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Host Genetic Variation in Susceptibility to Punta Toro Virus

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SUMMARY

Infection of small laboratory animals by Punta Toro virus (PTV), family *Bunyaviridae*, genus *Phlebovirus*, is a model for the study of the human pathogen Rift Valley fever virus (RVFV). We have identified inbred mouse strains with significant differences in host response to the Adames strain of PTV. Nine inbred strains of mice representing major branches in the *Mus musculus* phylogeny were inoculated subcutaneously with a high dose of PTV in survival experiments. Two inbred strains of mice, NZW/LacJ and 129S1/SvImJ, died ~4 days after PTV infection, whereas 7 other strains survived the challenge and showed no clinical signs of disease. Histologically, 129S1/SvImJ mice showed massive hepatocellular necrosis and had additional lesions in lung, brain, and spleen, whereas NZW/LacJ mice had mild piecemeal hepatocellular necrosis. PTV viral loads in the livers of infected mice were determined by reverse transcriptase quantitative PCR. Inbred mice from strains that showed clinical signs and succumbed to PTV infection had higher liver viral loads than did mice of resistant strains. Hybrid F₁ mice were generated by crossing susceptible 129S1 and resistant FVB/N mice and tested for susceptibility. The hybrid F₁ mice showed significantly higher viral loads in the liver than the resistant parental FVB/N mice, suggesting that susceptibility is dominant. These findings will enable an unbiased genetic approach to identify host genes mediating susceptibility to PTV.

Keywords

Bunyaviridae; phlebovirus; murine

INTRODUCTION

Infections by viruses of the family *Bunyaviridae* are a serious worldwide public health and agricultural concern. The human and livestock pathogen Rift Valley fever virus (RVFV) is the best-characterized virus in the genus *Phlebovirus* of the family *Bunyaviridae* (Schmaljohn and Nichol, 2007). RVFV infections of livestock produce fever, viremia, and

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leukopenia, and in severe cases, hepatic necrosis (Bird et al., 2009; Schmaljohn and Nichol, 2007). Transmission of RVFV to humans occurs via bites from infected mosquitoes or among people tending to infected animals at birth or during abortions of infected livestock (Schmaljohn and Nichol, 2007; Woods et al., 2002). RVFV is considered a major zoonotic threat, and it is of increasing concern because of its spread outside of Africa (Bird et al., 2009). Diseases caused by phleboviruses other than RVFV are less well documented, but Punta Toro virus (PTV), first isolated from febrile patients in Panama in 1966 and 1972, and sandfly fever Sicilian and Naples viruses cause phlebotomus fever in humans similar to illness caused by RVFV (Perrone et al., 2007; Sabin, 1948; Sabin, 1951). Prevalence of neutralizing antibodies to PTV in one study in Panama was 5% in children under the age of 20 and 27–40% in adults (Tesh et al., 1974).

PTV is genetically similar to RVFV and has been studied in small rodents as a model for RVFV (Anderson et al., 1990; Fisher et al., 2003; Gowen et al., 2006; Mendenhall et al., 2009; Perrone et al., 2007; Pifat and Smith, 1987). Two strains of PTV, Adames (PTV-A) and Balliet (PTV-B), differ in their virulence in hamsters and mice (Anderson et al., 1990; Mendenhall et al., 2009). PTV-A is highly virulent, while isolates of PTV-B are of lower virulence. PTV-A infections of hamsters and mice are very similar to RVFV infections, causing tissue damage and hepatic necrosis (Peters and Anderson, 1981; Ritter et al., 2000). PTV-B is avirulent in mice unless inoculated intracerebrally (Sidwell et al., 1988).

In both human and animal infections, host genetic determinants likely contribute to phlebovirus pathogenesis, as suggested by a range of individual disease outcomes from infection with viruses that have little or no amino acid variation (Bird et al., 2009; Gerdes, 2004; Peters and Slone, 1982). Two studies on RVFV susceptibility attempted to determine the role of host genotype on infection in rats (Peters and Anderson, 1981; Ritter et al., 2000). However, the results of those reports were equivocal, possibly due to poor genetic characterization of inbred rat strains (Ritter et al., 2000). Inbred mouse strains do not vary in their susceptibility to RVFV (Peters and Anderson, 1981), but a study utilizing strains from recently-trapped wild mice demonstrated a significant natural variation in RVFV susceptibility (do Valle et al., 2010). Susceptible (wild-derived) and resistant (classic inbred) mouse strains varied in (1) quantitative viral loads, (2) time between RVFV infection and death, and (3) activation of the type I interferon pathway. Susceptibility to PTV-A has also been examined in inbred mice (Pifat and Smith, 1987). Some mouse strains had high mortality when inoculated at 4 weeks of age, and mortality was lower when inoculated at 8 weeks of age, whereas other strains of mice appeared to be resistant to infection at both ages.

In this study we identified two strains of inbred mice, 129S1/SvImJ (129S1) and NZW/LacJ (NZW), that were susceptible to PTV-A infection when inoculated at 3–5 weeks of age. The infection resulted in severe hepatic necrosis and mortality in both mouse strains. Seven other strains, A/JCr, BALB/c, FVB, DBA/2, C57BL/6, NOD/ShiLt and C3H/HeJ, were resistant and showed no clinical signs following PTV-A infection. Using reverse transcriptase quantitative PCR (RT-qPCR), we showed significant differences in viral loads in livers of susceptible mice infected with PTV-A compared to resistant mice, confirming that susceptibility can be determined by a quantitative assay. Hybrid F₁ mice generated by crossing mice of a susceptible strain (129S1) and a resistant strain (FVB) had significantly higher viral loads in the liver when compared to the resistant strain. This indicates that genetic susceptibility to PTV-A is likely to be a dominant phenotype.

MATERIALS AND METHODS

Mice and Infections

Inbred mice were obtained from Jackson Laboratory (Bar Harbor, ME) (129S1/SvImJ [129S1], FVB/NJ [FVB], NOD/ShiLt/J [NOD], NZW/LacJ [NZW]) or from the National Cancer Institute (NCI, Frederick, MD) (C57BL/6NCr [C57BL/6], 129S1/SvImJ/Cr [129S1], FVB/NCr [FVB], A/JCr, BALB/cAnNCr [BALB/c], C3H/HeJCr [C3H], DBA/2NCr [DBA/2]). Hybrid (129S1/SvImJ $\times$ FVB/NJ) F₁ mice were bred in the animal facilities at the University of Michigan. All animal experiments were performed in accordance with federal and institutional policies for humane use and care. Animals were housed in ventilated isolator cages with food and water ad libitum. Mice were infected subcutaneously (s.c.) at 3–5 weeks old with 10^{-1} to 10^6 plaque-forming units (PFU) of PTV-A strain diluted in endotoxin-free phosphate-buffered saline in a total volume of 100 μ L.

Virus

PTV-A was obtained from Dr. Robert Tesh at The University of Texas Medical Branch at Galveston. The virus was passaged once in Vero E6 (African green monkey kidney) cells at low multiplicity to generate a virus stock that was used in all experiments. Virus was titrated on Vero E6 cells by plaque assay.

Quantification of Viral Loads

Mice were infected s.c. with PTV-A at a dose of 10^2 PFU (for histopathology) or 10^6 PFU (for histopathology and survival analysis) and euthanized at days 3 or 4 after infection. For 50% lethal dose (LD₅₀) determination, 129S1 mice were infected s.c. with 10^{-1} to 10^4 PFU of PTV-A (5 mice per dose) and observed over a 15-day period. The LD₅₀ was calculated by the method of Reed and Muench (1938). Viral loads were measured by analyzing RNA levels by RT-qPCR. Total RNA was isolated from livers using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. cDNA was synthesized using random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA). qPCR was performed on an Applied Biosystems 7300 real-time PCR machine (Foster City, CA). Primers used to detect PTV-A were PTVleft682 (5' CAATACGAGGTTACATCCAAAGAA3') and PTVright722 (5'CATTGTAGGGGAAGCCAGA3') and recognize the NSs gene. PCR products were detected using a fluorescent probe, Universal Probe #48 (Roche Applied Sciences, Indianapolis, IN). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was detected with primers and fluorescent probe (Cat. no. 4352339E, Applied Biosystems). Separate reactions with GAPDH primers were prepared in parallel with PTV-A primers for each sample. Two μ L of cDNA was added to 23 μ L reaction mixtures containing 2X TaqMan universal PCR mix (Applied Biosystems) with primers and probes for PTV-A or GAPDH. All reactions were for 40 cycles of 15 s at 90°C and 60 s at 60°C. Standard curves were generated using known amounts of plasmid containing the PTV-A NSs gene, pcDNA3.1-PTVA-NSs, or the mouse GAPDH gene, mGAPDH-TOPO (construction of these plasmids is described below). The standard curves were used to convert cycle threshold values for experimental samples to copy numbers of PTV-A and GAPDH. Copy numbers of viral gene transcript were normalized to GAPDH for each sample. Each sample was assayed in triplicate. pcDNA3.1-PTVA-NSs was constructed by performing RT-PCR on PTV-A RNA using PTVBAM5 (5'ATGCGGATCCATGTCCAACATAAACTATTATG3') and PTVXHO3 (5'ATCGCTCGAGTATGTCTTGATTTAGCATTG3') primers. The PCR product was then digested with BamHI and XhoI and cloned in similarly digested pcDNA3.1+ (Invitrogen). mGAPDH-TOPO was constructed by reverse transcribing GAPDH RNA from mouse 3T12 cells using random primers and PCR amplifying using mGAPDHfull-FO2: 5'CGGAATTCGACGGCCGCATCTTCTTG-3' and mGAPDHfull-RE2:

5'CGGGATCCTTGGGGGCCGAGTTGGGATAGG3' primers. The PCR product was directly cloned into pCR2.1-TOPO (Invitrogen) per the manufacturer's instructions.

Histology

Organs (liver, lung, brain, spleen muscle, sciatic nerve) were collected, immersion fixed in 10% neutral buffered formalin, and embedded in paraffin. Five-micron sections were stained with hematoxylin and eosin, randomized, and scored blind by a board-certified veterinary pathologist.

Statistical Analysis

Data were analyzed using Microsoft Excel v. 11.5.3 (Everett, WA) and Graph Pad Prism 5 (La Jolla, CA). Survival data were analyzed using SAS v. 9.1 (Cary, NC).

RESULTS

Inbred mouse strains vary in susceptibility to PTV-A infection

To investigate PTV infection in inbred mouse strains, we chose mice from the main branches of the inbred mouse genealogy (<http://www.niehs.nih.gov/research/resources/collab/crg/dendrogram.cfm>). Susceptibility to the PTV-A strain was previously shown to depend on age of the mice in several inbred mouse strains (Pifat and Smith, 1987). To reduce variability based on age, we infected mice within a limited age range of 3–5 weeks old. We assessed morbidity and mortality after infecting groups of 5 male mice with 10^6 PFU PTV-A. Following inoculation, 129S1 and NZW mice showed severe clinical signs by 3 days post infection (dpi), including tremor and bleeding from the mouth. The 129S1, NZW, and C57BL/6 mice rapidly succumbed to infection, with 90–100% mortality by 3–5 dpi (Fig. 1). We repeated the experiment with groups of five male mice of strains shown in Fig. 1 (129S1, NZW, C57BL/6, FVB, and DBA/2 mice). Again the 129S1 and NZW mice had significant mortality, with 100% death by 5 dpi (Table 1). In contrast to the first survival experiment where 4 out of 5 C57BL/6 mice died early, all five of the replicate C57BL/6 mice survived. To clarify this discrepancy, we infected a third group of five C57BL/6 mice and all of the animals survived. The summary of the survival experiments are tabulated in Table 1. Additional strains including A/JCr, BALB/c, FVB, DBA/2, NOD and C3H that were infected with a 10^6 PFU dose did not succumb (Fig. 1, Table 1) and showed no overt clinical signs of infection. We performed a LD_{50} experiment for the 129S1 strain using 30 mice and determined that the LD_{50} was $10^{2.7}$. In the two independent survival experiments, the FVB and DBA/2 mice had 100% survival to >14 days when inoculated with 10^6 PFU PTV-A (Table 1). Thus we estimated their LD_{50} to be $>10^6$ PFU. Based on these combined data, we conclude that 129S1 and NZW mice are susceptible and FVB mice are resistant.

Viral loads in susceptible and resistant mouse strains

We chose to further evaluate 129S1 (susceptible) and FVB (resistant) mice because of the extensive characterization of these strains in the literature. For genetic mapping, a reproducible quantitative phenotypic assay is essential. In other susceptibility studies, high viral loads correlated with susceptibility (do Valle et al., 2010; Spindler et al., 2001). We developed a phenotypic assay that correlated with PTV-A susceptibility measured in the survival experiment. 129S1 and FVB mice were inoculated with 10^2 PFU PTV-A and organs were collected 3 dpi. RNA isolated from the livers of these mice was quantitated by RT-qPCR. Resistant FVB mice had reproducible and clearly distinguishable low viral loads while susceptible 129S1 mice had high viral loads (Fig. 2).

Susceptibility of hybrid F₁ mice

We crossed resistant and susceptible mouse strains to obtain hybrid (129S1/SvImJ X FVB/NJ)F₁ progeny. Mice of the parental strains and progeny F₁ mice were infected with 10² PFU PTV and analyzed for liver viral loads 3 dpi to determine the dominance of susceptibility. Hybrid F₁ mice had significantly higher viral loads ($P < 0.001$) compared to the resistant parental FVB mice (Fig. 2). There was no significant difference between F₁ males and females. The 129S1 mice also showed high viral loads in the liver that were also significantly greater than those in FVB and F₁ mice ($P < 0.001$) and $P < 0.05$, respectively). The difference in liver viral loads between the F₁ and FVB mice indicates that genetic susceptibility is likely to be dominant.

Histopathological lesions induced by PTV-A infection

The 129S1 and NZW strains were both highly susceptible to PTV-A infection. Despite showing similar mortality after infection, 129S1 mice exhibited more severe pathology than NZW mice. At a dose of 10⁶ PFU PTV, livers from 129S1 mice showed extensive areas of acute hepatocellular coagulative necrosis affecting multiple liver lobes by 3 to 4 dpi (Fig. 3). The lungs from 129S1 mice had marked pulmonary vascular congestion and scattered perivascular hemorrhages (data not shown). Perivascular microhemorrhages were also seen multifocally throughout the brain. In addition, spleens from 129S1 mice had multifocal neutrophilic splenitis with diffuse red pulp congestion. In contrast, in the NZW mice hepatocellular necrosis was minimal and was characterized by individual necrotic hepatocytes often surrounded by small numbers of inflammatory cells (Fig. 3). When susceptible 129S1 mice and resistant FVB mice were inoculated with 10² PFU PTV, the 129S1 mice had mild piecemeal hepatocellular necrosis and centrilobular hepatocyte degeneration, whereas no pathological changes were observed in FVB mice (data not shown). The severe liver damage in the 129S1 strain after PTV infection is consistent with previous reports of PTV-induced hepatic damage in mice and hamsters (Fisher et al., 2003; Mendenhall et al., 2009; Pifat and Smith, 1987).

DISCUSSION

Host factors contributing to pathogenesis of phleboviruses are not well studied because the viruses infect humans, and most must be used under BSL-3 conditions. Genetic mapping of susceptibility provides an unbiased approach to identifying host factors involved in viral pathogenesis. With the availability of mouse genomics tools, positional cloning has identified genes and loci involved in susceptibility to a number of RNA and DNA viruses (Brown et al., 2001; Lee et al., 2001; Mashimo et al., 2002; Perelygin et al., 2002; Spindler et al., 2010). Genetic mapping is facilitated when there are rapid quantitative assays for the phenotype of interest. To investigate the genetics of susceptibility of mice to the phlebovirus PTV, a human pathogen, we sought to characterize strain differences in susceptibility, identify a quantitative assay for susceptibility, and determine whether the phenotype is dominant. We chose mouse strains that are part of the mouse genome-resequencing project (www.niehs.gov/crg/cprc.htm), thereby facilitating future studies with regard to fine mapping and candidate gene analysis.

We showed that two mouse strains representing different branches of the inbred mouse phylogeny, 129S1 and NZW, were highly susceptible to PTV-A. These strains had high viral loads in the liver, high mortality, and in the case of 129S1, an LD₅₀ nearly four log units lower than the LD₅₀s of resistant strains FVB and DBA/2. We crossed susceptible and resistant strains to obtain F₁ progeny. Viral loads in the F₁ mice were significantly different from those in the resistant FVB parental strain, indicating that susceptibility is likely to be dominant.

In both susceptible mouse strains, PTV-A infection resulted in hepatic necrosis and pulmonary hemorrhage. The pathological changes in the livers of susceptible mice correlate with those reported previously for PTV infection in mice and humans (Bird et al., 2009; Perrone et al., 2007; Sabin, 1948; Schmaljohn and Nichol, 2007). Extensive liver pathology has also been reported for mice infected with RVFV (do Valle et al., 2010; Mims, 1957). Even though PTV-A caused similar mortality in 129S1 and NZW mice, and both strains showed liver and lung damage, there was more severe pathology in 129S1 mice. This suggests that genetic differences between these strains may result in specific tissue damage and disease manifestations.

In contrast to previous reports examining PTV-A infections (Mendenhall et al., 2009; Pifat and Smith, 1987), we found that C57BL/6 mice were resistant to the virus. However, we tested C57BL/6NCr (from the NCI), whereas the other investigators used C57BL/6J mice (from Jackson Laboratory). Our differing results for C57BL/6 mice are likely due to genetic variation among the substrains. We have also observed differences in susceptibility to mouse adenovirus type 1 in these two C57BL/6 substrains (L.E. Gralinski, A.R. Welton, and K.R. Spindler, unpublished).

The genetic background of both humans and animals can result in variable outcomes to viral infection. The identification of genes involved in susceptibility and resistance to viruses has increased our understanding of many viral disease processes (Brinton, 1997). The investigation of genetic loci linked with susceptibility to PTV should provide important insight into the pathogenesis of this virus and other members of the Phlebovirus genus. Straightforward genetic crosses and mapping are needed to determine whether susceptibility is polygenic, monogenic, or the result of a small number of variants, and to identify the underlying gene(s). A positional cloning approach, performing linkage analysis on backcross or intercross mice, will be the first step in such an analysis (Welton et al., 2005). Ever more powerful genotyping resources are becoming available for genotyping mouse progeny, which will facilitate rapid genetic analysis. Further analyses will indicate whether one or more host genes for susceptibility are common to a number of different bunyaviruses.

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Abbreviations

dpi	days post infection
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
LD₅₀	50% lethal dose
PFU	plaque-forming units
PTV	Punta Toro virus
PTV-A	PTV-Adames
PTV-B	PTV-Balliet
RT-qPCR	reverse transcriptase quantitative PCR
RVFV	Rift Valley fever virus

s.c. subcutaneously

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Figure 1.

Survival of PTV-infected mice. Male mice (3–5 weeks old) of the indicated strains were inoculated s.c. with 10^6 PFU PTV; $n=5$ mice for all groups. Mice were euthanized when moribund or at the conclusion of the experiment.

Figure 2.

Virus loads in susceptible and resistant mouse strains. Mice of the indicated strains were inoculated s.c. with 10² PFU PTV and euthanized 3 dpi. RT-qPCR was performed on RNAs prepared from liver homogenates. Analysis of each sample was done in separate wells for PTV primers and GAPDH primers; quantities of RNA were determined with standard curves for PTV and GAPDH; and copies of PTV RNA were normalized to GAPDH for each sample. 129S1 and FVB mice were males. F1 designates (129S1 × FVB)F₁ mice (23 males, 13 females). The means and 95% confidence intervals are shown; n, number of mice per group. Kruskal-Wallis ANOVA was performed with a Dunn's multiple comparison posttest.

*, $P < 0.05$; ***, $P < 0.001$. There was no statistical difference between F_1 males and females.

Figure 3.

Histopathological lesions induced by PTV infection. Mice were inoculated s.c. with 10^6 PFU of PTV and organs were harvested 3 dpi. Livers were stained with hematoxylin and eosin. Mock 129S1: S, normal sinusoid, H, normal hepatocyte. Infected NZW: focal hepatocellular degeneration and hemorrhage; I, infiltration of inflammatory cells. Infected 129S1: N, necrosis. Size bar is 25 μ m.

Table 1

Survival of Inbred Mice Infected with PTV^a

Strain	% Mortality	Died/Total ^b	Day of death ^b							Mean ^c
			3	4	5	6	7	-		
129S1	100	10/10	4	6						3.6
NZW	90	9/10	4	3	1	1				4.9
C57BL/6	27	4/15	1	1	2					5.3
A/JCr	20	1/5	1							
BALB/c	20	1/5						1		
FVB	0	0/10								
DBA/2	0	0/10								
NOD	0	0/5								
C3H	0	0/5								

^aMale mice were inoculated s.c. with 10⁶ PFU PTV-A in 100 μ L.

^bKaplan-Meier analysis of survival curves for 129S1, NZW, and C57BL/6 indicated that we can reject the null hypothesis that the three curves are indistinguishable from each other ($P < 0.0001$). Subsequently, data from 129S1, NZW, and C57BL/6 were pooled and compared to data for the other six strains. Fisher's exact analysis rejects the null hypothesis that 129S1, NZW, and C57BL/6 have survival the same as the remaining six strains ($P = 0.0021$).

^cMice were euthanized if moribund or at the conclusion of the experiment (15 days).

^dThe mean day of death for 129S1 mice was statistically different from NZW and C57BL/6 mice ($P < 0.01$, $P < 0.05$, respectively) (one way ANOVA, Bonferroni posttest). There was no significant difference between NZW and C57BL/6.