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Prevalence of Rat Virus Infection in Progeny of Acutely or Persistently Infected Pregnant Rats

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Infant rats are susceptible to persistent rat virus (RV) infection, but risk of persistent infection after prenatal exposure to virus is unclear. We examined this aspect of RV infection in the progeny of dams inoculated with virus during or prior to pregnancy. Sprague-Dawley (SD) dams were infected during pregnancy (gestation day 9) by oronasal inoculation with 10^5 $TCID_{50}$ of the UMass strain of RV. SD rats were infected prior to pregnancy by oronasal inoculation of two-day-old females with 10^2 $TCID_{50}$ of RV-UMass, which induced persistent infection. They were mated to non-immune males after reaching sexual maturity. Rats were assessed for RV infection by virus isolation, in situ hybridization, contact transmission, or serologic testing. The progeny of dams inoculated with virus during gestation had high prevalence of infection through postpartum week 9 (9 of 12 rats were virus positive at week 3, and 7 of 10 were virus positive at week 9). Additionally, 2 of 10 rats were virus positive at least through postpartum week 15. The progeny from persistently infected, seropositive dams had no evidence of infection and did not transmit infection to contact sentinels. However, 12 dams were virus positive at necropsy and 9 had transmitted infection to their breeding partners. These results indicate that prenatal infection in non-immune dams can lead to RV persistence in their progeny. By contrast, the progeny of persistently infected dams are protected from infection, presumably by maternal antibody, although their dams can transmit infection to their breeding partners.

Rat virus (RV) is a common parvovirus of laboratory rats which replicates preferentially in mitotically active cells (1-3). Infection can disrupt experimentation with rats by causing disease, especially in fetal or neonatal rats (4, 5), or by distorting biological responses dependent on cell proliferation (6-9). The pathogenicity of RV for fetal rats, in particular, was exploited during early years of parvovirus research, to develop a rodent model for virus-induced congenital anomalies (6, 9, 10-12). Although RV infection also can be pathogenic for infant rats under experimental conditions, natural infection is often asymptomatic and is detected by seroconversion rather than clinical morbidity. However, asymptomatic infection can lead to viral persistence despite the onset of antiviral immunity (13-15). Persistent infection, in turn, extends risks for transmission of virus and interference with rat-based research.

Postnatal susceptibility to persistent RV infection is high during early infancy. For example, oronasal inoculation of two-day-old rats with the Y strain of RV caused persistent infection, lasting at least six months in some animals (14). Affected rats developed humoral immunity within three weeks, but transmitted infection for up to 10 weeks. By contrast, rats inoculated after weaning rarely remained infected for more than four weeks. More recent studies using the UMass strain of RV indicated that oronasal inoculation of six-day-old rats induced high prevalence of asymptomatic persistent infection (15). In situ hybridization, using virus strand-specific probes, revealed vascular and intestinal smooth muscle cells to be prominent sites of persistent infection, which included virus replication (16). Further, lung and kidney—sites conducive to virus excretion—harbored persisting virus in some rats.

Although persistent infection after non-lethal exposure to RV in infant rats is well documented, the outcome of non-lethal prenatal infection is unclear. Therefore, we assessed infection in the progeny of female rats exposed to virus under two conditions relevant to breeding colonies: acute infection induced in non-immune dams during pregnancy, and persistent infection induced in dams prior to pregnancy.

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Materials and Methods

Rats: Virus antibody-free, Sprague-Dawley (SD) rats (*Rattus norvegicus*) were obtained from a commercial vendor (Harlan Sprague Dawley, Indianapolis, Ind.). They included time-mated and randomly mated pregnant females, adult males which served as breeding partners, and adult males and females which served as contact sentinels for transmission of infection. Rats were housed and husbanded in microisolette cages as described (17). Male and female rats were co-housed until pregnancy was confirmed by abdominal palpation, then males were removed and housed individually. Pregnant dams and dams with litters were housed individually. Progeny were group housed by sex or housed individually after weaning. Rats were selected by random sampling to test for infection by virus isolation, in situ hybridization (ISH), contact transmission or serologic testing.

The care and use of rats in the experiments reported here was approved by the Yale Institutional Animal Care and Use Committee. They met or exceeded all guidelines and standards contained in the US Public Health Service *Guide for the Care and Use of Laboratory Animals*.

Virus inoculation and isolation: The UMass strain of RV (RV-UMass) was used for all experiments (18). Virus stocks were produced and quantified, using an established line of normal rat kidney (NRK) cells (15). To examine the effects of acute maternal infection during pregnancy, time-mated, non-immune pregnant rats were inoculated by the oronasal route at gestation day 9 or 12. To obtain persistently infected dams for determining

the effects of maternal infection induced prior to pregnancy, infant rats were inoculated by the oronasal route at two days of age with 10^2 $TCID_{50}$ of virus and were kept to breeding age. Lung, spleen, and kidney from selected dams and their progeny were assayed for infectious virus by explant culture (19).

Clinical observation and necropsy: Rats were observed daily for clinical signs of infection. Small blood samples (100 μl) were obtained by hypodermic puncture of the caudal artery in the tail under light methoxyflurane anesthesia. Larger blood samples were collected from the caudal vena cava after euthanasia. Serum was stored at –20°C pending assay for antiviral antibodies. Rats were euthanized by use of carbon dioxide gas at pre-scheduled time points or if clinical signs of infection developed. Adults, infants and fetuses were examined for gross lesions, during which pieces of lung, spleen, and kidney were collected aseptically for virus isolation by explant culture. The following tissues were fixed in freshly prepared periodate-lysine-paraformaldehyde solution (20) for 16 hours: brain, lung, heart, aorta, thymus, spleen, peripheral lymph nodes, salivary gland, liver, small intestine, kidney, gonads, mesenteric vessels, and gonadal vessels.

In situ hybridization (ISH): A randomly primed ^{32}P-labeled DNA probe was prepared from a commercial kit (New England Biolabs, Beverly, Mass.), using purified RV-UMass DNA, from nucleotide 1,086 to 4,300 as a template (21). Fixed tissues were embedded in paraffin and sectioned at 5-μm thickness. The ISH was performed as described elsewhere (21) and was assessed by light microscopy.

Serologic testing: Sera were tested initially for antibodies to RV by an immunofluorescence assay (IFA) (22). They were subsequently tested for IgM and IgG antibodies to RV VP2 (capsid) protein by an enzyme-linked immunosorbent assay (16, 23). Briefly, VP2 was expressed in bacteria and purified from the insoluble protein fraction using His-bind resin (Novagen; Madison, Wis.). Ninety-six well flat-bottom polystyrene microtitration plates (Nunc MaxiSorp; Roskilde, Denmark) were coated with 150 ng of RV VP2 or bacterial-expressed β-gal protein purified from the soluble fraction of bacteria as a negative control. Plates were incubated at room temperature for two hours, then overnight at 4°C. Plates were washed three times with phosphate-buffered saline (PBS) containing 0.5% Tween 20, and were blocked with 250 μl of 3% gelatin in PBS for 1 hour at 37°C. Plates were washed again, and 100-μl serial dilutions of sera, in 0.5% bovine serum albumin (BSA)-PBS were added to antigen- and β-gal-coated wells. Plates were incubated at 37°C for two hours and washed. Horseradish peroxidase-conjugated secondary antibodies, goat anti-rat immunoglobulins (IgG, IgA, IgM) used at a 1:10,000 dilution in PBS-0.5% BSA (ICN Pharmaceuticals, Inc.; Aurora, Ohio), or mouse anti-rat IgM, IgG1, or IgG2a at a 1:2,000 dilution in PBS-BSA (Serotec, Inc.; Raleigh, N.C.), were added to the appropriate wells, and incubation proceeded at 37°C for 1 hour. After washing three times, 3,3',5,5'-tetrameth-ylbenzidine (TMB) peroxidase substrate (Kirkergaard & Perry; Gaithersburg, Md.) was added to the wells for five minutes, followed by addition of 1*N* HCl to halt the reaction. Absorbance was measured in a plate reader at a setting of 450 nm. Titer endpoints were defined as the inverse dilution of the last absorbance value greater than mean β-gal absorbance plus two standard deviations for that dilution.

Representative sera from dams and sires collected prior to breeding, and from dams, sires, and progeny at necropsy, also were tested by IFA for antibodies to RV, rat parvovirus, rat coronavirus, Sendai virus, and *Mycoplasma pulmonis*. Uninoculated rats were seronegative for all antigens, and inoculated rats were seropositive only for RV.

Contact transmission of infection: To test for contact transmission by progeny of acutely infected dams, non-immune sentinel rats were co-housed with individual or paired index rats for one week, then were housed separately for two weeks and tested for anti-RV antibody by IFA. To test for contact transmission by the progeny of persistently infected dams, sentinels were co-housed with index rats for four to six weeks after the index rats had lost maternal immunity, as determined by results of IFA serologic testing, were then tested for anti-RV antibody.

Results

Prevalence of infection in the progeny of non-immune dams inoculated with RV during pregnancy: The pathogenicity of prenatal RV infection is strongly influenced by virus dose and the stage of gestation at which dams are inoculated. Susceptibility to transplacental infection is high during early to mid-gestation (6, 10, 15, 24-26). Therefore, we selected this interval to seek reliable conditions for induction of persistent infection in preliminary experiments (Table 1). Non-immune dams were inoculated oronasally on gestation day 9 with graduated doses of RV-UMass. Inoculation of dams with 10^7 $TCID_{50}$ of virus caused 100% mortality among their fetuses, as determined by gross and microscopic examination, by postinoculation day (PID) 10 (gestation day 19) whereas the progeny of dams inoculated with 10^5 $TCID_{50}$ of virus had low prenatal mortality. Dams inoculated with this dose, however, transmitted infection to all fetuses examined at PID 10 and to all pups tested at postpartum week 3. Dams inoculated with 10^4 $TCID_{50}$ of virus on gestation day 9 transmitted prenatal infection without fetal mortality, but some progeny were virus negative at postpartum week 3. To determine whether exposure of more mature fetuses to RV would further reduce prenatal mortality while retaining susceptibility to persistent infection, two dams were inoculated with 10^5 $TCID_{50}$ of virus on gestation day 12. No fetal deaths were observed and 6 of 10 randomly selected newborn pups from the two litters were virus positive. However, no virus was detected among pups tested at postpartum week 3.

On the basis of the foregoing results, 10 non-immune pregnant rats were inoculated with 10^5 $TCID_{50}$ of virus on gestation day 9 to induce prenatal infection. Their progeny were tested for infection at postpartum weeks 3, 8, 9, 15, and 16 (Table 2). All inoculated dams delivered live litters. The progeny were tested so that at least four litters were sampled at each of the time points and individual litters were sampled at least twice. Nine

Table 1. Effect of virus dose and time of maternal inoculation on the outcome of prenatal infection with the UMass strain of rat virus (RV-UMass)

Dams inoculated on gestation day	Virus dose ($TCID_{50}$)	No. of dams	Outcome among progeny[a]			
			Prenatal[b]		Postnatal[c]	
			Deaths	Infected[d]	Deaths	Infected[d]
9	10^7	4	48/50	ND	ND	ND
	10^5	4	6/29	4/4	0/8	8/8
	10^4	4	0/32	9/9	0/23	3/6
12	10^5	2	0/24	6/10	0/14	0/6

[a]No.of affected/total No. of rats.
[b]Ten days after inoculation.
[c]Three weeks after parturition (42 to 44 days after inoculation).
[d]Determined by explant culture and in situ hybridization (ISH).
ND = Not determined.

of 12 rats (75%) examined at week 3 by explant culture and ISH had virus in multiple tissues. The primary site of virus-positive cells was vascular smooth muscle. All rats had serum anti-RV IgG, but not IgM. No virus was detected by explant culture or ISH in 10 rats examined at week 8. Therefore, we tested an additional cohort for persistent infection by contact exposure to sentinel rats to examine the possibility that infection was still present in this age group. Five female and five male rats scheduled for necropsy at week 16 were paired with age- and sex-matched sentinels for one week at week 9 and with a fresh group of sentinels at week 15. The sentinels were housed individually for two weeks after contact exposure, then tested for antibodies to RV. Seven of 10 sentinels exposed at week 9 seroconverted, indicating persistent infection in a majority of the index rats during this interval. One of 10 sentinels introduced at week 15 also seroconverted and the corresponding index rat was seropositive, although no virus was detected in this rat by explant culture when it was necropsied at week 16. However, virus was detected in one other seropositive index rat at week 16. Thus, two of 10 rats tested at the 15- to 16-week interval were persistently infected.

The serum of all index rats necropsied at week 3 contained only IgG antibody to RV. All 10 rats necropsied at week 8 had anti-RV IgG, and two also had anti-RV IgM, consistent with active immunity against RV. At week 16, eight rats had anti-RV IgG and three of these also had anti-RV IgM. However, the two remaining rats in this cohort were seronegative.

Prevalence of persistent infection in the progeny of persistently infected, seropositive dams: Seropositive dams in which persistent infection had been induced during infancy were mated at 9 to 11 weeks of age with virus antibody-free males. Their progeny were tested for infection during late gestation (day 17 through 20), at birth, postpartum week 3, or after loss of maternal immunity. Multiple litters were tested at each time point. Additionally, dams were tested for infection after their litters were weaned and male breeders were tested for seroconversion.

Thirteen of 17 dams delivered litters of 6 to 14 pups. Two dams delivered small litters (four and five pups) that contained several mummified fetuses at necropsy. Two dams died from dystocia at parturition, and two remained barren. Fourteen of the dams were tested for persistent infection at necropsy. Twelve had one or more virus-positive tissues on the basis of results of ISH, and five of these had infectious virus in lung, spleen, or kidney (Table 3). One additional dam died from dystocia, but tissues were not suitable for explant culture or ISH. However, it had transmitted infection to its breeding partner, which suggests that the dam was persistently infected. The predominant sites of persistent infection in the dams, determined by ISH, were vascular smooth muscle cells, although signal also was detected in pneumocytes and renal tubular epithelium of several rats. Nine of 14 male breeders tested developed antibodies to RV (Table 3), indicating that they had been infected by their breeding partner.

Twenty-five late-term fetuses, 37 neonates, and 4 weanlings from the persistently infected dams were tested for infection by explant culture and ISH, but none yielded infectious virus or had tissues that tested positive for viral DNA (Table 3). Because RV antibody can suppress detection of virus in infected rats (27), 10 additional rats were co-housed with contact sentinels for four to six weeks after they had lost maternal immunity (postpartum weeks 14 through 16), as determined by IFA serologic testing. None of the index or sentinel rats developed antibodies to RV.

Table 2. Prevalence of infection in the progeny of dams inoculated oronasally with 10^5 $TCID_{50}$ of RV-UMass on gestation day 9

Postpartum week	No. of litters	Infection detected by[a]: Explant[b] culture	ISH[c]	Contact transmission	Total of infected progeny
3	4	6/12	9/12	–	9/12
8	7	0/10	0/10	–	0/10
9	5	–	–	7/10	7/10
15[d]	5	–	–	1/10	1/10
16	5	1/10	0/10	–	1/10

[a]No. positive/No. tested.
[b]One or more of the following tissues was virus positive: lung, spleen, kidney.
[c]Tissues examined are indicated under Materials and Methods.
[d]These 10 rats were evaluated at week 16 by explant culture and ISH as shown in the next line.

Discussion

Induction of prenatal RV infection by parenteral inoculation of dams with high doses of virus during early to mid-gestation often causes fetal pathologic changes, a preferred outcome when prenatal RV disease was being assessed as a model for human prenatal virus infection. However, more recent pathogenesis studies indicated that inoculation of dams oronasally with a high dose (10^7 $TCID_{50}$) of RV-UMass at gestation day 12 caused fetal mortality in some litters, whereas others were clinically normal at birth (15). Additionally, more than half of the pups tested (9/14) remained infected for at least three weeks after birth. This result implied that prenatal RV infection can be non-pathogenic and persistent. The experiments reported here extend this concept. Oronasal inoculation of non-immune pregnant females during mid-gestation (gestation day 9) with a dose 100-fold lower (10^5 $TCID_{50}$) than that used previously resulted in low prenatal mortality and persistent postnatal infection. Seventy-five percent (9/12) of randomly selected rats from multiple litters remained

Table 3. Prevalence of RV infection in the breeding partners and progeny of dams inoculated with 10^2 $TCID_{50}$ of RV-UMass during infancy to establish persistent infection

Dam	Age (wk) at breeding	Age (wk) at necropsy	Seroconversion in breeding Dam[a]	partner[b]	Infected progeny[c,d] Fetus	Neonate	3 wk
1	9	13	+	+[e]	Dystocia		
2		13	+			0/2	
3		18	+	+[f]		0/5	0/2
4		14	+			0/6	
5		21	-	+		0/5	0/2
6			ND	+	Barren		
7	11	13	+	-	0/5		
8		14	+	-	0/5		
9		14	+	+	0/5		
10		14	+	-	0/5		
11		14	+	-		0/5	
12		24	-	-		0/5	
13		15	+	+		0/5	
14		15	+	+		0/4	
15		17	+	+		0/5	
16			ND	+	Dystocia		
17			ND	ND	Barren		

[a]Tested for infection by explant culture and ISH.
[b]Tested for infection by serologic procedure.
[c]No. tested/No. positive. Tested for infection by explant culture and ISH, except as noted in ([d]).
[d]Ten additional progeny from litters 3, (3 rats) 5 (3 rats), and 12 (4 rats) were tested for infection by pairing them with sentinel rats for 4 to 6 weeks after they had lost maternal immunity (14 to 16 weeks of age). None of the sentinels developed anti-RV antibody.
[e]Same male paired with dams 1 and 2.
[f]Same male paired with dams 3 and 4.
ND = Not determined.

infected at postpartum week 3, and 70% (7/10) of rats tested transmitted infection to contact sentinels 9 weeks after they were born. Furthermore, infection persisted in these animals despite the presence of humoral immunity to RV. We note that the accuracy of detecting persistent infection was increased when explant culture and ISH were supplemented by tests for contact transmission. Apart from demonstrating the diagnostic value of multiple detection methods, this result supports the concept that persistent infection is maintained by small quantities of virus (14). Our findings also are consistent with the relationship between the onset of infection and immunity during RV infection in suckling rats; that is, infant rats are susceptible to persistent infection if exposure to virus precedes onset of passively acquired antiviral immunity (28). Additionally, once infection is established, virus can persist despite the onset of actively acquired antiviral immunity (14, 15). Extending this concept to prenatal infection, virus can initiate fetal infection within five days after inoculation of pregnant dams, probably as a result of viremia (10, 26), whereas humoral immunity in adult rats develops seven to 10 days after inoculation (17). Therefore, it is unlikely that newly acquired maternal immunity can prevent fetal infection or subsequent persistence of virus.

Other factors may contribute to persistent infection, but their roles are unclear. Results of pathogenesis studies in rats inoculated as infants, indicate that RV may evade immune elimination through mechanisms ranging from viral sequestration to weakened antiviral immunity caused by lymphocytotropic infection (16). Results of this study imply that immunologic tolerance to RV is not a factor, however, because some prenatally infected rats developed anti-RV IgM antibody, indicative of active immunity to virus, and no virus was detected in rats that had lost maternal immunity.

There was progressive reduction in the number of virus-positive rats, most evident after week 9, implying that prenatally infected rats can eventually eliminate infection. Indeed, neither virus nor antibody was detected in two rats at week 16. Although we cannot rule out that these rats were not infected in utero, the high prevalence of infection in rats tested at earlier time points weighs against this possibility. The factors favoring termination of infection are unknown, but on the basis of prior observations in experimentally infected infant and adult rats, may include age- related resistance due to reduction in mitotic activity among RV-susceptible target cells, constraints on virus replication modulated by humoral immunity, and initial exposure to small doses of virus (14, 15, 17, 28). Nevertheless, two seropositive rats remained infected through at least week 15, including one that transmitted virus to a contact sentinel. Thus, prenatally infected rats present some risk for prolonged transmission of infection. Although virus was transmitted by animal-to-animal contact in experiments reported here, it also could occur by fomite because RV is highly resistant to environmental inactivation (29).

Inoculation of a small virus dose (10^5 $TCID_{50}$) was a key factor for inducing asymptomatic persistent infection. Nevertheless, it still exceeded concentrations likely to be encountered by pregnant rats during natural infection. Therefore, future studies should assess inoculation of smaller doses of virus than those reported here, which may require inoculation of dams earlier in pregnancy, but after the onset of placental development, which begins at about gestation days 4 to 5 (30). Additionally, timing of inoculation should favor fetal exposure before the protective effects of maternal immunity intercede. These considerations may explain the recently reported failure to induce to prenatal infection in dams inoculated 5 to 7 days prior to mating (31).

One of our earlier studies suggested that rats born to persistently infected, seropositive dams were protected from infection by maternal immunity (24), as determined by loss of RV antibody (i.e., maternally derived immunity) to indicate absence of infection. The experiments of the study reported here also used explant culture, ISH, and contact transmission to document that the progeny of persistently infected dams had no evidence of pre- or postnatal RV infection. This outcome can be attributed to anti-RV humoral immunity, which was well-established in persistently infected females prior to mating. We speculate that maternal immunity abrogated fetal exposure to virus by preventing maternal viremia. Progeny were afforded additional protection by transplacental and colostral transfer of maternal antibody. This interpretation is consistent with the protective role attributed to humoral immunity during acute RV infection. Convalescent RV immune serum protected infant rats from infection and disease if it was administered prior to or coincident with inoculation of virus (27). Additionally, infants born to seropositive dams were protected from challenge with RV (25). Furthermore, athymic and euthymic pups nursed by RV immune dams were protected from lethal doses of RV, administered shortly after birth, whereas pups from non-immune dams had high morbidity and mortality (28, 32). Although persistently infected dams did not transmit infection to their progeny, many infected their breeding partners. This effect had been found in a prior investigation (24), but current use of ISH helps to explain the cause: persistent virus was found (at low levels) in lung and kidney, sites conducive to viral excretion.

Our findings suggest several risks presented by RV infection in rat breeding colonies. New infection among non-immune breeding rats could be spread by persistently infected offspring relocated after weaning. A similar hazard could be imparted, at least transiently, by male rats recently exposed to persistently infected females. By contrast, the results reaffirm that pre-existing maternal immunity is protective against prenatal RV infection. Therefore, infected females that are seropositive at mating appear to deliver uninfected offspring. Reproductive problems developed in 6 of 17 females inoculated as infants to produce persistently infected breeders. Two had small litters and some mummified fetuses, two developed dystocia, and two remained barren; conditions which can develop spontaneously in breeding females. Pathologic examination of these dams, which included the genital tract, did not reveal lesions attributable to parvoviral infection. Nevertheless, we cannot rule out a causal relationship between RV infection initiated during infancy and subsequent reproductive problems.

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