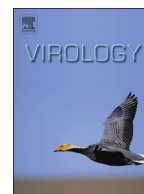Contents lists available at ScienceDirect

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Limited susceptibility of mice to Usutu virus (USUV) infection and induction of flavivirus cross-protective immunity

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ABSTRACT

Flaviviruses are RNA viruses that constitute a worrisome threat to global human and animal health. In Europe, West Nile virus (WNV) outbreaks have dramatically increased in number and severity in recent years, with dozens of human and horse deaths and a high avian mortality across the continent. Besides WNV, the only clinically relevant mosquito-borne flavivirus detected so far in Europe has been the Usutu virus (USUV), which after being reported for the first time in Austria in 2001, quickly spread across Europe, causing a considerable number of bird deaths and neurological disorders in a few immuno-compromised patients. Even though USUV infects multiple avian species that develop antibodies, there is little information about USUV susceptibility, pathogenicity and cross-reactive immunity. Here, the susceptibility of suckling and adult mice to USUV infection and the induction of cross-protective immunity against WNV challenge have been addressed.

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Introduction

Flaviviruses constitute a group of arboviruses that, in many cases, represent a worrisome threat to global human and animal health. For instance, West Nile virus (WNV) outbreaks have recently increased in number, frequency, and severity in Europe and the Mediterranean Basin causing a considerable number of cases of neuroinvasive disease in animals and humans, with dozens of human and horse deaths across the continent (Gray and Webb, 2014; Martin-Acebes and Saiz, 2012).

Besides WNV, the only clinically relevant mosquito-borne flavivirus detected so far in Europe has been the Usutu virus (USUV) that was described for the first time in Austria in 2001 (Weissenbock et al., 2002), although it was probably present since 1996 or even earlier (Weissenbock et al., 2013). The virus has quickly spread across the continent (Pauli et al., 2014), causing a considerable number of bird deaths. USUV circulation has already been detected in mosquitoes (Rizzo et al., 2014), birds (Garigliany et al., 2014), bats (Cadar et al., 2014), and equids (Lupulovic et al., 2011).

Even more, although only two USUV human cases had been described in Africa during the 1980s (Nikolay et al., 2011), nowadays, USUV antibodies have been detected in blood donors in Germany (Allering et al., 2012), in Italy (Gaibani et al., 2012), where it has been associated with neurological disorders in two immunosupressed patients (Cavrini et al., 2009; Pecorari et al., 2009), and in clinically WNV suspected patients from Croatia (Vilibic-Cavlek et al., 2014), where the cases of first human have been recently reported (Santini et al., 2014). Thus, as WNV, USUV seems to have also become a European resident pathogen.

Nowadays, there is limited information regarding the susceptibility and pathogenicity of USUV in naturally or experimentally infected animals. Only one study has reported that USUV infection of mice younger than one week of age causes the death of the animals, which present some neuronal disorders (Weissenbock et al., 2004), and very recently, addressing the protective capability of WNV vaccine candidates, no mortality due to USUV infection was observed in a limited number of adult mice (Merino-Ramos et al., 2014). In the case of birds, seroepidemiological studies have shown that multitude of species can be infected and develop antibodies against the virus, without showing signs of the disease (Pauli et al., 2014), and that the virus presents limited pathogenicity for domestic geese (*Anser anser f. domestica*) or chickens (*Gallus domesticus*) (Chvala et al., 2005; Chvala et al., 2006).

To date, and although human vaccines are available for different flaviviruses, including Tick-borne encephalitis virus (TBEV), Yellow fever virus (YFV) and Japanese encephalitis virus (JEV), no human

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licensed vaccine is available for many others, such as DENV, WNV, or USUV (Beasley, 2011; Dauphin and Zientara, 2007; Martin-Acebes and Saiz, 2012; Thomas and Endy, 2011). Infection with a flavivirus of the JE serocomplex, as USUV and WNV, may elicit cross-reactive antibodies that can protect against other flavivirus infections (Lobigs and Diamond, 2012). However, cross-protection after vaccination with one flavivirus against other members of the family can be limited (Lobigs and Diamond, 2012). Thus, it is probable that vaccination against different related flaviviruses will not be as efficient in inducing protection (Heinz and Stiasny, 2012). Even more, cross-reactive immunity has also been associated with enhanced infection and disease outcome in DENV, mainly due to antibody dependent enhancement, ADE (Lobigs and Diamond, 2012). Experimental studies have shown that ADE after WNV infections can be induced in vitro (Beck et al., 2013), although no evidences of this effect have been documented in vivo for members of the JE serocomplex (Lobigs and Diamond, 2012). In any case, data available about cross-reactivity between USUV and WNV and its possible consequences are scarce (De Madrid and Porterfield, 1974; Rushton et al., 2013).

In the present report, we have assessed the susceptibility of suckling and adult mice to USUV infection and the induction of cross-protective immunity against WNV challenge.

Results

Mortality rates

Adult mice (8 weeks old) were i.p. infected with different doses (10^2 or 10^4 pfu/mouse) of USUV or WNV. All adult mice survived to USUV infection, independently of the infecting dose inoculated, contrary to what was observed upon WNV infection, where 16.6% and 8.3% surviving rates were recorded, respectively (Fig. 1A). Surviving mice to primary infection with either virus were challenged with a high dose (10^7 pfu) of WNV. Only six (25%) of the mice that survived to USUV infection with the lower dose (10^2 pfu) died between 9 and 11 days after challenge with WNV,
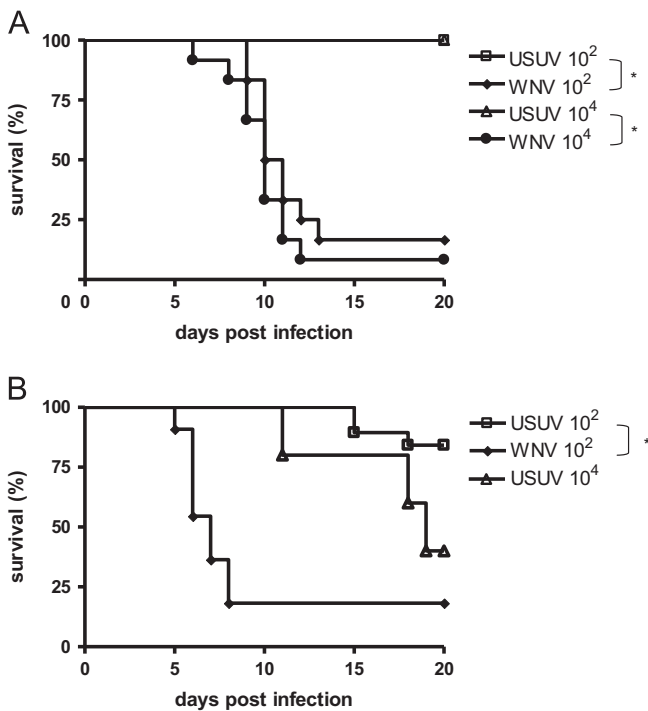
Fig. 1. Survival rates (days post infection) in mice infected with 10^2 or 10^4 PFU/mouse of either USUV or WNV. (A) Adult mice; (B) suckling mice. Statistically significant differences are indicated with asterisk ($P < 0.05$).

Table 1
Percentage and antibody titers* of positive sera at different time after primary infection from adult mice infected with USUV and challenged with WNV.

Infecting virus (pfu/mouse)	Days post USUV infection						Challenging virus (pfu/mouse)	Days post USUV infection (days post WNV NY-99 challenge)					
	Antigen							Antigen					
	d+4		d+7		d+15			d+22 (d+4)		d+25 (d+7)		d+35 (d+17)	
	USUV	WNV	USUV	WNV	USUV	WNV		USUV	WNV	USUV	WNV	USUV	WNV
PBS	0%	0%	0%	0%	0%	0%	WNV (10^7)	0%	0%	66.67% (3.3 ± 0.5)	100% (4.5 ± 0.8)	100% (5.19 ± 1.91)	100% (5.65 ± 1.66)
USUV (10^2)	0%	0%	0%	0%	55.6% (3.28 ± 0.5)	0%	WNV (10^7)	100% (5.25 ± 0.81)	100% (4.37 ± 1.16)	100% (4.63 ± 2.1)	100% (4.25 ± 1.93)	100% (8.97 ± 2.82)	100% (5.52 ± 2.41)
USUV (10^4)	0%	0%	0%	0%	66.65% (3.91 ± 1.34)	0%	WNV (10^7)	100% (8.07 ± 1.83)	100% (3.40 ± 1.31)	100% (8.13 ± 1.64)	100% (3.87 ± 1.23)	100% (9.86 ± 1.40)	100% (5.16 ± 2.45)

Three mice per group were tested at days +4, +7, +22 and +25 post primary USUV infection; and 18 and 10 mice per group at days +15 and +35, respectively.
* Titers are expressed as the P/N average ± S.D.

while the remaining surviving animals, either to USUV (42/48) or WNV infection (3/3), were protected.

Contrary to that observed in adult mice, suckling animals infected with either 10^2 or 10^4 pfu of USUV survived to the infection in a dose dependent manner, 84.2% and 40%, respectively (Fig. 1B), rates that are much higher than that recorded (18%) among animals infected with a low dose (10^2 pfu) of WNV. All suckling mice that survived to USUV infection were protected against challenge with WNV.

Antibody induction

Over 50% of the adult mice inoculated with USUV presented specific anti-USUV IgG by day 15 p.i. (Table 1). No cross-reactive antibodies against WNV were detected up to 15 days post initial USUV infection. In contrast, 66.67% of the PBS inoculated mice infected with WNV elicited USUV cross-reactive antibodies from 7 d.p.i., and by day 15 all sera were cross-reactive (Table 1). All USUV infected mice elicited detectable IgG antibodies against both antigens as early as 4 days after WNV challenge. Overall, P/N ratios were infecting-dose dependent and increased after challenge (Table 1). In suckling mice, anti-USUV IgG antibodies were only detected from 15 d.p.i. (data not shown), similarly to what has been previously observed in WNV infected suckling mice (Rodriguez-Pulido et al., 2012).

Neutralization capability of seropositive samples from adult mice was tested against USUV and WNV by PRNT. After primary infection with USUV, no neutralizing antibodies were detected against any of the two viruses. However, after WNV challenge, all tested mice infected with USUV, either with 10^2 pfu/mice ($n=6$) or 10^4 pfu/mice ($n=7$), neutralized both viruses with average $PRNT_{90}$ titers of 130 (± 60) and 215 (± 104) for USUV and WNV, respectively. On the other hand, none of the 8 mice infected only with WNV presented neutralizing antibodies against USUV, but they showed the highest $PRNT_{90}$ titers against WNV (average 325 ± 155).

Viral genomic detection

Although the number of analyzed mice by time point and dose was limited, no positive samples (0/25) for USUV-RNA were detected in euthanized adult mice at any tested time (4 to 35 days post USUV infection). In contrast, WNV-RNA was amplified in 50% (3/6) of those USUV infected adult mice euthanized 7 days post-WNV challenge. Among those euthanized mice infected only with WNV tested, 25% (1/4) and 66% (2/3) were WNV-RNA positive at 4 and 7 days post infection, respectively, with titers ranging from 10^3 to 10^7 pfu/gram of tissue (data not shown).

On the other hand, the 2 suckling mice tested 7 days post USUV infection resulted USUV-RNA positive, but no positivity was recorded in any of the 6 mice tested earlier (4 days) or later (15 days) after infection.

Discussion

In Europe, besides WNV, the only other flavivirus circulating is the USUV, which has been responsible for a noteworthy number of bird deaths (Pauli et al., 2014), and a few human cases of neurological disorders (Cavrini et al., 2009; Pecorari et al., 2009). However, data about USUV pathogenesis are very limited. Availability of an animal model is important not only to assess viral pathogenicity, but also flaviviral antigenic cross-reactivity, as protection against antigenically related viruses has been previously reported (Beck et al., 2013); however, this is not always the case, since protection after vaccination against one flavivirus not always imply protection against infection with another flavivirus (Lobigs and Diamond, 2012). In search for a possible animal

model, we have analyzed the susceptibility of adult and suckling mice to USUV infection, and tested whether USUV infection elicited cross-protection against WNV infection.

Previous description of USUV pathogenesis reported a high mortality in suckling mice (Weissenbock et al., 2004). Here, a dose dependent mortality was observed, as 84% and 40% of the suckling mice survived to the infection with 10^2 and 10^4 pfu, respectively. Differences in mortality rates between both studies could be due to the different mice and virus strains, and doses used.

In contrast, as very recently described in a limited number of animals (Merino-Ramos et al., 2014), no mortality was recorded in adult mice at any of the doses tested. Both suckling and adult mice similarly infected with WNV showed significantly higher mortality rates; thus confirming the limited pathogenicity of USUV in mice.

Antibody mediated immunity is considered a major player on protection against flavivirus infections (Vaughan, Roghanian, and Cragg, 2010). In our study, USUV infected mice, either suckling or adults, showed detectable IgG against the virus only from 15 d.p.i. Even more, while none of the USUV-IgG positive samples neutralized USUV, all WNV-infected mice presented high neutralizing titers against WNV by day 17 p.i. that did not cross-neutralized USUV.

Differences between USUV infected suckling and adult mice were further observed by testing the presence of viral RNA in the brains of a limited number of animals. No USUV-RNA could be amplified from adult mice at any tested time after infection (4 to 35 days), but positive amplification was recorded in suckling mice tested 7 d.p.i., indicating that USUV reaches suckling mice brains.

Cross-reactive antibodies can induce cross-protection against infection with related flaviviruses in some instances (Fang and Reisen, 2006; Goverdhan et al., 1992; Nemeth, Bosco-Lauth, and Bowen, 2009; Price and Thind, 1972; Tarr and Hammon, 1974; Tesh et al., 2002), but this is not always the case (Thomas et al., 2006). In this study, no cross-reactivity against WNV was observed in USUV-infected adult mice at any time post infection, while all sera from WNV infected animals cross-reacted with USUV by day 17 post-infection, confirming the different antibody response elicited against the two viruses. Even more, after WNV challenge, mice sera neutralized both viruses, although titers were higher against WNV. Heterologous virus challenge of pre-infected animals uses to boost neutralizing antibodies against the immunizing virus to a higher level than to the challenging one, but, when major differences in virulence between the two viruses exist, the immune response against the more virulent tends to be of greater magnitude, consistent with the greater antigenic load produced (Lobigs and Diamond, 2012), as it seems to be the case in this study.

Almost all USUV-infected mice were protected against challenge with a high dose of a WNV neuroinvasive strain. The only exceptions were six adult mice infected with the lower dose of USUV. On the other hand, WNV-RNA was amplified 7 days post WNV-challenge from 50% of the tested USUV pre-infected adult mice, numbers similar (66%) to that recorded in animals infected only with WNV, thus, indicating that USUV infection protects mice against WNV disease and death, but not against infection.

The results here presented demonstrate a different susceptibility of adult and suckling mice to the infection with the flavivirus USUV. The cross-protective immunity elicited by USUV-infected mice against challenge with the heterologous neurovirulent WNV highlights its potential as a low pathogenic flaviviral model, which may also help to develop cross-protective flavivirus vaccine candidates.

Materials and methods

Viruses

The USUV SAAR 1776 (GenBank acc. no. AY453412.1, (Bakonyi et al., 2004)) and a WNV NY99 strains (Gen Bank acc. no. KC407666,

(Cordoba et al., 2007; Martin-Acebes and Saiz, 2011)) were propagated and titrated on Vero cells as described (Martin-Acebes et al., 2011).

Mice

Groups ($n=12$) of 8 weeks-old Swiss female mice were intraperitoneally (i.p.) inoculated with either 10^2 or 10^4 plaque forming unit (pfu)/mouse of each flavivirus (USUV and WNV) in 100 µl of PBS. As control, a group of mice was inoculated with PBS alone. Surviving mice were i.p. challenged with a high dose (10^7 pfu/mouse) of neurovirulent WNV NY-99 strain eighteen days after primary infection. USUV-infected animals were bled prior to infection, 4, 7 and 15 days post primary infection (d.p.i.), and 4, 7 and 17 days post challenge with WNV (corresponding to 22, 25, 35 d.p.i.). On days 4 and 7 post USUV infection and 4, 7 and 17 post WNV challenge, 1–4 mice/group were anesthetized and euthanized to collect blood and brains. Viral infections and samples collection was conducted as described (Alonso-Padilla et al., 2011; Blazquez and Saiz, 2010; Cordoba et al., 2007).

Additionally, litters ($n=5$ to 19) of Swiss suckling mice (4–7 days-old) were i.p. infected with similar doses of USUV (10^2 and 10^4 pfu/mouse). Another group was i.p. infected with 10^2 pfu/mouse of WNV, and a control group was sham-infected with PBS alone. A similar scheme of bleeding and tissue collection as that of adult mice was followed, including the challenge with WNV.

During the experiments all animals were monitored daily and received water and food *ad libitum*. Those mice showing signs of disease were anesthetized and euthanized, as were all surviving animals at the end of the experiment (35 days after initial USUV infection). All mice were handled in strict accordance with the guidelines of the European Community 86/609/CEE. The protocols were approved by the Committee on Ethics of animal experimentation of our Institution (INIA's permit numbers 2011–35 and 2012–05). All experiments with infectious viruses were conducted in the biosafety level 3 facilities of our institution.

Immunological assays

Heat-inactivated sera (1:100 dilution) were assayed for anti-WNV IgG antibodies by enzyme-linked immunosorbent assay (ELISA) using as antigens heat-inactivated viruses (WNV or USUV) produced in Vero cells (Alonso-Padilla et al., 2009; Cordoba et al., 2007). The positive cut off value was assigned using a positive/negative (P/N) ratio ≥ 2, calculated by dividing the mean absorbance of the test serum by the absorbance of the negative control serum (Escribano-Romero et al., 2013).

Plaque reduction neutralization tests (PRNT) were conducted on Vero cells with WNV NY-99, or USUV SAAR 1776, using 2-fold serial sera dilutions (Alonso-Padilla et al., 2009; Petrovic et al., 2013). Titers were calculated as the reciprocal of the serum dilution, diluted at least 1:40, which reduced plaque formation $\geq 90\%$ (PRNT$_{90}$), relative to samples incubated with negative control pooled sera.

Virological assays

Viral RNA was extracted from the processed brains using a NucleoSpin viral RNA isolation kit (Macherey-Nagel, GmbH & Co., Düren, Germany). WNV-RNA was quantified by real time qRT-PCR as described (Cordoba et al., 2007; Lanciotti et al., 2000). Quantification was calculated by generating a standard curve with previously titrated WNV (10^6–10^{-1} pfu/reaction) and samples were considered negative when Ct ≥ 35, equivalent to 10^2 pfu/gram of tissue (Blazquez and Saiz, 2010; Cordoba et al., 2007).

For quantitative analysis of USUV-RNA, a specific qRT-PCR was developed, using primers forward 5′-GCGCCACTTCGCCAAGGAGT-3′ and reverse 5′-CGTCTGTTCGCTTGGGCCGT-3′, and 5′FAM-ACTGGGTTA-CCAAAGCCGAAAGGCC-3′TAMRA as probe, corresponding to positions 10718–10737, 10808–10789 and positions 10761–10785, respectively, according to the USUV strain SAAR 1776 (GenBank acc. no. AY453412. 1). The probe and the primers, which amplified a 91 bp fragment of the 3′–NC region, were designed upon alignment of 6 USUV and 6 WNV sequences available at GenBank (nucleotide accession numbers: AY453411.1, KF573410.1, AY453412.1, HM569263.1, EF206350.1, KC754954.1, AF404757.1, AF260968.1, KC407673, AY532665.1, KC407666, GQ851606.1) using the ClustalW (version 2.0.12, http://www.ebi.ac.uk/tools/clustalw2). The specificity of the primers and probe was tested by BLAST analysis (http://www.ncbi.nlm.nih.gov/blast); (Altschul et al., 1990). Real-time qRT-PCR amplification was carried out in one step using High Scriptools-Quantimix Easy Probes kit (Biotools, Madrid, Spain), according to the manufacturer's protocol. Reverse transcription was carried out for 30 min at 48 °C, followed by an incubation step for 10 min at 95 °C and 40 amplification cycles (15 s at 95 °C, 1 min at 60 °C). Quantification was determined with the construction of a plasmid with the prototype strain SAAR 1776 cloned into PCRII-Topo® (LifeTechnologies, Carlsbad, CA) using the primers described above. Plasmid DNA was purified using Purelink® HiPure Plasmid Filter maxiprep kit (LifeTechnologies), and *in vitro* transcription was performed with the mMESSAGE mMACHINE® T7 kit (Ambion, Austin, Tx), followed by a precipitation with phenol-chloroform. Standard curves and validation of number of copies obtained by transcription were generated by comparing 10-fold serial dilutions of the purified transcript with previously titrated USUV (10–10^6 pfu/reaction), and samples were considered negative when Ct ≥ 35, equivalent to 10^3 pfu/gram of tissue. The specificity of the real-time qRT-PCRs was assayed by testing dilutions of different WNV strains of lineage 1 and 2, and USUV of known titers.

Statistical analyses

Kaplan-Meier survival data were analyzed by a logrank test using GraphPad PRISM v.2.01 (GraphPad Software). Asterisks in the figure denote statistically significant differences ($P < 0.05$).

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