Much viral antigen was observed in the lungs and the heart from both developmentally delayed and apparently normal fetuses. In one of the abnormal (developmentally delayed) fetuses, most of the hepatocytes were extremely degenerated and destroyed, though there was less viral antigen in the liver than in the lungs and heart. The inconsistency between the location of the viral antigen and the lesion was also mentioned previously [21]. The reasons for these inconsistent findings are considered to be the following. The antisera used for immunostaining in our present study was prepared by injection into guinea pigs of purified virus which contained capsid protein but not NS protein. Accordingly, the present findings indicate the distribution of capsid protein, but not of NS protein. Recently, Brandenburger et al. [2] demonstrated that the NS protein of MVM revealed cytotoxic activity when overexpressed. The pathogenesis of RV may be closely related to the levels of NS protein expression.

The reduction in the number of fetuses and the absence of implantation sites found in Exp. I may indicate embryonic death at an early stage of embryogenesis, but morula cells from the infected pregnant females were free from viral transmission in Exp. II. The embryos surrounded by zona pellucida, from the 1-cell to the blastocyst in the embryonic stage, are considered to be protected from some viruses [5]. Some researchers reported that mouse embryos from which zona pellucida was removed were infected with mouse hepatitis virus [13] and Sendai virus [11]. In the present study, we demonstrated that RV infected the cultured cells derived from ICM and caused cell death. These results suggest the possibility that hatched blastocytes are infected with RV and degenerate in the pre- or post-implantation stage.

In conclusion, vertical transmission of RV varies according to the stage of maternal viremia and gestation. When pregnant females cause viremia early in gestation, some hatched oocytes may be infected with RV and embryogenesis is disturbed. When viremia is caused in the middle gestation, almost all the fetuses are transplacentally infected and organogenesis is strictly disturbed. When viremia is caused late in gestation, almost all the fetuses develop normally and are born carrying the virus. The transplacental infection of rodent parvovirus and its implications for embryogenesis should be an interesting animal model for human parvoviral abortion.

On the other hand, apparently healthy fetuses were also infected with the virus and harbored a low titer. These animals may become immunotolerant and con-
continuously shed the infectious virus after birth. Hence, rederivation by hysterectomy in RV-infected rats may not be completely reliable. The present findings on vertical transmission suggest that much attention should be paid to using the rederivation technique.

Acknowledgment

This work was supported by Grant-in-Aid (No. 04680041) for Scientific Research (C) from the Ministry of Education, Science and Culture, Japan.

References