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Fetal death persists through recurrent pregnancies in mice following Ljungan virus infection

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Abstract

Objectives—Laboratory mice infected with Ljungan virus (LV) early in pregnancy suffer from perinatal deaths. Here we investigate the persistence of that effect through the outcome of consecutive pregnancies in LV infected mice.

Study design—CD-1 mice were infected while pregnant and their adult female offspring were followed in parallel with uninfected control mice during repeated pregnancies. Three mating attempts resulted in two or three pregnancies per dam. The outcome of the last pregnancy was carefully monitored.

Results—Both the dams infected as adults and their adult female offspring suffered perinatal deaths during the last pregnancy which occurred approximately six months after the original LV exposure and acute infection. The non-infected control animals experienced no perinatal deaths.

Conclusions—Perinatal death persists across recurrent pregnancies in this mouse model of LV infection, both in animals infected as adults and in females exposed to the virus in utero. This implies that LV persists in mice long after initial infection, is maintained in a quiescent state, but can remain pathogenic in later pregnancies.

Keywords

Ljungan virus; mice; animal model; intrauterine death; consecutive pregnancy

INTRODUCTION

Ljungan virus (LV), a member of the Parecho virus genus, family *Picornaviridae*, was originally isolated from one of its wild rodent reservoirs, the bank vole (*Myodes glareolus*), by the Ljungan river in Sweden (Niklasson et al., 1999). LV is associated with diabetes and myocarditis in different species of wild voles and lemmings (Niklasson et al., 2003; Niklasson et al., 2006a). We have previously reported that laboratory mice infected with LV two days after conception resulted in a congenital infection with a high frequency of neonatal death (Samsioe et al., 2006). Signs of early abortions and malformations, predominately of the brain and skull, were observed. The majority of offspring death

occurred at the time of birth. Encephalitis was found in offspring born dead or dying soon after birth and in surviving offspring without symptoms.

We have also found that surviving male offspring develop a type 2-like diabetes condition as adults, with high serum insulin levels and impaired glucose tolerance (Niklasson et al., 2006b). The perinatal death and the type 2-like diabetes conditions were dependent on the combination of infection and stress. Infection or stress alone had no or little adverse effect on the pregnancy, while infection in combination with stress resulted in high frequencies of intrauterine death, stillbirth and malformation (Niklasson et al., 2006b; Samsioe et al., 2006). It has also been discovered recently that the incidence of human intrauterine fetal death (IUFD) in Sweden tracks the fluctuations in rodent populations in the same region (Niklasson et al., 2007). In addition, LV has been observed in half of the investigated IUFD cases (Niklasson et al., 2007).

The findings that male mice exposed to LV infection during pregnancy develop diabetes later in life raised the question of whether LV causes a persistent infection in these animals. The present study examines viral persistence by investigating the outcome in LV-infected mice during consecutive pregnancies.

MATERIALS AND METHODS

Animal treatment

Out bred CD1 mice (Charles River Laboratories, Sulzfeld Germany) were used in this study. A total of 15 mice were infected intraperitoneally (i.p.) on gestational day (GD) two (Niklasson et al., 1999) with 1,000 ID₅₀ of LV strain 145SL passed six times in suckling mouse brain. This first group of mice was followed during three mating attempts. A second group consisted of 20 randomly selected female offspring from the first group of 15 dams. These mice (exposed to the virus in utero) were followed as adults through three mating attempts. A third non-infected control group consisted of 15 female mice given normal saline i.p. on GD two. This control group was also followed during a total of three mating attempts. Eight non-infected male CD-1 mice were used for mating. At mating, two females shared one randomly selected male in a cage for a maximum of four days or until a positive sperm plug was registered. Males used for mating with non-infected control females were kept separate from other males. The same males were used for all three mating attempts.

All animals were kept in individual cages with free access to food and water and kept on a 12:12 hour light cycle.

A previous study found that the combination of infection and mild stress is necessary for pathogenic effect (Samsioe et al., 2006). The present study protocol uses the same stress regime as the previous study. This consisted of repeated measurement of BW, glucose in drinking water and a glucose tolerance test during pregnancy.

The body weight of each pregnant dam was measured on GD 2; 4; 6; 9; 11; 13; 16; 17; 19 and a glucose tolerance test was performed on GD 17. The glucose tolerance test was performed as follows: 2 g glucose per kg body weight was administrated i.p. (as 100g glucose/l solution). Non-fasting blood glucose was measured (Accu Check® blood glucose meter, Roche) immediately before the glucose injection and at 60 and 120 minutes post administration. An additional metabolic stress was imposed in this experiment by the addition of 100 g of glucose/l to the drinking water of all animals for the duration of the experiment.

The experimental procedures were approved by the animal ethical committee in Stockholm (N283/05) and in accordance with international guidelines (NIH publications no. 85-23, revised 1985).

Animal follow up

Each of the 50 experimental dams was subjected to three mating attempts. This resulted in either two or three pregnancies for all dams. Offspring of the first and second mating were removed from the dam 10 days after birth and a new mating initiated three weeks later. The outcome of the third mating attempt was recorded for all three groups (i.p. infected, exposed to infection in utero and controls).

Each pregnancy cycle included mating (4 days) and pregnancy (21 days). The offspring were removed at the age of 10 days and a new mating attempt was begun 21 days later. This sequence of events was repeated three times meaning that the offspring from the last pregnancy were sacrificed just short of 6 months (168 days).

All pregnant mice delivered naturally and the delivery phase was monitored continuously in order to detect all offspring born dead or alive. All females were sacrificed one week after the last delivery, and the uterus was investigated macroscopically for signs of fetal resorptions and/or implantation sites.

The number of females suffering from perinatal loss and the total number of pups born dead or alive were recorded. Perinatal death was defined as stillbirth or death during the first 24 hours. Pregnancy in females was defined based on a positive sperm plug or a weight increase of more than 5 gram per week followed by a similar weight decrease.

Pregnant females failing to deliver were investigated for termination of pregnancy. In all cases clear signs of embryo/fetal resorptions and/or implantation sites were determined at autopsy after expected delivery.

Tissues (brain, lung, liver and spleen) from four of the *group 1*, females (infected i.p.) and four of the *group 2*, females (in utero exposed) were collected for analysis of LV RNA by a real-time RT-PCR (Mantke et al., 2007). Pups born dead were also investigated. The placenta, brain, heart/lung and liver/intestine were collected from four offspring born dead from i.p. infected females and five offspring born dead from females exposed to infection in utero.

RESULTS

Thirteen dams out of 15 from the i.p. infected *group 1* became pregnant during the third pregnancy attempt, and 11 of these had poor pregnancy outcomes. Ten dams in this group experienced perinatal deaths (one animal lost the entire litter) and one female suffered from fetal death diagnosed by resorptions. (Table 1). In the *group 2* (in utero exposure), all 20 dams became pregnant and eight experienced complications. Four females gave birth to dead pups (one female lost the entire litter). Four of the pregnant females did not deliver and suffered from perinatal death / embryo resorption diagnosed at autopsy (Table 1). In the control *group 3*, nine out of 15 females became pregnant and each pregnant dam gave birth to live offspring. Neither perinatal deaths nor resorptions nor implantation sites were noted within the control group. There was a significant difference between the number of the *group 1* dams (infected i.p.), suffering perinatal loss and/or resorptions compared to the control *group 3* ($p < 0.0001$; Fishers exact test). Likewise, there was a significant excess of perinatal death and resorptions in the *group 2* (in utero exposure), as compared to the *group*

3 (controls) dams ($p=0.0033$; Fishers exact test). All animals had a normal glucose tolerance tests.

The PCR test was positive for LV in three out of four of the *group 1* dams (infected i.p), in one or more organs, while one out of four females exposed to LV in utero were PCR positive (Table 2A). PCR testing of the dead pups revealed that in those from the *group 1* dams three out of four were found positive and in the *group 2* pups (in utero exposure), three out of five were PCR positive in one or more organs (Table 2). None of the infected dams were positive for the brain samples, but in contrast the offspring were often found positive in the brain samples (Table 2)

DISCUSSION

We previously reported that over half of the mice infected with LV virus during pregnancy and exposed to stress produce malformed pups that die in the perinatal period (Samsioe et al., 2006). This earlier study found stress to be important for the development of disease in the infected animals. Infection or stress alone had a minimal negative effect on the outcome of a pregnancy while animals subjected to the combination of infection and stress suffered from a high frequency of dead offspring. The study also demonstrated that valuable information can be gained by constant monitoring of the delivery phase. While malformed offspring and resorptions can be diagnosed by sacrificing the pregnant mice 2-3 days before expected delivery, this procedure will not necessarily identify all pups that will die perinatally. Comprehensive information can only be obtained by observing the animals during the entire period of expected delivery (Samsioe et al., 2006).

The discovery that LV is associated with severe disease in its natural reservoir and the finding that the same virus causes disease during pregnancy in laboratory mice initiated studies to clarify whether LV may have a role in disease during pregnancy in humans. A striking association between small rodent abundance and the number of human cases of intrauterine fetal death (IUFD) has been found in Sweden. The coefficients of determination found in this study was very high ($R^2=82$) suggesting that LV is likely responsible for a substantial fraction of all cases in the region studied (Niklasson et al., 2007). This study also diagnosed LV in infant brain tissue and in the placenta in half of the IUFD cases investigated by immunohistochemistry (IHC). A follow up study has now confirmed the presence of LV in human IUFD cases using both IHC and LV specific real-time RT-PCR (Samsioe et al submitted Birth Defects).

The recurrence of stillbirth and intrauterine fetal death in humans is well recognized (Freeman et al., 1985; Frias et al., 2004; Greenwood et al., 1994; Samueloff et al., 1993; Sharma et al., 2007). The risk for subsequent stillbirth increases by two to tenfold compared to birth in women who have not experienced stillbirth (Greenwood et al., 1994; Samueloff et al., 1993). Previous observations, that LV in the acute phase of the infection causes perinatal death in laboratory mice and that mice exposed to the virus several months earlier also suffer from disease stimulated the present study aimed at following infected mice over consecutive pregnancies. We used the same regimen to monitor the pregnancy outcome and the same stress regimen of repeated body weight measuring, metabolic stress with glucose in the drinking water and a glucose tolerance test established in the previous study (Samsioe et al., 2006).

This present study clearly shows that LV infected mice also suffer from recurrent perinatal death, not only during the acute phase of the infection following initial exposure, but also in consecutive pregnancies.

Reproductive problems expressed as with perinatal death was found to persist in both dams infected i.p. and in dams exposed to the virus in utero over the six-month-long follow up to the third pregnancy. This implies that LV persists in mice long after initial infection, is maintained in a quiescent state, then can become pathogenic to disrupt later pregnancies. LV is difficult to cultivate, making traditional virological methods for estimating virus load very difficult (Niklasson et al., 1999). However, a recently developed real-time RT-PCR assay has provided an important tool to measure presence of viral RNA as indirect evidence of infectious virus in tissue (Mantke et al., 2007). The fact that LV viral RNA can be detected in female mice six months post viral i.p. inoculation points to a persistent infection. Low levels of viral RNA could be detected in male mice infected with LV early in life during the entire six months follow up (Kallies et al. personal communication or in press). Presence of viral RNA in the brain of dead offspring supports the hypothesis that LV infection acquired in utero in combination with maternal stress can cause recurrent perinatal death.

Viral RNA was detected in one of the four females tested that had been exposed to viral infection in utero. This finding, in combination with the observation of PCR-positive dead offspring, shows that at least some of these females also suffer from persistent infection. No pathology was performed on the females or their offspring. The limited number of specimens available for PCR analysis does not allow any conclusions regarding presence of virus or virus load in any particular animal or organ nor the outcome of the pregnancy. It is necessary to investigate this animal model in more detail to understand what role LV plays in the pathogenesis and whether intervention or prophylaxis might be possible. Parallel studies suggesting that LV may cause IUFD in humans, make this animal model an important tool for developing therapy and prophylaxis.

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Pregnancy outcome in CD 1 dams infected i.p. exposed to Ljungan virus in utero and controls at the third pregnancy

Table 1

	Total no females	Pregnant	Mean number of pups per pregnancy	Number Dams				Total no pups lost
				Pregnant, delivering dead pups	Pregnant, loss of entire litter	Pregnant, fetal resorption		
Infected i.p.*	15	13	11	10	1	1	>21	
Exposed <i>in utero</i> **	20	20	12,5	4	1	4	>15	
Controls	15	9	12,5	0	0	0	0	

* The number of dams infected i.p suffering perinatal loss and/or resorptions compared to the controls: $p<0.0001$; Fishers exact test.

** Excess of perinatal deaths and resorptions in group exposed in utero compared to the controls: $p=0.0033$; Fishers exact test.

Table 2A

PCR results of females infected i.p. and females exposed to LV in utero

Female	Brain	Lung	Liver	Spleen
Nr. 1 i.p. infected	neg.	pos.	neg.	pos.
Nr. 2 i.p. infected	neg.	neg.	neg.	neg.
Nr. 3 i.p. infected	neg.	neg.	pos.	pos.
Nr. 4 i.p. infected	neg.	pos.	pos.	pos.
Nr. 5 exposed in utero	neg.	neg.	neg.	pos.
Nr. 6 exposed in utero	neg.	neg.	neg.	neg.
Nr. 7 exposed in utero	neg.	neg.	neg.	neg.
Nr. 8 exposed in utero	neg.	neg.	neg.	neg.

Table 2B

PCR results of offspring born dead from i.p. infected dams and offspring born dead from dams exposed in utero. (n.d = not done)

Pups	Brain	Lung/heart	Liver/intestine	Placenta
Nr. 1 i.p. infected	pos.	n.d.	n.d.	n.d.
Nr. 2 i.p. infected	pos.	n.d.	pos.	pos.
Nr. 3 i.p. infected	pos.	neg.	pos.	n.d.
Nr. 4 i.p. infected	neg.	neg.	neg.	n.d.
Nr. 5 i.u. exposed	neg.	n.d.	neg.	n.d.
Nr. 6 i.u. exposed	pos.	n.d.	pos.	pos.
Nr. 7 i.u. exposed	pos.	n.d.	n.d.	n.d.
Nr. 8 i.u. exposed	pos.	neg.	neg.	neg.
Nr. 9 i.u. exposed	neg.	neg.	neg.	n.d.