Pregnancy increases the risk of mortality in West Nile virus-infected mice

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West Nile fever outbreaks in the USA have caused over 700 human deaths, primarily due to neurological disease. The usual transmission route of West Nile virus (WNV) involves mosquito bites; however, alternative routes, including intrauterine infection, have also been reported. Here, the pathogenicity of WNV in mice during gestation has been investigated. An extremely high mortality rate was observed in pregnant mice (98%, 60/61) compared with non-pregnant mice (52%, 28/53; P<0.001), independent of the infecting dose or the week of pregnancy. Antibody titres were similar between pregnant and non-pregnant mice and between surviving and non-surviving animals. WNV RNA titres in brains were also similar between pregnant and non-pregnant mice. WNV RNA could be detected in placentas and fetuses. These observations suggest strongly that, in the mouse model, pregnancy increases the risk of severe WNV infection and may help to understand the pathogenic mechanisms involved in WNV infection during pregnancy.

Since it was first detected in New York in 1999 (Anderson et al., 1999; Lanciotti et al., 1999), West Nile virus (WNV), a mosquito-transmitted flavivirus, has spread all over the USA, some provinces of Canada, northern Mexico, parts of the Caribbean and Latin America, causing thousands of deaths among wild birds and horses. In humans, WNV infection is frequently unapparent or causes a relatively mild febrile condition, but it may also cause fatal encephalitis (Granwehr et al., 2004; Hayes & O’Leary, 2004). Thus far, around 20 000 human cases have been reported in the USA (http://www.cdc.gov). More than 8000 were classified as neuroinvasive, which resulted in over 700 deaths. Up to 10 000 cases were classified as West Nile fever and more than 400 had an unspecified clinical presentation. To date, no licensed human vaccine or prophylactic therapy is available (Granwehr et al., 2004; Hayes & O’Leary, 2004). Even though exposure to infected mosquitoes is the most important risk factor for acquiring WNV infection, virus transmission to humans through blood transfusion (Pealer et al., 2003), organ transplantation (Iwamoto et al., 2003) and breastfeeding (CDC, 2002a), as well as transplacental infection during pregnancy (CDC, 2002b; Hayes & O’Leary 2004), has also been reported. Data from a recent study in pregnant women shows detection of WNV in infants within a month of delivery from WNV-positive mothers and suggests congenital transmission of the virus (O’Leary et al., 2006). However, little is known about the mechanisms of transmission and it is unclear whether and how the virus causes some of the abnormalities observed in infants (O’Leary et al., 2006).

Mice are a suitable animal model for the study of WNV infection in humans as some signs in WNV-infected mice parallel those exhibited by humans with severe neuroinvasive disease, such as confusion, tremor of extremities and paralysis (Granwehr et al., 2004). Moreover, in mice, pregnancy somewhat resembles that of humans and the first, second and third weeks of pregnancy are, in many aspects, equivalent to the first, second and third trimesters of the human gestational period. In an attempt to gain insights into the incidence of WNV infection during gestation, we infected pregnant mice (at different weeks of gestation) with various doses of WNV. As controls, groups of male and non-pregnant female mice were also infected.

WNV strain NY99 flamingo 382-99 (Lanciotti et al., 1999), kindly provided by Dr H. von Briesen (Georg-Speyer-Haus, Frankfurt, Germany), was propagated and titrated on Vero cells (Tardei et al., 2000). Eight- to ten-week-old BALB/c (H-2d) mice were infected by intraperitoneal (i.p.) injection with different doses of virus (105–108 p.f.u. per mouse) in 200 μl Dulbecco’s modified Eagle’s medium (DMEM) containing 5% fetal bovine serum (FBS). Non-infected contact-control cage-mate mice were inoculated with DMEM containing 5% FBS. Virus manipulation and mouse experimentation were carried out in our Biosafety
Level 3 (BSL-3) containment facilities and were approved by and performed according to the guidelines for animal experimentation of the Animal Safety Committee of CISA (Madrid, Spain). In some instances, mice were bred in house and pregnancy was checked by the presence of vaginal plugs.

Mice were monitored daily for signs of illness. Typical clinical signs of WN disease were observed among infected mice, and animals that died of the disease presented ruffling, hunchback posture and hindlimb weakness and paralysis 24–48 h prior to death. In contrast, none of the non-infected contact-control cage mates developed disease signs. Mortality rates and mean survival time (MST) values were recorded and statistical comparisons between groups were made by using $\chi^2$ or Fischer's tests for categorical variables and the Mann–Whitney test (unpaired samples) for quantitative variables. Values of $P<0.05$ were considered significant. At indicated time points, animals were anaesthetized with halothane before bleeding or euthanasia. Collection of tissue and blood was performed under sterile conditions as reported by Julander et al. (2005).

Mortality rates and MST values recorded among non-pregnant mice dying of WNV disease (Table 1) were not statistically significantly different from those described by Diamond et al. (2003). However, at the lower doses, mortality rates were slightly higher than those reported in a further study (Wang et al., 2003). The slightly higher mortality rates reported here could be due to differences either in virus strain (NY99 or Sarafend), mouse strain (BALB/c or C57BL76J), administration route (i.p., intravenous or subcutaneous) or a combination thereof. In fact, the NY99 strain, which belongs to WNV lineage I, is more virulent than some strains isolated in other continents (Beasley et al., 2002). Actually, the MST recorded in the present study was similar between the different groups of non-pregnant mice and, in general, lower than those described for the WNV lineage II Sarafend strain (Wang et al., 2003).

Humoral and cellular response against viral proteins contributes to protection and recovery from WN disease (Granwehr et al., 2004). Serological analysis by ELISA (Ebel et al., 2002) using heat-inactivated WNV as antigen (Blitvich et al., 2003) showed that specific IgM and IgG antibodies were elicited in all infected mice but, at a given infecting dose, no significant differences in antibody titres were observed between mice that died of WN disease and those that survived (Fig. 1). IgG antibodies were detectable in the latter for up to 4 months post-infection (p.i.), and these animals were protected against challenge with a lethal dose of $10^8$ p.f.u. per mouse inoculated within 21–95 days p.i.

On the other hand, none of the non-infected contact-control cage mates developed specific antibodies. Thus, contrary to what has been described in birds and alligators in laboratory settings (Komar et al., 2003; Klenk et al., 2004), no horizontal transmission of WNV was observed in mice.

Mortality rates in pregnant mice were extremely high (98 %, 60/61) compared with those in non-pregnant animals (52 %, 28/53; $P<0.001$; Table 1), independent of the infecting dose administered (92 vs 55 % at a dose of $10^2$ p.f.u. per mouse, $P=0.038$; 100 vs 58 % at $10^3$ p.f.u. per mouse, $P=0.023$; and 100 vs 48 % at $10^4$ p.f.u. per mouse, $P<0.001$) or the week of pregnancy (first, second or third) at which they were infected (data not shown). In contrast, MST values, whilst slightly higher in non-pregnant mice dying of WN disease, were not statistically significantly different (Table 1).

Our results also showed that, at any given infecting dose, specific IgM and IgG titres elicited in pregnant and non-pregnant mice were similar (Fig. 1). Therefore, it seems unlikely that the high mortality found here in pregnant mice

### Table 1. Mortality rates and survival time recorded in WNV-infected mice

<table>
<thead>
<tr>
<th>Dose (p.f.u. per mouse)</th>
<th>Non-pregnant</th>
<th>Pregnant</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Percentage of dead mice</td>
<td>MST* (days)</td>
</tr>
<tr>
<td></td>
<td>(no. dead/total)</td>
<td></td>
</tr>
<tr>
<td>$10^2$</td>
<td>55.5 (10/18)</td>
<td>9.4±2.93</td>
</tr>
<tr>
<td>$10^3$</td>
<td>58.3 (7/12)</td>
<td>10.2±1.66</td>
</tr>
<tr>
<td>$10^4$</td>
<td>47.8 (11/23)</td>
<td>9.7±1.71</td>
</tr>
<tr>
<td>$10^5$</td>
<td>50.0 (7/14)</td>
<td>9.1±1.45</td>
</tr>
<tr>
<td>$10^6$</td>
<td>66.6 (8/12)</td>
<td>9.2±0.78</td>
</tr>
<tr>
<td>$10^7$</td>
<td>83.3 (5/6)</td>
<td>8.6±0.49</td>
</tr>
<tr>
<td>$10^8$</td>
<td>100.0 (23/23)</td>
<td>8.2±1.29</td>
</tr>
</tbody>
</table>

*Values represent mean survival time (MST)±SD of mice that died up to 15 days post-WNV infection.

†$P=0.038$.

‡$P=0.023$.

§$P<0.001$. 

http://vir.sgmjournals.org
Fig. 1. Scatter plot of the levels of specific IgG (squares) and IgM (circles) antibodies at day 7 after infection with $10^4$ p.f.u. WNV per mouse. Data correspond to pregnant (empty symbols) and non-pregnant (shaded symbols) mice that died of WN disease, and to surviving mice (filled symbols). Titres were determined by ELISA and are expressed as positive/negative (P/N) values of each sample, calculated by dividing the mean absorbance of positive antigen-containing wells by the absorbance of the negative antigen-containing wells (Ebel et al., 2002). The dotted line represents the positive cut-off P/N value below which results are considered negative. Thin and thick solid lines represent the mean titre corresponding to animals that survived or died of WN disease, respectively. Thin and thick dashed lines show mean titres of pregnant and non-pregnant mice, respectively. No significant differences were observed between the different groups analysed.

Fig. 2. Scatter plot of the levels of WNV RNA in the brains of pregnant (empty symbols) and non-pregnant (shaded symbols) mice infected during the first (triangles), second (squares) or third (circles) week of gestation, and non-pregnant mice (filled symbols). Brains were harvested at the time of death (8–10 days p.i.), homogenized and subjected to real-time RT-PCR (Lanciotti et al., 2000) using a positive-control sample of known titre. Values are given as genomic equivalents (g tissue)$^{-1}$. The dotted line indicates the limit of sensitivity of the assay.

Murray Valley encephalitis virus (MVEV) (Aaskov et al., 1981), but the proportion of dead animals did not reach that observed here. In WNV infection, a recent analysis of the effect of reactive immunoglobulin in fetal virus infection has shown high mortality in a limited number of untreated dams (Julander et al., 2005). Congenital infection of mice with SLEV 8 days post-coitus (p.c.) resulted in infection of both the placenta and the fetus (Aaskov et al., 1981). Likewise, JEV (Andersen & Hanson, 1975) and WNV (Julander et al., 2005) infect mouse fetuses more efficiently during the first week of pregnancy than thereafter, suggesting that fetal infection may differ at different stages of placental development. Furthermore, WNV titres in the placenta were higher and were detectable earlier after infection than in other maternal organs (Julander et al., 2005). Consistent with these observations, no WNV RNA was detected in the brains, placenta or fetuses of a few dams infected during the second or third week of gestation that were euthanized 2–4 days p.i. In contrast, WNV RNA was detected in the placentas (between $1.1 \times 10^5$ and $1.6 \times 10^5$ genomic equivalents g$^{-1}$) and the fetuses (between $8.9 \times 10^4$ and $2.7 \times 10^5$ genomic equivalents g$^{-1}$) of two mice infected during the first week of pregnancy (6 days p.c.), euthanized after 4 and 5 days respectively. Lack of virus detection in the brain at this early time point is not surprising as, at the dose administered ($10^8$ p.f.u. per mouse), it is too early in the infection process for invasion of the brain. Replication of WNV in the placenta might increase viral load in pregnant mice early after infection, even before it could be detected in the brains of the dams, favouring a high mortality rate.
Virus infection during pregnancy could have serious consequences for fetuses and newborns (Koi et al., 2001). Intrauterine fetal infection with several flaviviruses is often associated with fetal mortality, abortion, preterm delivery of stillborns and death of newborns at or shortly after birth, but most babies showed an apparently normal life (Aaskov et al., 1981; Andersen & Hanson, 1975; Julander et al., 2005; Mathur et al., 1981). In the present report, all animals infected during the first week of pregnancy died before delivery. On the other hand, five of the 14 mice infected during the second week of gestation survived to deliver pups, but only one dam, infected with the lowest dose (10^2 p.f.u.), survived to the end of the experimental period (90 days). This animal was actively infected, because specific antibodies were present in its serum and it was protected against challenge with a lethal dose of WNV administered 2 months after the initial infection. No overt signs of WN disease were observed during the follow-up of infants born 3 days p.i. to this surviving dam, and they showed specific IgG 1 month after birth. In fact, they were protected against challenge with a lethal dose of WNV inoculated 60 days after infection of their mother.

Maternal infection by WNV during pregnancy has been reported in humans (CDC, 2002b; Hayes & O'Leary, 2004). Infection of the placenta and intrauterine transmission of WNV to the fetus were first documented in a woman with signs of WN disease, who was later diagnosed with meningocencephalitis (CDC, 2002b; Hayes & O'Leary, 2004). Her infant was born at term with choriorretinitis and severe cerebral abnormalities, although such abnormalities could not be associated conclusively with the virus infection (Alpert et al., 2003). A case of a premature delivery has also been documented in a WNV-infected woman and, although her infant presented with neonatal respiratory distress, no tests for WNV were performed (Hayes & O'Leary, 2004). In another three pregnancies complicated by WNV infection, no apparent abnormalities have been observed in the newborns (Hayes & O'Leary, 2004). Lately, it has been reported that none of 71 WN-infected pregnant women included in a retrospective study died of WN disease and that most of their children were born healthy (O'Leary et al., 2006). In this study, three cases of infant malformation were observed, suggesting the possibility of congenital infection with WNV. In any case, and because the mechanisms of non-mosquito-borne transmission and the effects and abnormalities seen in the infants remain largely unknown, assessment of the fetus or child is recommended when mothers are infected by WNV (CDC, 2004). Even more, the CDC and the state health departments of the USA are currently collecting clinical and laboratory data on outcomes of pregnancies of WNV-infected women, and clinicians are encouraged to report known or suspected cases (O'Leary et al., 2006). Nevertheless, all of these observations indicate that, in contrast to the elevated mortality found here in WNV-infected pregnant mice, no increased mortality is observed in pregnant women (O'Leary et al., 2006). Susceptibility to WNV infection in inbred mice has been linked to the presence of point mutations in the 2'-5'-oligoadenylate synthetase gene (Mashimo et al., 2002), of which regulation by interferon can be affected during pregnancy and, thus, these mutations may account, to some extent, for the differences observed between humans and mice.

In summary, and although care should be taken before extrapolating our data to WNV-infected women, the high risk of severe WN disease observed in pregnant mice deserves further investigations, which should help to understand better the pathogenic mechanisms implicated in WNV infection during pregnancy in mice.

**Acknowledgements**

We are indebted to A. Canals and E. Domingo for making possible for us the use of the BSL-3 facilities and for their continuous support. The work was supported in part by a grant (AGL2004-06071) from the Spanish Ministerio de Educación y Ciencia (MEC) to J.-C. S. and by the Research Foundation, University of Connecticut, for support through a Faculty Small Research Grant to A.G. L. C. has been supported by a scholarship from INIA and EER by the ‘Juan de la Cierva’ programme (MEC).

**References**


