Increasing West Nile virus antibody titres in central European plasma donors from 2006 to 2010

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We analysed by neutralisation assay 55 intravenous immunoglobulin preparations produced from human plasma collected in three central European countries, specifically Austria, Germany and the Czech Republic, from 2006 to 2010. The preparations from 2009 and 2010 contained increasing titres of neutralising antibodies against West Nile virus (WNV) in the absence of reported human WNV cases in these countries.

Introduction

Clinical cases of West Nile virus (WNV) infection in the European Union (EU) have so far been limited to sporadic outbreaks (Table), while serological studies in sentinel horses and birds, as well as humans, have shown WNV circulation for decades, particularly in the southern EU Member States [1]. However, as exemplified by the WNV outbreak in Greece in summer 2010, the pathogenicity of this virus for humans can change. A study in 2007 detected 1% (4 of 392) WNV seropositivity in human serum samples collected from asymptomatic individuals living in Greece [2], whereas in 2010, WNV caused the second largest outbreak of human infections recorded in the EU since the outbreak in Romania in 1996 [3].

In order to assess how many people in central European countries may have been exposed to the virus, including individuals with subclinical disease, we analysed the WNV neutralising antibody titre of intravenous immunoglobulins (IVIG), a blood product containing pooled immunoglobulins from the plasma of more than a thousand donors from Austria, Germany and the Czech Republic. The suitability of this approach for assessing the cumulative number of WNV infections in a large donor population has recently been demonstrated through the use of IVIG to follow the spread of WNV in the United States [35].

Methods and results

Fully validated neutralisation assays were used to determine WNV and tick-borne encephalitis virus (TBEV) neutralisation titres (NT₅₀) in 55 individual lots of IVIG manufactured from plasma pools (≥1,000 donors) collected in three EU countries (KIOVIG, Baxter), Austria

(ca. 40-45%), Germany (ca. 40-45%) and to a lesser extent the Czech Republic (ca. 15%). The IVIG lots tested were produced in the years 2006 (n=10), 2007 (n=9), 2009 (n=20) and 2010 (n=16). For the year 2008, IVIG lots produced exclusively from EU plasma were not available for testing. The NT₅₀ were determined, at least in duplicate, as described [35], using WNV strain 385-99, lineage 1 or TBEV strain Neudoerfl. When tested against WNV, IVIG samples were used undiluted and titrated on Vero cells (ECACC 84113001); against TBEV, samples were initially diluted 1:5 and titrated on A549 (ATCC CCL-185) cells. After seven days, each well was assessed for virus-induced cytopathic effect, and the NT₅₀, i.e. the reciprocal dilution resulting in 50% virus neutralisation, is reported as mean±standard deviation of two or more replicates (Figure 1B). Plotting the WNV neutralisation titres of the respective IVIG lots against the year of product release, a statistically significant increase over the years 2006 to 2010 was observed (p=0.0269 one way ANOVA and p=0.004, post test for linear trend; Figure 1A).

To specifically discriminate antibodies induced by WNV infections of EU plasma donors from possibly cross-reactive antibodies against TBEV, a Flavivirus related to WNV and widely endemic in central Europe, the TBEV neutralisation capacity of the IVIG lots was also determined, after the first lots with higher WNV activity had been detected. This was done for 30 of the 55 IVIG lots (Figure 1B). We assessed a potential contribution of TBEV neutralising antibody titres to the measured WNV antibody titres by correlation analysis and found a significant correlation (r²=0.5, p=0.002) between the TBEV and WNV neutralisation titres for 19 of 30 IVIG lots analysed (Figure 1B, open circles). The apparent WNV neutralisation capacity of the majority of IVIG lots produced from plasma collected in Austria, Germany and the Czech Republic therefore resulted, at least partially, from cross reactive antibodies as induced by the wide-spread use of TBEV vaccines or TBEV infections. The IVIG lots produced from plasma collected more recently, six lots in 2009 and five lots released for 2010 until April, did not fit this correlation. In general, plasma collection pre-dates release of IVIG lots

TABLE

Cases of West Nile virus infection and seropositivity in EU countries, Russia and Israel, by year of occurrence, 1962–2010

Year	Country	Cases	WNND	Fatalities	Seropositivity	CFR [%]	Lineage	Reference
1962–1964	France	13	NI	0	NI	NI	NI	[4]
1973	Portugal	NI	NI	NI	0.5% (1,649 sera anti-WNV-positive)	NI	NI	[5]
1973	Spain	NI	NI	NI	17% (701 sera anti-Flavivirus-positive)	NI	NI	[6]
1975–1979	France	NI	NI	NI	5% (235 sera anti-WNV-positive)	NI	NI	[7]
1975–1976	Spain	NI	NI	NI	8% (1,037 sera anti-WNV-positive)	NI	NI	[8]
1980	Spain	NI	NI	NI	8% (130 sera anti-Flav-positive)	NI	NI	[9]
1982	France	1	1	NI	NI	NI	NI	[10]
1996	Romania	393	352	17	NI	4	1	[11] [12] [13] [14]
1997	Czech Republic	5	0	0	2.1% (619 sera anti-WNV-positive)	NI	NI	[15]
1997–1998	Romania	NI	13	1	4% (959 sera anti-WNV-positive)	NI	NI	[12] [16]
1999	Czech Republic	4	NI	NI	NI	NI	NI	[15]
1999	Czech Republic	NI	NI	NI	2% (619 sera anti-WNV-positive)	NI	NI	[15]
1999	Russia	318	84	40	NI	13	1	[11] [17] [18]
2000	Russia	56	20	0	NI	NI	NI	[12] [17]
2000	Israel	417	307	35	NI	8	NI	[19] [20]
2001	Russia	64	NI	NI	NI	5–10	NI	[12] [17]
2002	Czech Republic	1	0	0	NI	NI	NI	[21]
2002	Spain	NI	NI	NI	1% (797 sera anti-Flavivirus-positive)	NI	NI	[22]
2003	France	4	2	0	NI	NI	NI	[23]
2003	Hungary	NI	14	0	NI	NI	NI	[24]
2007	Greece	NI	NI	NI	1.02% (4 sera anti-WNV-positive)	NI	NI	[2]
2007	Russia	54	NI	2	NI	4	2	[25]
2008	Hungary	NI	14	0	NI	NI	NI	[26]
2008	Italy	13	8	0	NI	NI	NI	[27] [28]
2009	Italy	NI	17	3	NI	NI	1	[29] [30]
2010	Hungary	3	NI	NI	NI	NI	NI	[31]
2010	Russia	448	26	6	NI	1	NI	[25] [32]
2010	Romania	41	NI	4	NI	10	NI	[25] [31]
2010	Greece	261	191	35	1.5% (392 sera anti-WNV-positive)	18	2	[31] [33] [34]

CFR: case fatality rate; NI: no information available; WNND: case with West Nile neuroinvasive disease; WNV: West Nile virus.

FIGURE 1

West Nile virus and tick-borne encephalitis virus neutralisation by intravenous immunoglobulin lots produced from plasma collected in Austria, Germany and the Czech Republic (N=55)

IVIG: intravenous immunoglobulins; NT₅₀: the reciprocal dilution resulting in 50% virus neutralisation; TBEV: tick-borne encephalitis virus; SD: standard deviation; SEM: standard error of the means; WNV: West Nile virus.

A: WNV neutralisation titres determined in IVIG lots produced between 2006 and 2010. WNV neutralisation titres within (open circles) and out of (black diamonds) the correlation slope as shown in B. Oneway ANOVA analysis revealed a systematic ($p=0.0269$) mean increase in NT₅₀ of IVIG sorted by production year.

B: Correlation analysis ($r^2=0.5$, $p=0.002$) of EU plasma-derived IVIG lots (N=30) tested at least in duplicate for WNV and TBEV neutralisation. Results are given as mean NT₅₀ ± SD.

IVIG lots exclusively collected from EU plasma were not available for the year 2008.

by approximately 6–8 months. The IVIG lots containing significantly higher WNV-neutralising capacity (Figure 1, black diamonds) did not follow a seasonal pattern.

The non-structural flavivirus protein NS1 is only expressed during infection, but not present in the inactivated whole virus vaccines that are used in the countries analysed here. In addition, reactivity of antibodies with the NS1 protein is specific to the flavivirus serotype, and thus infections with either WNV or TBEV can be differentiated [36]. We therefore analysed the antibody specificity of six randomly selected IVIG lots, produced in 2007 (n=1) and 2009 (n=5), by Western blot. TBEV- or WNV-infected Vero cells as well as recombinant WNV NS1 antigen were used as a positive and uninfected Vero cells as a negative control [37, 38]. The blots were incubated with IVIG produced from plasma collected in Austria, Germany and the Czech Republic (Figure 2A), or with a control serum from TBEV-infected mice (Figure 2B). IVIG interacted with the flavivirus structural envelope protein E as well as the WNV-specific NS1 (Figure 2A). In contrast, the control mouse serum reacted strongly only with the E protein of TBEV-infected cells and weakly with WNV-infected cells (Figure 2B).

Discussion and conclusion

We found that IVIG preparations manufactured from plasma collected in Austria, Germany and the Czech Republic, contained neutralising antibodies against WNV at titres which have increased significantly since 2009. As WNV and TBEV are related flaviviruses, albeit distantly, we quantified neutralising antibody titres against TBEV in these IVIG lots, to investigate cross-reactivity between these two viruses as a potentially confounding variable. Indeed, very high TBEV NT₅₀ titres of between 400 and 3,000 were observed in all

FIGURE 2

Reactivity of intravenous immunoglobulin lots produced from plasma collected in Austria, Germany and the Czech Republic with viral proteins

HRP: horseradish peroxidase; IVIG: intravenous immunoglobulins; NT₅₀: the reciprocal dilution resulting in 50% virus neutralisation; TBEV: tick-borne encephalitis virus; PVDF: polyvinylidene fluoride; SD: standard deviation; SDS PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; WNV: West Nile virus.

SDS PAGE transferred to Immobilon PVDF membranes and probed with (A) EU IVIG (1:10; WNV NT₅₀ mean ± SD: 1.6 ± 1.2, n=4) and HRP-coupled anti-human IgG (1:10,000) or (B) with TBEV-infected mouse serum (1:200) and HRP-conjugated anti-mouse IgG (1:10,000).

Lanes 1: Supernatants of TBEV-infected Vero cells; lanes 2: uninfected Vero cells (negative control); lanes 3: WNV-infected Vero cells; lanes 4: *E. coli*-expressed recombinant NS1 (positive control).

The figure shows one representative result of six IVIG lots tested.

investigated years, a result of vaccination and possibly subclinical TBEV infections. Despite a significant correlation of TBEV and WNV neutralisation titres in approximately 60% of the IVIG lots, 11 of the 30 lots contained significantly higher ($p < 0.0001$) neutralisation titres against WNV (mean $\pm$ standard error of the means (SEM): 6.5 ± 0.6 , $n=11$, compared with 2.8 ± 0.1 , $n=19$) that did not correlate with the TBEV-neutralising capacity. IVIG lots produced from plasma collected in Austria, Germany and the Czech Republic were shown by Western Blot to contain specific antibodies against WNV NS1, the most useful differentiation marker for flavivirus infections in humans [36], which provided further evidence for past WNV infections in plasma donors from the central part of the EU. As the detection of antibodies to the WNV NS1 protein is serotype-specific, a possible contribution of antibodies against dengue virus can be excluded. The theoretical possibility of a contribution of neutralising antibodies to Usutu virus (USUV) [39], a virus that belongs to the same serocomplex as WNV, was not evaluated in vitro, as even the highest USUV activity in Austria as observed during the summer of 2003 [40] had no impact on the WNV-specific neutralisation titres in IVIG lots [41]. Moreover, despite a comprehensive surveillance programme for dead birds and mosquitoes in Austria [42], where around 45% of the plasma is collected for production of the IVIG lots tested in this study, no evidence of human exposure to USUV could be found [43], which makes a significant contribution of USUV antibodies to WNV neutralisation titres determined in the present study unlikely. However, additional studies would be relevant to describe this issue more precisely.

These results demonstrate WNV seropositivity in asymptomatic plasma donors from Austria, Germany and the Czech Republic, although some of the seropositivity could be due to travel-related infections. The present epidemiological situation in these countries is thus similar to the one in Greece before the recent epidemic. The causative agent of the outbreak in Greece, WNV lineage 2, has only once before been isolated from humans with severe clinical progression, in an outbreak in Volgograd, Russia in 2007 [18]. Before those two outbreaks, this lineage was considered to be less virulent in humans compared to isolates belonging to the lineage 1 [44]. It has been suggested that the evolution from low to high human pathogenicity is associated with mutations of only a few amino acids, most likely in the non structural proteins [45].

The WNV neutralisation capacity of IVIG lots produced from Austria, Germany and the Czech Republic has increased from a mean NT_{50} of 2.5 in 2006 and 2007 to 4.2 at the beginning of 2010, which accounts for an increase of 1.7 in the WNV neutralising capacity (Figure 1). The TBEV neutralisation titres of IVIG have not changed over time, and thus the increase in WNV neutralisation titres is most likely a true reflection of increased virus circulation. Using the same, fully validated assay, reconvalescent sera from human WNV

cases in North America have earlier been shown to have a mean NT_{50} of 208 [41], 120-fold higher than the increase now observed. This would indicate past WNV exposure of just under 1% of the population of Austria, Germany and the Czech Republic. The increasing WNV seropositivity in these countries marks this virus as a potential public health concern in this area, and a future epidemic associated with human morbidity and mortality similar to that observed in summer 2010 in Greece cannot be excluded.

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Conflict of interest

The authors of this manuscript have conflicts of interest to disclose: All authors were employees of Baxter AG and the work reported in this article was funded by Baxter AG.

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