Functional Activity of Virus Antibodies in Immune Globulins: West Nile Virus, for example

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Submitted by:
Yvonne Hofmeister
(0510206024)

Thesis coach: Dr. Thomas R. Kreil, Baxter BioScience Vienna
Thesis assessor: Dr. Beatrix Kuen-Krismer, FH Campus Vienna
Biotechnology

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Table of contents

Aknowledgements I

Table of contents II

Index of Figures IV

Index of Tables V

Index of Abbreviations VI

Abstract .... - 1 -

Zusammenfassung .... - 2 -

2. West Nile Virus - Introduction .... - 4 -

1.1. Virology .... - 4 -
1.2. Epidemiology .... - 5 -
1.3. Clinical Manifestation and Diagnosis .... - 8 -
1.4. Treatment and IGIV .... - 10 -
1.5. IgG subclasses .... - 11 -

3. Materials and Methods .... - 14 -

3.1. Test articles .... - 14 -
3.2. Virus and cells .... - 14 -
3.3. IgG subclass separation .... - 15 -
3.3.1. FPLC program .... - 15 -
3.3.2. ÄKTA program(1) .... - 17 -
3.3.3. ÄKTA program(2) .... - 19 -
3.4. Elisa .... - 20 -
3.5. RID .... - 22 -
3.6. HPLC .... - 24 -
3.7. WNV infectivity and neutralization test .... - 25 -
3.7.1. $TCID_{50}$ assay .... - 26 -
3.7.2. WNV micro neutralization assay .... - 26 -
3.8. *In vivo* protection against WNV challenge .... - 28 -

3.8.1. *In vivo* protection against a WNV challenge - IGIV and the respective ....
IgG subclasses diluted to identical protein concentration .... - 29 -
3.8.2. *In vivo* protection against a WNV challenge - IGIV and the respective ....
IgG subclasses diluted to certain identical WNV neutralization capacity........... - 29 -

4. Results .... - 30 -

4.1. IgG subclass separation .... - 30 -
4.1.1. FPLC program .... - 30 -
4.1.2. ÄKTA program (1) .... - 32 -
4.1.3. ÄKTA program (2) .... - 34 -
4.2. Elisa/RID .... - 37 -
4.3. HPLC .... - 40 -
4.4. WNV micro neutralization assay .... - 42 -
4.4.1. IGIV lots released in 2008 .... - 42 -
4.4.2. *In vitro* Neutralization capacity of parent IGIV and derived IgG ....
subclasses .... - 44 -
4.5. *In vivo* protection against a WNV challenge .... - 47 -
4.5.1. *In vivo* protection against a WNV challenge - IGIV and the respective
IgG subclasses diluted to identical protein concentration .... - 47 -
4.5.2. *In vivo* protection against a WNV challenge - IGIV and the respective
IgG subclasses diluted to identical WNV neutralization capacity .... - 50 -

5. Discussion .... - 55 -

Curriculum Vitae .... - 61 -

Index of Figures

Figure 1 Phylogenetic Tree .... - 5 -
Figure 2 Transmission cycle of West Nile Virus .... - 6 -
Figure 3 Separation of IgG Subclasses by rProtein A Sepharose (FPLC) .... - 31 -
Figure 4 Separation of IgG subclasses by rProtein A Sepharose (ÄKTA) .... - 32 -
Figure 5 Separation of IgG subclasses by rProtein A Sepharose (ÄKTA) .... - 35 -
Figure 6 *In-vitro* WNV NT determined for IGIV final container lots released in the year 2008 and for plasma from donors past WNV infection .... - 43 -
Figure 7 *In-vitro* WNV NT determined for IGIV final container and subsequent IgG subclasses .... - 45 -
Figure 8 *In vivo* protection by US-IGIV and IgG subclasses at 60mg/ml (A) or 30mg/ml (B) against a WNV challenge (10E5TCID$_{50}$; s.c.) .... - 49 -
Figure 9 *In vivo* protection by US-IGIV and IgG subclasses at NT 2 (A) or NT 0.2 (B) against a WNV challenge (10E5TCID$_{50}$; s.c.) .... - 52 -
Figure 10 *In vitro* neutralization at same protein concentration, i.e. 30mg/ml, and *in vivo* protection at suboptimal neutralization capacity, i.e. NT 0.2 .... - 54 -

Index of Tables

Table 1 Affinity of rProtein A for human IgG .... - 12 -
Table 2 Dilution for standards, positive control and samples .... - 22 -
Table 3 10% and 5 % IGIV products and subsequent IgG subclasses diluted to same protein concentration, i.e. 60 or 30 mg/ml..................................... - 29 -
Table 4 Analyzed Peaks by calculation of separated IgG subclasses by ÄKTA.. - 33 -
Table 5 Biochemical characteristics of separated IgG subclasses by ÄKTA ...... - 36 -
Table 6 ELISA/RID data of IGIV and subsequent IgG subclasses .... - 38 -
Table 7 Correlated data for the IgG1 enriched fraction (LE 12 G 031) and IgG2 enriched fraction (LE 12 G 031) .... - 39 -
Table 8 HPLC-data of IGIV and subsequent IgG subclasses .... - 41 -
Table 9 *In-vitro* WNV NT determined for IGIV final container and subsequent IgG subclasses .... - 46 -

Index of Abbreviations

CNS	Central Nervous System
CPE	Cytopathic Effect
dest.	distilled
ELISA	Enzyme-linked Immuno Sorbent Assay
Env	Envelope
FPLC	Fast Protein Liquid Chromatography
HIVIG	HIV Hyper Immune Globulin
HIVIG	Hyper Immune Globulin Intravenous
HPLC	High Performance Liquid Chromatography
IgG	Immune Globulin G
IGIV	Immune Globulin Intravenous
IGIV-IL	Immune Globulin Intravenous - Israel
IgM	Immune Globulin M
IPC	In Process Control
JEV	Japanese Encephalitis Virus
kDa	kilo Dalton
LOD	Limit of detection
mM	Millimolar
MOI	Multiplicity of Infection
MVEV	Murray Valley Encephalitis Virus
n.d.	not determined
NEAS	nonessential amino acid
NT	Neutralization titer
PBS	Phosphate buffered saline
R&D	Research and Development
RID	Radial Immune Diffusion
RT	Room temperature
sc	subcutaneous
SD	Standard Deviation
SEM	Standard Error of the Mean
SLEV	Saint Louis Encephalitis Virus
$TCID_{50}$	50% Tissue Culture Infective Dose
US	United States
WN	West Nile
WNF	West Nile Fever
WNIG	West Nile Immune Globulin
WNV	West Nile Virus

Abstract

West Nile virus (WNV) was newly introduced into the US in 1999. Since then the virus spread across the entire North American continent and caused mostly asymptomatic infections in approximately 1% of the population. Consequently Immune Globulin Intravenous (IGIV), which is produced from plasma of thousands of healthy US donors, contains WNV neutralizing antibodies. WNV neutralization titers in individual IGIV lots vary significantly and range between non-reactive to highly protective, as was shown by pre-exposure protection in a lethal WNV mouse model (Planitzer et al., 2007).
The underlying molecular basis (i.e. effector functions) of the protection afforded by WNV antibodies within IGIV is so far poorly understood.
Better understanding of antibody mediated protection might allow mechanistically targeted medical intervention to treat WNV infections and provide guidance for the development of a vaccine.

Methods
Immune Globulins in IGIV lots of high WNV-neutralizing capacity were separated into IgG subclasses by fractionated rProtein A Sepharose affinity chromatography. The resulting IgG 1, 2 and 3 fractions were tested for ***in vitro*** neutralization capacity and ***in vivo*** protection in a mostly lethal WNV challenge mouse model.

Results
At identical antibody protein concentrations, the IgG1 subclass had significantly higher ***in vitro*** WNV neutralization capacity than the other subclasses, or even the parent IGIV preparation. When tested for protection at suboptimal conditions (i.e. IgG subclass was diluted to minimal WNV antibody neutralization capacity) IgG1 subclass was significantly more protective than the other IgG subclasses.

Conclusions
After human WNV infection, neutralizing antibodies of predominantly the IgG1 subclass are raised on response. When adjusted to equivalent neutralization titers, the IgG1 subclass is the most protective, which might reflect a more effective adaptor function of the IgG1 subclass with other parts of the immune system. For the development of a WNV vaccine, it might therefore be desirable to specifically induce WNV neutralizing antibodies of the IgG1 subclass.

Zusammenfassung

Das West Nil Virus (WNV) ist 1999 zum ersten Mal in Nordamerika aufgetreten und hat seitdem zu Infektionen in ungefähr 1% der ursprünglich naiven Bevölkerung geführt. Als Antwort auf die meist asymptomatisch verlaufende Infektion werden WNV neutralisierende Antikörper gebildet. Diese WNV neutralisierenden Antikörper kann man in Immun Globulin Intravenös (IGIV) Produkten nachweisen, wobei die Antikörpertiter sehr stark variieren und von nicht-reaktiv bis hoch-schützend reichen, was anhand von einem tödlichen WNV Maus Modell gezeigt wurde (Planitzer et al., 2007).
Wenig ist über die molekulare Basis der Antikörper-vermittelten Protektion (Effektorfunktion) bekannt und es gibt noch viele offene Fragen.
Vertieftes Wissen der durch Antikörper vermittelten Protektion könnte die Behandlung von WNV durch mechanistisch gezielte medizinische Intervention verbessern und auch eine Orientierungshilfe für die Entwicklung von Impfstoffen darstellen.

<u>Methoden</u>
IGIV Lots mit hoher WNV Neutralisationskapazität wurden mittels rProtein A Sepharose Affinitätschromatographie in die einzelnen IgG Subklassen aufgetrennt. Die daraus resultierenden Subklassenfraktionen IgG1, 2 und 3 wurden ***in vitro*** auf ihre Neutralisationskapazität und ***in vivo*** auf ihre Protektion in einem meist letalen WNV Challenge Maus Modell getestet.

<u>Resultate</u>
Von Fraktionen mit ähnlicher Antikörper Protein Konzentration zeigte die IgG1 Subklassenfraktion eine signifikant höhere ***in vitro*** WNV Neutralisationskapazität gegenüber den anderen Subklassenfraktionen und auch dem IGIV aus welchem die Subklassenfraktionen gewonnen wurden.
Bei Testung auf Protektion in suboptimalen Konditionen, d.h. wenn die IgG Subklassenfraktionen auf minimale WNV Antikörper Neutralisationskapazität verdünnt wurden, zeigte die IgG1 Subklassenfraktion eine signifikant höhere Protektion als die anderen Subklassenfraktionen.

<u>Fazit</u>
Nach einer humanen WNV Infektion werden hauptsächlich Antikörper der IgG1 Subklasse induziert. Werden die Subklassen in ähnlichen Neutralisationstitern

verabreicht, ist die IgG1 Subklasse ebenfalls am protektivsten, was möglicherweise auf eine effektivere Adapterfunktion der IgG1 Subklasse mit anderen Teilen des Immunsystems erklären lässt.

Basierend auf diesen Resultaten wäre eine effektive Induktion der IgG1 Subklasse durch einen WNV Impfstoff wünschenswert.

1. West Nile Virus - Introduction

1.1. Virology

West Nile Virus (WNV), a positive sense single-stranded RNA virus is a member of the JEV antigenic complex in the family ***Flaviviridae***, genus ***Flavivirus*** (Petersen and Marfin, 2002). The JEV antigen complex includes four antigenically related viruses that are important for causing encephalitis in humans: JEV, WNV, SLEV, MVEV (Petersen and Marfin, 2002).
Virions are small (~35nm in diameter), spherical and enveloped (Petersen and Roehrig, 2001) and the genome is about 11kb in length and encodes three structural proteins including a capsid protein (C) for binding viral RNA, a pre-membrane protein (prM, 18-20 kDa) that blocks premature viral fusion and an envelope protein (E, 53 kDa) as well as seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) which regulate viral transcription, replication and attenuate host antiviral response (Samuel and Diamond, 2006; Petersen and Roehrig, 2001).
The E protein, which is Immunologically the most important structural protein (Mukhopadhyay et al., 2003; Samuel and Diamond, 2006; Petersen and Roehrig, 2001), mediates viral attachment, membrane fusion, viral assembly (Samuel and Diamond, 2006; Petersen and Roehrig, 2001).

1.2. Epidemiology

WNV was first isolated 1937 from a blood specimen of a febrile woman in the West Nile district of Uganda (MacKenzie et al., 2004) and was newly introduced to the Western Hemisphere into the US in 1999 (Petersen and Roehrig, 2001).

West Nile virus can genetically be divided into two lineages as shown on Figure 1 (Lanciotti et al., 2002). The WNV responsible for the US outbreak (NY 99) is genetically a virus of the lineage 1 (Petersen and Marfin, 2002). So far only members of lineage 1 have been associated with major human outbreaks (Petersen and Roehrig, 2001).

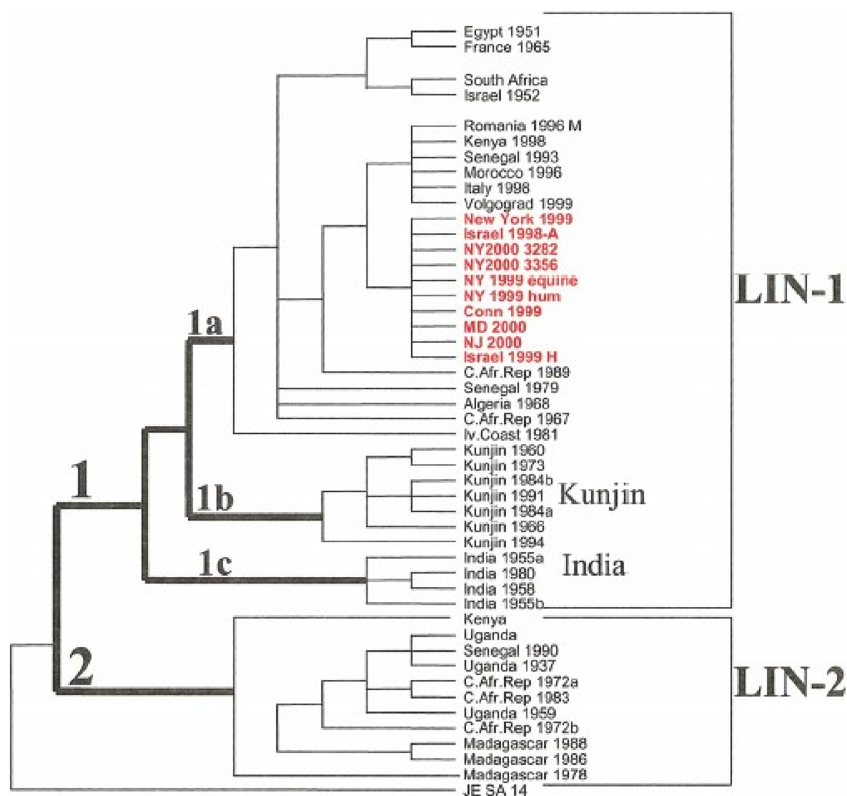


Figure 1 Phylogenetic Tree

Phylogenetic tree based on nucleic acid sequence data (Lanciotti et al., 2002)

West Nile virus is maintained in a transmission cycle between mosquito vectors and bird reservoir hosts (CDC, 2009). Infectious mosquitoes carry virus particles in their salivary glands and infect susceptible bird species during blood-meal feeding (CDC, 2009) as shown in Figure 2.

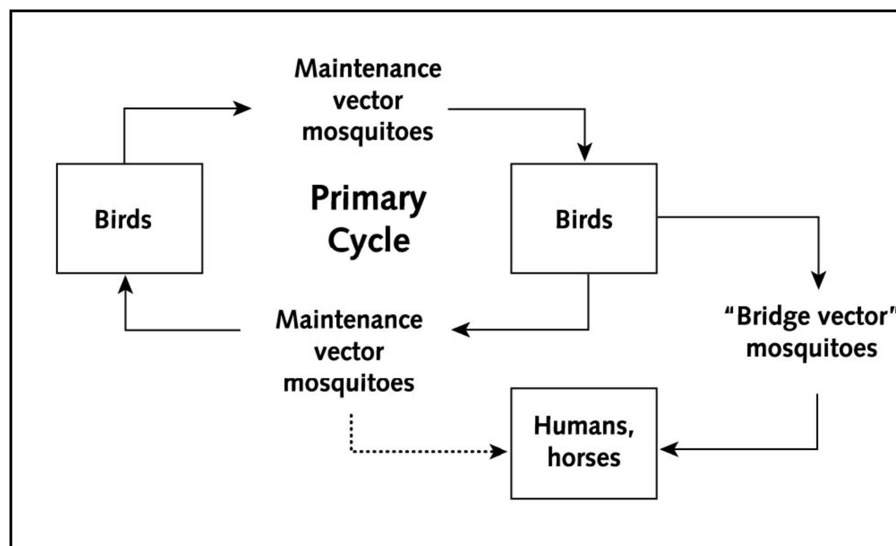


Figure 2 Transmission cycle of West Nile Virus
Transmission cycle of WNV in North America is shown (Petersen and Marfin, 2002)

Humans, who do not develop infectious-level viremias, are so called "dead-end" or incidental-hosts (CDC, 2009).
Infections in humans mostly occur by the bite of an infected mosquito, but infrequent transmissions via organ transplantation and blood transfusion have also been reported although plasma products are safe as described earlier (Kreil et al., 2003).

The 1999 outbreak in the US point out an epidemiologically rare process, i.e. the exposure of a formerly naïve population to a now widely prevalent virus (Planitzer et al., 2007).

During the years 1999 to 2008, a total number of 28,960 cases were reported to the national surveillance system in the US (ArboNET reported by CDC, last updated April 10, 2009).

WNV outbreaks are also reported in other countries than the US. The two largest epidemics occurred in Israel (1951-1954 and 1957) and in South Africa (1974) (Petersen and Roehrig, 2001).
Severe confirmed human infections were further reported 1996 in Romania (393 cases) and 1999 in Russia (942 cases) (Petersen and Roehrig, 2001).
Saint Louis Encephalitis virus (SLE) also a member of the JEV serocomplex, caused an isolated outbreak in the US with approximately 2,000 cases in 1975 (CDC, 09) and never since, due to unknown reasons.

1.3. Clinical Manifestation and Diagnosis

After an incubation period of 3 to 14 days (Petersen and Marfin, 2002), approximately 20 to 30% of those infected develop WNF (Samuel and Diamond, 2006), i.e. West Nile fever, and less than 1% of infected people (1 out of 150) experience neuroinvasive disease (encephalitis, meningitis) (MacKenzie et al., 2004). The majority of WNV infections, i.e. approximately 80% do not develop any symptoms.

The mortality rate following neuroinvasive course of disease is about 10% (Samuel and Diamond, 2006). Age is one of the most important risk factors for developing a severe course of disease, i.e. people older than 80 years have a 30-times higher risk to develop a neuroinvasive disease (Chowers et al., 2001).
Beyond that, Immunocompromised persons have an increased risk to develop a severe course of disease after WNV infection. Mortality rate among Immunocompromised people is approximately 31% whereas not Immunocompromised people have a rate of 13% (Chowers et al., 2001).

Other manifestations of WNV infection are rather rare, like myelitis, optic neuritis and polyradiculitis (Campbell et al., 2002).West Nile fever is typically characterized with symptoms as general weakness, drowsiness, headache and pains mostly in the chest and lumbar region.

Patients suffering from a neuroinvasive disease have symptoms of fever and headache. Moreover symptoms of depressed deep tendon reflex, diffuse muscle weakness, flaccid paralysis and respiratory failure are common in encephalitis.
Encephalitis, the more common neurologic disease can lead to cerebral dysfunction and in 15% of the cases to coma (Campbell et al., 2002). Mortality appears more often in WN encephalitis in older age groups (median age, 70 years) (Campbell et al., 2002), whereas meningitis is more frequent in younger age groups (median age, 35 years) (Sejvar et al., 2003).

In general WNV infections are confirmed by detection of WNV antibodies or by virus genome sequence in serum or cerebrospinal fluid (CSF).
The most efficient diagnostic tool for confirmation WNV infection is the detection of specific IgM antibodies to WNV by MAC-ELISA (Petersen and Marfin, 2002).
As IgM antibodies normally do not cross the blood-brain barrier, the detection in the CNS is an important clinical indication for an infection of the CNS (Petersen and Marfin, 2002).
However, false-positive ELISA results are reported due to cross-reactivity with related Flaviviruses (Dengue, Yellow Fever) (Marfin and Gubler, 2001).
In addition, IgM persists for six months and longer which can lead to the result that people in endemic areas have persistent IgM antibody levels from previous infections (Petersen and Marfin, 2002).
To sum up, a serological assay for antibody function, e.g. a PRNT or a NT_{50} for confirmation of WNV infections are the gold standards (Petersen and Marfin, 2002).

1.4. Treatment and IGIV

To date, no specific treatment for WNV infection is available.
Current options for the clinician are administration of purine, pyrimidine analogues (e.g. ribavirin) and interferon **α**, and it has been suggested that also IGIV containing high-titer anti-WNV antibodies might be beneficial clinically (Petersen and Marfin, 2002).

Ribavirin has ***in vitro*** activity against many RNA and DNA viruses including flaviviruses, i.e. West Nile virus. It is suggested that high dose inhibit the replication and cytopathogenicity of the virus (Campbell et al., 2002).
It has been shown that interferon **α, applicated before or after WNV** infection, increases the s**urvival of Vero cells and the levels of interferon α** can be easily used in human beings too(Campbell et al., 2002).
A double-blinded, placebo-controlled trial for interferon-**α**-n3 is in progress (MacKenzie et al., 2004).
IGIV produced from the plasma of thousands of healthy US donors contain WNV neutralizing antibodies and may therefore be a good source for another antiviral agent.

As WNV circulates in Israel since the early 1950ies, IGIV produced from Israeli donors contain high WNV neutralizing antibody titers. OMRIX Biopharmaceuticals have developed a strategy to process positive WNV antibody titers into a pharmaceutical relevant grade, so-called WNV IGIV (WNIG). The result of this WNIG was a 10 times higher neutralization capacity obtained by NT as compared to IGIV-IL and US IGIV at a time when WNV had not been endemic (Ben-Nathan et al., 2009).

IGIV lots with high titers against West Nile virus are a good option for substitution treatment for people with immunodeficiencies (MacKenzie et al., 2004) , as those people have an increased risk to develop severe case of disease (Chowers et al., 2001). Also high titer IGIV could be used for substitution therapy when traveling in high risk areas or during WNV season.

1.5. IgG subclasses

There are four different IgG subclasses classified as IgG1, IgG2, IgG3 and IgG4 in humans, whereas in mice the classification is the following: IgG1, IgG2a, IgG2b and IgG3.
All four subclasses have approximately the same size, i.e. 150kDa.

Immune Globulin G (IgG) is not only abundant in serum (>98% of total serum antibodies) but even more so in purified preparations of Immune Globulin Intravenous (IGIV). The serum distribution of the subclasses IgG1 to IgG4 is given in the following relation 9:3:1:0.5. According to textbook, IgG anti-viral antibodies are highly restricted to IgG1 and IgG3 subclass, while antibodies of the IgG4 and IgG2 subclass are found less often in viral clearance (Skvaril, 1986), with little primary data to substantiate the claim though. Complement activation by classical pathway is claimed to be performed by the IgG1 and IgG3 subclass. IgG2 and IgG4 do not activate compliment at all (Mehlhop et al., 2007).

Due to different binding affinities of IgG subclasses to FcγR, the effector functions of IgG subclasses vary (Nimmerjahn and Ravetch, 2008).
Both, human IgG1 and IgG3, which are considered to be the most important proinflammatory IgG subclasses and dominate antiviral immunity, bind to the activating FcγRI that is widely expressed on human as well as mouse cells of the haematopoetic system, with a high affinity of 10^8 to 10^9 M^{-1} (Nimmerjahn and Ravetch, 2008).
Similarly in the mouse the IgG2a subclass binds the FcγRI with high affinity, establishing mouse IgG2a and human IgG1 and IgG3 as functional homologous (Nimmerjahn and Ravetch, 2008).

Some strains of ***Staphylococcus aureus*** synthesize protein A, a ligand with specifity for the Fc region of IgG. The pH of the elution chromatography is ranging from pH 6.5 to 3.5. IgG3 has a very low to no binding affinity to protein A and is therefore found in the flow through of the elution chromatography. Both, IgG1 and IgG2 subclasses have a very high binding

affinity at pH 6.0 to 7.0, however the two subclasses elute at different pH as can be seen in Table 1. As result, IgG2 subclass is eluted first and shortly afterwards the IgG1 subclass is eluted.
The IgG4 subclass elutes at the whole range of pH elution and therefore can be found in IgG1 and IgG2 subclass fractions. However, IgG4 is present in very low concentrations in IGIV and does not influence the results of the separated IgG subclasses.

Human IgG			
Antibody	**Affinity**	**Binding pH**	**Elution pH**
IgG1	very high	6.0 - 7.0	3.7 - 4.5
IgG2	very high	6.0 - 7.0	4.55 – 4.7
IgG3	low - none	8.0 - 9.0	≤ 7.0
IgG4	low - high	7.0 - 8.0	3.0 - 6.0

Table 1 Affinity of rProtein A for human IgG
Given is the monoclonal antibody, the affinity of the antibody, the range of binding pH and the range of elution pH (GE Healthcare product information; Scharf et al., 2001)

IgG subclass virus neutralization data are rather limited.
In vitro the efficiencies of human IgG subclasses from polyclonal HIVIG in the neutralization of HIV-1 strains was compared earlier. A HIV-1 fusion assay, ELISA and neutralization assay was performed to analyze the IgG subclass differences in viral clearance. IgG1, IgG2 and IgG3 bound HIV-1 proteins as shown by ELISA. However, ELISA is just a method to detect anti-HIV-antibodies, but does not give information about the functionality of the subclass. Using the HIV-1 fusion assay, it was shown that the IgG3 subclass blocked the fusion more efficiently than the other two subclasses. By performing a neutralization assay IgG3 subclass showed the highest neutralization capacity. The more flexible hinge region of the IgG3 subclass was claimed to be the reason for the more effective binding to HIV-1 proteins and neutralization capacity (Scharf et al., 2001).
The second known ***in vitro*** data is about binding and neutralization activity of human IgG1 and IgG3 subclass. A neutralization assay and a reactivity assay were performed to analyze the IgG subclasses 1 and 3. ELISA was

used as read out for the neutralization assay by detecting the HIV-1 p24 antigen. It was shown that the IgG3 subclass from individual sera of patients showed very low to none neutralization capacity to HIV-1. The IgG1 subclass had nearly the same neutralization capacity as purified total IgG. The results for reactivity, performed as FACScan flow cytometry, showed similar results, IgG3 has the lowest binding reactivity (Cavacini et al., 2003).

In summary, the ***in vitro*** neutralization of human IgG1 and IgG3 was so far shown by two papers. The results of them are contradictory, possibly due to using plasma pools (Scharf et al., 2001) or sera of HIV-1 infected individuals (Cavacini et al., 2003) for testing.

In vivo protection data are only available for mouse IgG2a in an Ebola virus infection model. Monoclonal antibodies to the Ebola glycoprotein were generated and tested for their efficacy. All IgG2a monoclonal antibodies, a homologous to the human IgG1 and IgG3 subclass, were completely protective. It was claimed that mouse IgG2a binds complement more effectively, which may result in better resolving of Ebola infections by activating the classical pathway (Wilson et al., 2000).

So far, not a single experimental study using human IgG subclasses in an *in vivo* challenge model has been published.

Information on the ***in vivo*** protection by human IgG subclasses might help to further improve strategies for vaccine development, in providing guidance as to which IgG subclass might be most desirably induced.

2. Materials and Methods

2.1. Test articles

The following Immune Globulin Intravenous (Human) (IGIV) products manufactured by Baxter BioScience were used in the study: (1) 10% Gammagard Liquid or KIOVIG (Baxter Healthcare Corp., Westlake Village, CA); (2) 5% Endobulin from Source Plasma collected in the US (Baxter Healthcare Corp., Westlake Village, CA, US).

2.2. Virus and cells

The WNV (isolate "385-99", obtained from the liver of a Snowy owl found dead in New York in 1999; kindly provided by Dr. Robert E. Shope; University of Texas Medical Branch, Galveston, TX), was propagated in and titrated on Vero cells (ECACC 84 11 3001) as described (Planitzer et al., 2007), grown in TC Vero Medium (Baxter, Vienna) supplemented with 5% fetal calf serum, 1% L-glutamine, 1% gentamycin sulfate, 1% sodium pyruvat, 1% sodium carbonate and 1% NEAS (all GIBCO, Invitrogen Corporation, Carlsbad).

2.3. IgG subclass separation

2.3.1. FPLC program

The IgG subclass separation was executed by a pH gradient elution from a rProtein A-Sepharose, as previously described (Scharf et al., 2001). Protein A Sepharose is an affinity chromatographic matrix coupled to Sepharose 4 Fast Flow for optimal purification conditions.

rProtein A-Sepharose™ Fast Flow (Amersham Pharmacia Biotech AB, Uppsala, Sweden) was packed into a 1.5cm diameter column to a bed height of 25 cm. (V=23ml)

10mg protein/ml gel of the high titer 10% parent IGIV final container lot (LE 12 G 031) was separated by the described method.

The column was equilibrated in 5 column volumes (CV).
For the pH gradient elution (FPLC Controller LCC-501 Plus GE Healthcare, Bio-Science, Vienna, Austria) an equilibration buffer at pH 6.5 (0.2M $Na_2HPO_4{}^{*}2H_2O$) and an elution buffer at pH 3.5 (0.2M $Na_2HPO_4{}^{*}2H_2O$) was used. For all FPLC runs, the 10% parent IGIV final container lot (LE 12 G 031) was diafiltrated in order to keep the protein concentration constant at a changing pH (from ph 4.0 to pH6.5). N ml of 10% parent IGIV final container lot (LE 12 G 031) and 10xN of 0.2M $Na_2HPO_4{}^{*}2H_2O$ at pH 6.5 were pipetted into an Amicon stirred cell (Millipore, Billerica, MA01821).
In certain intervals probes were taken to determine the pH and the conductivity.
After the product application, the pH gradient elution was performed as following:
8CV 55% to 70% buffer B
2CV 70% to 85% buffer B
2CV hold 85% buffer B

Flow rate was held at 34cm/h for the equilibration, wash and regeneration and at 11cm/h for sample application and elution. The whole FPLC system was held at RT.

The flow through, the wash and the elution were collected each as 5ml fractions.
Pooled fractions were stored at +2°C to +8°C and an ELISA for pooled fractions was performed.

2.3.2. ÄKTA program(1)

For the IgG subclass separation the same product and the same column (1.5cm diameter; bed height of 25 cm; V=23ml) as for the FPLC was used, rProtein A-Sepharose^{TM} Fast Flow (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

The two buffers used for the pH gradient elution are 0.2M $Na_2HPO_4{}^*2H_2O$ at pH 6.5 (equilibration buffer) and pH 3.5 (buffer B).
The elution was performed by ÄKTA Explorer 10S (GE Healthcare, Bio-Science, Vienna, Austria).
The column was equilibrated in 5 column volumes (CV).
The high titer 10% parent IGIV final container lot (LE 12 G 031) was diluted at a 1:5 ratio with the equilibration buffer and 10mg protein/mg gel was applied to the column after the dialysis in the equilibration buffer.
After the product application, the column was washed with 7 CV equilibration buffer.
The pH gradient elution was changed to that of FPLC and performed as following:
3CV 55% to 70% buffer B
6CV Hold 70% buffer B
5CV 70-100% buffer B
3CV hold 100% buffer B

Flow rate was held at 60cm/h for the equilibration and wash at 38.4cm/h for the elution and sample application. The ÄKTA system was held at 15°C, whereas the FPLC system was kept at RT.
The flow through, the wash and the elution were collected each as 5ml fractions.
The chosen fractions were pooled and stored at +2°C to +8°C for further ELISA experiments.

IgG subclass separation was confirmed by ELISA. As results obtained by ELISA analysis demonstrated inaccurate separation, already fractionated IgG subclasses were used for further experiments.

In addition the yield of each fractionated subclass was low and there was not enough time to perform serial runs to reach the enough mg/ml amounts. Therefore already fractionated IgG subclasses were used for further experiments.
About 28mg/ml of each IgG subclass would have been needed to perform the experiments. As per run only 71µg/ml IgG3 have been yielded approximately 400 runs would have been performed to result in 28mg/ml. Moreover, the already fractionated subclasses were earlier used in animal experiments, i.e. in rabbits in order to test pyrogenity of 10% parent IGIV final container lot (LE 12 G 031) and the subsequent IgG subclasses.

2.3.3. ÄKTA program(2)

The IgG subclass separation was executed by a pH gradient elution from a different rProtein A-Sepharose, Mab select 200 ProteinA (GE Healthcare, Bio-Science, Vienna, Austria).
rProtein A-Sepharose was packed into a 5cm diameter column to a bed height of 25 cm (V=490ml).
The following high titer 10% parent IGIV products manufactured by Baxter BioScience were used in the study: Gammagard Liquid (Baxter Healthcare Corp., Westlake Village, CA) lots (LE 12 G 031, LE 12 G 030).

The same buffers were used as described for ÄKTA program (1).
The elution was performed by ÄKTA Explorer 100 Air 2 (GE Healthcare, Bio-Science, Vienna, Austria).
The column was equilibrated in 5 CV.
10% parent IGIV final container lot was diluted at a 1:5 ratio with the equilibration buffer and 20mg protein/mg gel was applied to the column after the dialysis in the equilibration buffer.
After the product application, the column was washed with 7 CV equilibration buffer.
The pH gradient elution was performed the same way as for the first ÄKTA program.

Flow rate was held at 60cm/h for the equilibration and wash and at 38.4cm/h for the elution and sample application. The system was held at RT.
The wash after the application of the sample and the three elution blocks were collected each as one fraction.
The fractions were sterile filtrated and concentrated by 30kD ultra-/diafiltration (Millipore, Billerica, MA01821). To the subclass-enriched fractions 0,25M glycine was added to stabilize the product and to set a pH of 4.7.

2.4. Elisa

Two different methods were used to detect the quantity of antibodies. ELISA (responsible person: Dr. Alfred Weber, Sr Mgr R&D, Baxter BioScience Vienna) was used at the Preclinical R&D Laboratory and at the IPC Laboratory RID (responsible person: Dr. Geoffrey Pot, Mgr Quality Control, Baxter Global Quality Lessines, Belgium) was the method of choice at Lessines.

Human IgG1, IgG2, IgG3 and IgG4 subclasses were measured with ELISA using commercially available paired-polyclonal sheep antibodies provided by The Binding Site (TBS, Birmingham, UK).
In particular, sheep anti-human IgG1 (TBS AU006, affinity pure; lot no. 6790) and sheep anti-human IgG1 peroxidase (TBS AP006, affinity pure; lot no. 239703), sheep anti-human IgG2 (TBS AU007, affinity pure; lot no. 6730) and sheep anti-human IgG2 peroxidase (TBS AP007, affinity pure; lot no. 240871), sheep anti-human IgG3 (TBS PC008, lot no. 39808) and sheep anti-human IgG3 peroxidase (TBS AP008, affinity pure; lot no. 237350), sheep anti-human IgG4 (TBS PC009; lot no. 29111) and sheep anti-human IgG4 peroxidase (TBS AP009, affinity pure; lot no. 227031) were used for the measurement of IgG1, IgG2, IgG3 and IgG4, respectively.
The reference preparation and the samples were diluted with phosphate-buffered saline containing Tween 20 (0.8% NaCl, 0.02% KCl, 0.02% KH_2PO_4, 0.126% Na_2HPO_4, pH 7.2-7.4, 0.05% Tween 20), 2 mM benzamidine and 1 mg/ml gelatin (Bio-Rad, EIA-grade).
The following linear dose-response ranges were determined for the IgG subclass ELISA: IgG1 251 – 16 ng/ml, IgG2 171 – 11 ng/ml, IgG3 58 – 4 ng/ml, and IgG4 38 – 2 ng/ml.
The test samples were diluted according to their estimated IgG subclass concentrations but at least 1/10 to obtain as many as possible dilutions with IgG subclass concentrations within the ranges of the respective calibration curve.
96-well Nunc Maxisorp F-96 polystyrene plates (VWR) were incubated overnight at +2 to +8°C with the non-conjugated antibody at a protein

concentration of about 10 µg/ml in 0.1 M $NaHCO_3$, 0.1 M Na_2CO_3, pH 9.5 (100 µl/well). The plates were washed with PBS-containing Tween 20 and inactivated by incubation with dilution buffer (200 µl/well; 60 min at +37°C). After a washing step, 100 µl/well of dilution buffer was added to each well and the standards/samples were diluted serially directly on the plate.

All dilution series were prepared in duplicates. The positions A11 and A12 of the plate were used for the assay blank and were incubated with dilution buffer only. The dilutions (100 µl/well) were then incubated 15 min at room temperature. Then 100 µL/well of the respective peroxidase conjugate were added, diluted to about 2 µg/ml with dilution buffer.

The plates were incubated for 60 min at room temperature, incubation was stopped by washing and finally the bound peroxidase activity was measured with the ready-to-use peroxidase substrate SureBlue (KPL; 100 µl/well). Reaction was stopped with 1.5 M sulfuric acid (100 µl/well) and measured the plate in an ELISA reader (EL 808, Bio-Tek) at 450 nm using a reference wavelength of 620 nm.

The further evaluation was done using a calibration curve approach: the logarithms of the blank-corrected mean optical densities measured for the standard dilutions were correlated to the logarithms of their known IgG subclass concentrations.

2.5. RID

RID is a technique to detect the quantity of antigen by measuring the radius surrounding samples of the antigen, which marks the boundary between the antigen and the antibody.

A Human IgG subclass Combi RID kit (Biomedical Diagnostics; RK021) was used for the test. In addition, human IgG1/IgG2/IgG3/IgG4 RID plates were used. (SOP number: LE-13-A 14006/02)

The cover of the plate was removed to let the plate dry at RT till no humidity is visible in the wells. The concentration [c] of the standards, given as mg/ml, were determined by the [c] given on the labels.

The [c] of the positive control is given on the label and the range was set at ±15%.

Standards, controls and samples are diluted as following:

	IgG		
	Dilution	BSA 7% [µl]	Product [µl]
Standard	1/1 (100%)	Standard	
	6/10 (60%)	40	60
	1/10 (10%)	225	25
Positiv control	1/10	225	25
Sample	*	*	*

Table 2 Dilution for standards, positive control and samples

*The dilution of the standard depends on the each sample.

Given is the dilution ratio for standards and positive control, the volume of needed BSA 7% [µl] and the volume of needed product [µl]

BSA is used as blocking buffer

5µl of samples, control and standards were pipette on the plates. The pipette should approach the bottom of the well, but should not damage the gel on the bottom of the wells. The samples, control and standards should be pipette slowly to avoid bubbles.

As soon as the diffusion started, the cover of the plate was put on the plate. In addition the plate was given into an aluminum vessel, where a wet sponge was in.

The plate was incubated at RT for at least 72 hours.

The plate reader was calibrated by a standard plate.

The radius of the diffusion zone was measured for standards, samples and control.

The radius of the standard for 100% was stated 9.5mm$\pm$0.5.

The results were calculated by linear regression, following the formula below:

$y=ax+b$

Legend:

y...[c]

x... radius of measured samples

The radius of each subclass is recommended.

IgG1 and IgG2: 10-12mm^2

IgG3 and IgG4: 16-21mm^2

2.6. HPLC

The 10% parent IGIV final container lot (LE 12 G 031) and the subsequent IgG subclasses, which had to be tested, were filtrated by a 0.45 µm syringe-filter (Sartorius Minisart) and diluted to 20 mg/ml by elution buffer(0.02 mol/l $NaH_2PO_4*H_2O$, 0.15 mol/l NA_2SO_4, 0.02% NaN_3, 10% DMSO, pH 6.8).
Gel filtration standard (BIO-RAD 151-1901) is a lyophilized mixture of molecular weight markers in the range of 1.35 to 670 kDa. The standard consists of Thyroglobulin (cow, 5mg/vial), IgG (cow, 5mg), Ovalbumin (chicken, 5mg), Myoglobin (horse, 2.5mg)and Vitamin B12 (0.5mg). This will be reconstituted with 0.5ml Aqua dest.
The gel filtration standard was packed into a 7.5mm diameter column (TOSOH; TSKgel G3000SW) to a bed height of 600mm.

The following 10% IGIV product manufactured by Baxter BioScience was used in the study: 10% parent IGIV final container lots (LE 12 G 031, LE 12 G 030) and the subsequent IgG subclasses.

20µl of the sample were applicated by a syringe filter (Sartorius Minisart) onto the column. Flow rate was held at 1.6 cm/min at RT.
The protein distribution was measured by UV-detector (BIO-RAD 1790) by reading the extinction at 280nm.
The peaks aggregates, dimers, monomers were specified.

Testing was performed at Preclinical R&D Laboratory (responsible person: Dr. Alfred Weber, Sr Mgr R&D, Baxter BioScience Vienna).

2.7. WNV infectivity and neutralization test

Negative control, WNV WS 2 and virus dilution containing 2x10E3 $TCID_{50}$/ml were titrated for the analysis of WNV micro neutralization assay.
Negative control and WNV WS 2 were titrated for the analysis of $TCID_{50}$ assay.
96 well plates were incubated for 7 days at 36$\pm$2°C and 4.5$\pm$0.5% CO_2.

After seven days wells were inspected visually using a microscope (Eclipse TS100; Nikon; Netherlands). Using the following symbols, the pattern of titration was recorded:
+ cells are infected, CPE is visible
- cells are not infected, no CPE visible

Titers of controls (negative control, positive control, virus dilution) were calculated using Clickit version 2.1.
Overall the following criteria must be fulfilled:

- The negative control has to be negative.
- The positive control has to be ranged between 7.50 and 8.50 $TCID_{50}$/ml.
- The virus dilution has to be in the range of 2.30-3.80 $TCID_{50}$/ml.

The samples were tested at least as duplicates. The test was performed on different days and performed by different operators.

2.7.1. $TCID_{50}$ assay

Infectious particles are quantified by virus titration. Infection of cells is determined by the induction of a CPE.

Serial 0.5 log dilutions of the samples were prepared in tissue culture medium, and 100µl of each dilution were added to each of 8 wells of a 96 well plate seeded with 2x10E5 Vero cells/ml. The cells were then incubated for 7 day before the CPE was evaluated by visual inspection of the cells under the microscope (Eclipse TS100; Nikon; Netherlands).

2.7.2. WNV micro neutralization assay

The test is used for semi-quantitative determination of West Nile Virus (WNV) neutralizing antibodies of either IGIV or subsequent IgG subclasses, utilizing strain 385-99 and Vero cell line, which is susceptible to infection. Test samples are serially diluted in cell culture medium and incubated with equal volume of 2x10E3 $TCID_{50}$/ml WNV. The sample-virus mixture is incubated for one hour at room temperature and titrated on Vero cells. Wells are inspected for cytopathic effect on the 7th day after infection.

Either IGIV or subsequent IgG subclasses were applied undiluted to micronic tubes and serially 2-fold diluted.

One row of a micronic rack represents one sample.

500µl of the WNV dilution (containing 2x10E3 $TCID_{50}$/ml) were added to each tube, gently mixed and incubated for 70min$\pm$20min at room temperature.

After the incubation time the virus-sample mixture was titrated onto 96 well plates. Using a multi-channel pipette 8x100µl of each tube was pipetted onto one column of a 96 well plate.

The MicroNT test should be done in such a way, that the difference from mean number of negative wells of a duplicate determination must not exceed the number of $\pm$ 8 negative wells.

The micro NT for samples was calculated using the following formula:

$$X = \frac{V}{2} \cdot 2^{(\frac{Nneg}{8}+0,5)}$$

Legend:

X…NT$_{50}$; i.e. serum dilution is required for 50% neutralization

V…test article, 1:2 dilution; diluted in the used test setup

N_{neg}**…number of negative wells on one 96**-well-plate

The LOD is defined as the lowest concentration of the test article which gives rise to a signal that is significantly different from the negative control. In case that the number of negative wells is zero (all wells show a CPE), the NT is determined as the half of the titer if there is one negative well.

When test article is diluted 1:2 in the test setup, the LOD is $\leq$0.8.

Differences in WNV NTs between individual test articles were evaluated by unpaired **student's** t-test and data are expressed as mean±SEM.

2.8. *In vivo* protection against WNV challenge

6 to 8 week old female BALB/c mice were challenged with WNV by subcutaneous (sc) injection of 0.2 ml containing 100,000 or 5 log_{10} $TCID_{50}$ WNV.

Two hours before WNV challenge, mice received a sc injection of 0.2 ml of prophylaxis, i.e. 10% parent IGIV final container lot (LE 12 G 031) or subsequent IgG subclasses.

Survival as well as symptom free survival was monitored for 28 days.

Survival with symptoms means that the animals showed symptoms of being ill (ruffled hair, paralysis), but did not die over the period of 28 days.

There was a daily control of mice and the symptoms were scored.

Survival curves were created by the Kaplan-Meier method, and compared by log rank test (GraphPad Prism v5.0 software, San Diego, CA, USA).

2.8.1. *In vivo* protection against a WNV challenge - IGIV and the respective IgG subclasses diluted to **identical protein concentration**

Two hours prior to WNV infection, mice received a sc injection of 0.2 ml of either IGIV, IgG1 enriched (LE 12 G 031), IgG2 enriched (LE 12 G 031), IgG3 enriched (LE 12 G 031) or Endobulin, diluted to the desired protein concentration, on the contra-lateral side.

Test article (LOT number)	[mg/ml]		
IGIV (LE 12 G 031)	100	60	30
IgG1 enriched (LE 12 G 031)	---	60	30
IgG2 enriched (LE 12 G 031)	---	60	30
IgG3 enriched (LE 12 G 031)	---	60	30
Endobulin	---	50	---

Table 3 10% and 5 % IGIV products and subsequent IgG subclasses diluted to same protein concentration, i.e. 60 or 30 mg/ml
Given is the test article and the protein concentration as mg/ml as used in *in vivo* experiment

2.8.2. *In vivo* protection against a WNV challenge - IGIV and the respective IgG subclasses diluted to certain **identical WNV neutralization capacity**

Mice received a sc injection of 0.2 ml of 10% parent IGIV final container lot (LE 12 G 030, LE 12 G 0 31), IgG1 enriched (LE 12 G 031) fraction, IgG2 enriched (LE 12 G 031) fraction and IgG3 enriched (LE 12 G 030) fraction, diluted to the desired Neutralization titer.
For NT 2, two independent experiments with 5 mice per group were used, i.e. in total 10 mice/group
At NT 0.2, three independent experiments with 5 mice per group were used, i.e. in total 15 mice/group

3. Results

3.1. IgG subclass separation

3.1.1. FPLC program

The 10% IGIV final container lot (LE 12 G 031) was fractionated into the IgG subclasses by rProtein A Sepharose separation using a FPLC-system.

IgG3 does not bind to the column and is therefore present in the flow through, IgG2 elutes at pHs 4.70 to 4.55 and IgG1 elutes at pHs 4.50 to 3.70(GE Healthcare product information; Scharf et al., 2001). IgG4, which amounts for 1-3% of the total IgG cannot be separated and is therefore present in the IgG1 as well as IgG2 fraction.
Measuring the optical density at 280nm allows to determine the protein concentration in each collected fraction and to differentiate between the individual IgG peaks.

It was expected that the first peak contains the IgG3 subclass, peak two the IgG2 subclass and peak three the IgG1 subclass. The second and third peaks are eluted sequently and peak 3 is present with a long tail.
Separation was not satisfying as IgG1 and IgG2 subclass were not fully separated and resulted in a long tailing of elution as can be seen on Figure 3. Therefore separation using FPLC-system was stopped and separation using ÄKTA was performed.

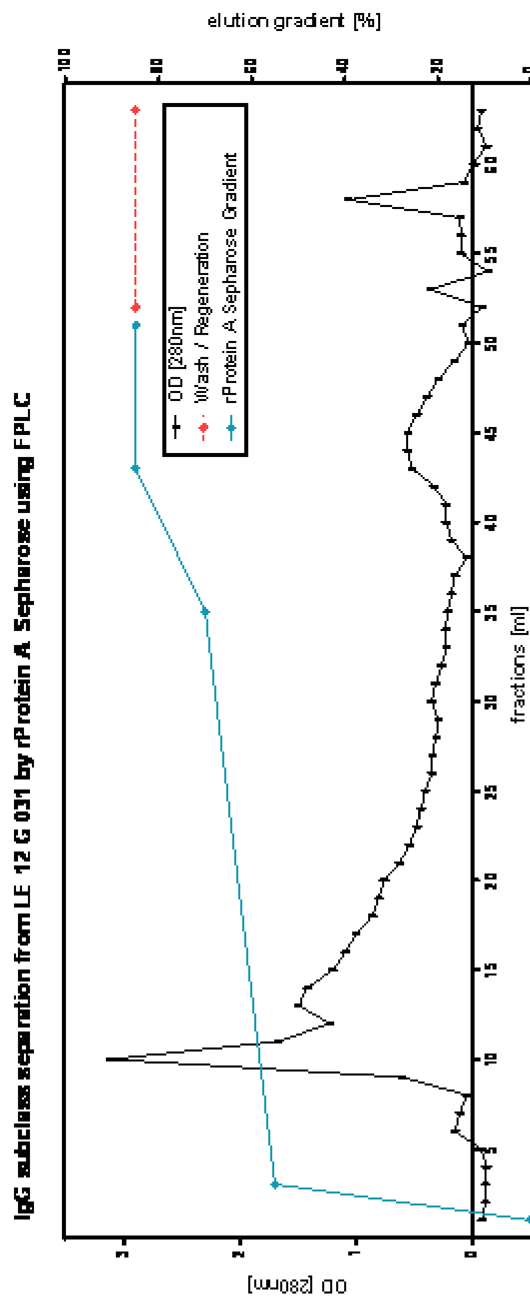


Figure 3 Separation of IgG Subclasses by rProtein A Sepharose (FPLC)
IgG subclass separation of 10% IGIV final container lot (LE 12 G 031) by FPLC-system. The protein concentration was measured at 280nm (black line) and the flow rate was held at 1.2 ml/min at room temperature.
The elution gradient for the rProtein A Sepharose separation is given in green and the dotted red line represents the wash and the regeneration respectively.

3.1.2. ÄKTA program (1)

Using the ÄKTA, the 10% IGIV final container lot (LE 12 G 031) was fractionated.

The flow through, peak 1 mainly consists of the IgG3 subclass (81%) as confirmed by ELISA (Table 4). IgG2 (86.6%) is primarily found in the peak 2. Peak 3 and 4 primarily contain IgG1 (peak 3: 84.8%; peak 4: 88.2%). Peak 5 was not further analyzed, as the fraction was not collected.

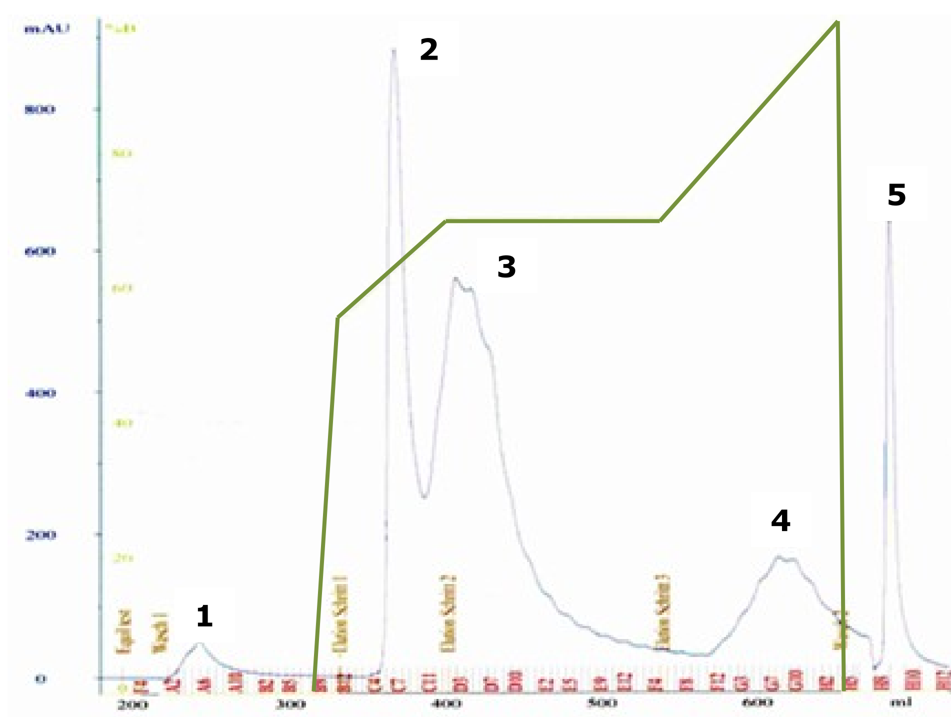


Figure 4 Separation of IgG subclasses by rProtein A Sepharose (ÄKTA)
IgG subclass separation of 10% IGIV final container lot (LE 12 G 031) by rProtein A Sepharose using ÄKTA. Given is the OD measured at 280nm (blue line) and the elution gradient (green line) for the rProtein A Sepharose separation. 5ml fractions were collected.
As confirmed by ELISA the flow through, peak1 contains IgG3 subclass, peak2 IgG2, peak 3 IgG1, peak 4 IgG1 and peak 5 the wash

Test article	conc. [µg/ml]	conc. calculated to the total volume [µg]	subclass conc. [%]
Peak 1 (total volume 40ml)			
IgG 1	4.8	192	5.5
IgG 2	11.5	460	13.1
IgG 3	71	2840	81.1
IgG 4	0.27	10.8	0.3
		3503	
Peak 2 (total volume 30ml)			
IgG 1	88.9	2667	6.1
IgG 2	1270	38100	86.6
IgG 3	83.9	2517	5.7
IgG 4	23.9	717	1.6
		44001	
Peak 3 (total volume 75ml)			
IgG 1	936	70200	84.8
IgG 2	118.7	8902.5	10.8
IgG 3	17.9	1342.5	1.6
IgG 4	30.9	2317.5	2.8
		82763	
Peak 4 (total volume 75ml)			
IgG 1	320.7	24052.5	88.2
IgG 2	35.6	2670	9.8
IgG 3	3.4	255	0.9
IgG 4	4.1	307.5	1.1
		27285	

Table 4 Analyzed Peaks by calculation of separated IgG subclasses by ÄKTA
Yield of IgG subclasses separated from IGIV final container lot (LE 12 G 031), after separation by ÄKTA. Given are the peaks 1 to 4 (shown in Graph #3), the concentration of each IgG subclass as [µg/ml], the concentration of each IgG subclass in total volume as [µg], the total IgG amount of all subclasses in total volume and the calculated IgG subclass content in each peak as [%]. Shaded values indicate main fraction within the respective peak.

3.1.3. ÄKTA program (2)

The protein concentrations of individual lots/fractions were analyzed after separation by ÄKTA (rProtein A Sepharose separation).
The typical lower IgG3 concentration obtained after separation, i.e. 0.53% in comparison with ~7.6% of IgG1 and IgG2 respectively, is a matter of necessary high wash volumes in order to separate IgG3 properly from the other IgG subclasses.

The yield of IgG1 enriched (LE 12 G 031) fraction (7.72%) and IgG2 enriched (LE 12 G 031) fraction (7.54%) are comparable with the IGIV lot (10%). (see Table 5)
The protein yield [%] was calculated in terms of total protein concentration in 10% IGIV final container (98,8g) versus protein concentration of the respective fraction relation.
The flow through is diluted by many column volumes of wash to reach IgG3-free IgG1 and IgG2 fractions and therefore the yield of IgG3 enriched (LE 12 G 031) fraction (0.5%) and IgG3 (Flow Through; LE 12 G 031) fraction (1.6%) is much lower than the concentration of IgG1 and IgG2 fractions.

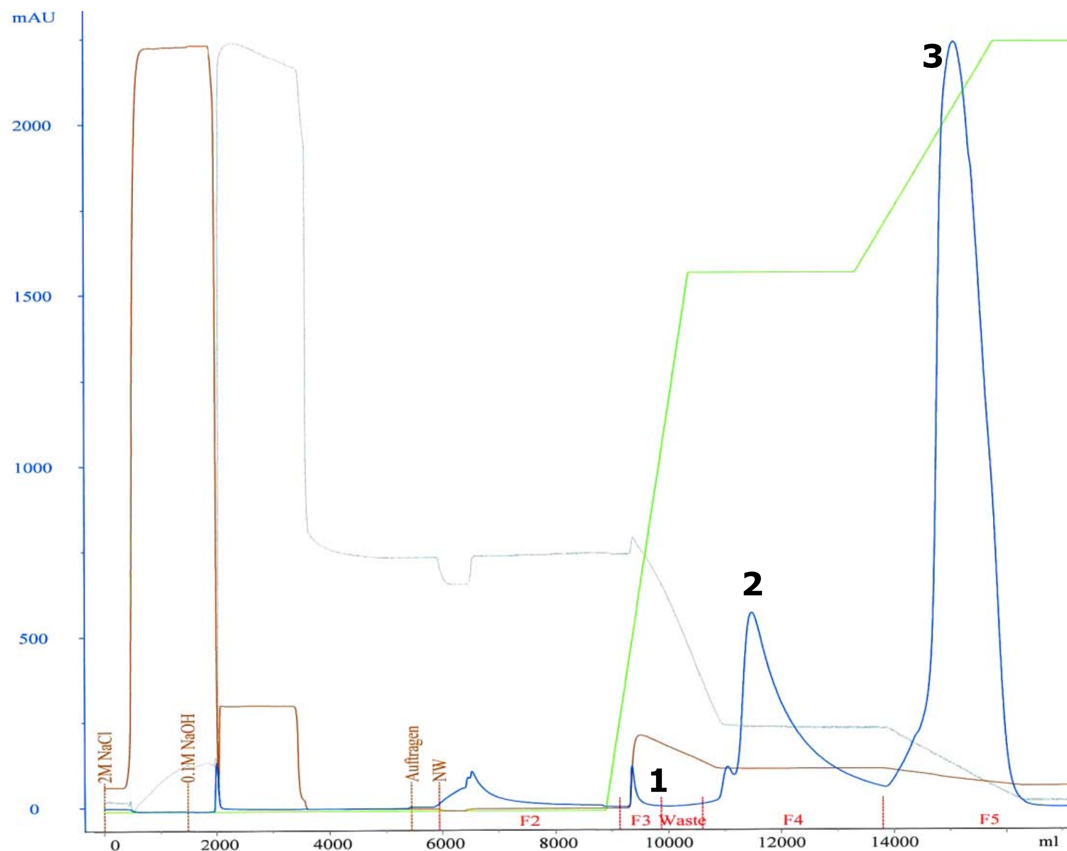


Figure 5 Separation of IgG subclasses by rProtein A Sepharose (ÄKTA)
IgG subclass separation of 10% IGIV final container lot (LE 12 G 031) by rProtein A Sepharose using ÄKTA. Given is the OD measured at 280nm (blue line), the elution gradient (green line) for the rProtein A Sepharose separation, the conductivity (brown line) and the pH (grey line). 5ml fractions were collected.
As confirmed by ELISA the flow through, peak1 contains IgG3 subclass, peak2 IgG2, peak 3 IgG1.

Biochemical Characteristics			
	protein conc [%]	protein conc [g]	yield [%]
10% IGIV (LE 12 G 031)			
10% IGIV	10	98.8	**100% = 98.8g**
IgG 1 enriched	7.72	47.37	48
IgG 2 enriched	7.54	16.74	17
IgG 3 enriched	0.53	0.47	0.5
IgG3 (Flow Through)	2.365	1.59	1.6
10% IGIV (LE 12 G 030)			
10% IGIV	10	98.8	**100% = 98.8g**
IgG 3 enriched	2.41	1.52	1.5

Table 5 Biochemical characteristics of separated IgG subclasses by ÄKTA
IgG subclass yield of two different 10% parent IGIV final container lots (LE 12 G 031, LE 12 G 030) after separation by ÄKTA. Given are the IGIV and subsequent fractions (IgG1, IgG2, IgG3) and the protein concentration as [%]. Final results are given as yield [%].

3.2. Elisa/RID

IgG subclasses separated by ÄKTA have been further analyzed by ELISA and RID to confirm purity of the individual subclasses.

Different methods were used and compared. The RID assay performed at Baxter's Quality Control in Lessines, resulted in comparable values determined by ELISA assay which was performed at Baxter's Preclinical R&D laboratory as well as Baxter's IPC laboratory.

The distribution of IgG subclasses in the 10% parent IGIV final container lot has been determined for better comparing of IgG values to fractionated lots.

Values determined by Lessines, Preclinical R&D and IPC Labor of IgG1 enriched (LE 12 G 031) fraction are comparable, as well as the ascertained values of IgG2 enriched (LE 12 G 031) fraction are comparable for Lessines and Preclinical R&D but not for IPC Labor.

The IgG3 enriched (LE 12 G 030) fraction was only determined by Preclinical R&D laboratory.

IgG3 fractions (LE 12 G 031) in comparison, the IgG3 content in the IgG3 flow through (28.4) was slightly higher than in the IgG3 enriched fraction (7.89; 6.71).

The yield of IgG3 enriched (LE 12 G 031) fraction is lower than IgG1 enriched fraction (7.89) and IgG2 enriched fraction (6.71).

	Lessines				Preclinical R&D				IPC Laboratory			
	IgG1	IgG2	IgG3	IgG4	IgG1	IgG2	IgG3	IgG4	IgG1	IgG2	IgG3	IgG4
	[µg/ml]											
10% IGIV (LE 12 G 031)												
IGIV*	n.d.	n.d.	n.d.	n.d.	60.5	27.6	5	2.2	n.d.	n.d.	n.d.	n.d.
IgG 1 enriched*	61.2	8.56	0.52	1.83	56.6	6.76	0.43	2.05	77	7.09	0.28	3.3
IgG 2 enriched*	2.51	61.26	2.11	1.85	<3.14	69.8	1.86	0.83	68.4	0.52	1.39	1.54
IgG3 enriched	n.d.	n.d.	n.d.	n.d.	0.16	<0.04	28.4	0.02	n.d.	n.d.	n.d.	n.d.
10% IGIV (LE 12 G 030)												
IGIV	n.d.	n.d.	n.d.	n.d.	71.8	33.19	5.38	0.02	n.d.	n.d.	n.d.	n.d.
IgG 3 enriched*	n.d.	n.d.	n.d.	n.d.	0.56	0.18	37.6	0.34	n.d.	n.d.	n.d.	n.d.

Table 6 ELISA/RID data of IGIV and subsequent IgG subclasses
IgG subclass yield of two 10% IGIV lots (LE 12 G 031, LE 12 G 030) after separation by ÄKTA. The values of IgG1, IgG2, IgG3 and IgG4 content [mg/ml] in 10% parent IGIV final container and the subsequent IgG subclasses was determined by RID/ELISA assay by three different laboratories.
* were used for in-vivo protection against WNV challenge – IGIV and respective IgG subclasses diluted to identical NT

The subclasses have been determined by three different ELISA operations and the mean value was built after correlation of all known data set ups. The data of Lessines and IPC have been correlated to Pre R&D data. Correlation analysis of all data sets for the IgG1 enriched (LE 12 G 031) fraction is significant and therefore a mean value of all available data sets can be build. (Lessines: PreR&D r^2=0.9994, IPC: PreR&D r^2=0.9991) Correlation analysis of two data sets (Lesssines, PreR&D) for the IgG2 enriched (LE 12 G 031) fraction is significant and therefore a mean value of the two data sets can be build. (Lesssines: PreR&D r^2=0.9996)

Using ELISA, purification and enrichment of IgG subclasses is confirmed. The mean value of IgG1 enriched (LE 12 G 031) fraction averages from all three data set ups (Lessines, IPC, Pre R&D) (64.93 mg/ml).
The mean value of IgG2 enriched (LE 12 G 031) fraction averages from two data set ups (Lessines, Pre R&D) (65.53 mg/ml).

	IgG1 enriched (LE 12 G 031)					**IgG2 enriched (LE 12 G 031)**				
	IgG1 [µg/ml]	IgG2 [µg/ml]	IgG3 [µg/ml]	IgG4 [µg/ml]	mean value IgG1	IgG1 [µg/ml]	IgG2 [µg/ml]	IgG3 [µg/ml]	IgG4 [µg/ml]	mean value IgG2
Preclinical R&D	56.6	6.76	0.43	2.05	64.93	<3.14	69.8	1.86	0.83	65.53
Lessines	61.2	8.56	0.52	1.83		2.51	61.26	2.11	1.85	
IPC Laboratory	77	7.09	0.28	3.30		68.4	0.52	1.39	1.54	---

Table 7 Correlated data for the IgG1 enriched fraction (LE 12 G 031) and IgG2 enriched fraction (LE 12 G 031)
Data for IgG1 and IgG2 fractions determined by either a laboratory in Lessines or generated by the IPC laboratory were correlated to data generated by ELISA in the Pre R&D laboratory.

3.3. HPLC

Structural functionality of the IgG subclasses was determined using HPLC analysis.

The sizes of IgG Dimers are in the range of 270kDa and 350kDa and IgG Monomers are approximately 160kDa in size.

A size of $\leq$100kDa confirms fragmentation of the IgG (Pier Gerald B.; Immunology, Infection and Immunity; 2004 - 1st Edition).

Several analyses of similar IgG subclass fractions have been performed between November 2007 and February 2008. Structural integrity has been confirmed by HPLC analysis in January 2009.

Analysis was performed shortly after IgG subclass separation, i.e. 2007 and 2008 and one to two years later. Therefore the structural integrity of the IgG subclasses has been monitored over a two year time span.

As IgG1 subclass, IgG2 subclass, IgG3 subclass and IgG4 subclass are nearly the same size, both 10% parent IGIV final container lots (LE 12 G 031, LE 12 G 030) show that no fragmentation has taken place (see Table 7).

The results confirm that IgG1 subclass enriched (LE 12 G 031) fraction has not been fragmented and is therefore functional (2009: 87.69%, 2008: 90.47%), also the IgG2 subclass enriched (LE 12 G 031) fraction is not fragmented. (2009: 96.09%, 2008: 98.85%)

The HPLC analysis revealed that IgG3 subclass enriched (LE 12 G 031) is structurally impaired as 70.71% are <60kDa.

IgG3 subclass enriched (LE 12 G 031) fraction shows that in February 2008 (160kDa: 98.27%) the IgG3 was present as Monomers and in January 2009 (270kDa: 97.81%) IgG3 subclass is present as Dimer.

IgG3 subclass enriched (LE 12 G 030) fraction is predominantly present as Dimer. (2009: 74.92%, 2007: 82.96%)

	Peak 1 >450kDa %area	Peak 2 ~350kDa %area	Peak 3 ~270kDa %area	Peak 4 ~160kDa %area	Peak 5 ~100kDa %area	Peak 6 <60kDa %area	Date of analysis
LE 12 G 031							
10% IGIV	0.19	11.00	-----	88.16	-----	65	Jan 09
IgG1	0.49	11.62	-----	87.69	-----	0.2	Jan 09
	0.33	9.20	-----	90.47	-----	-----	Feb 08
IgG2	0.27	3.49	-----	96.09	-----	0.15	Jan 09
	0.1	1.05	-----	98.85	-----	-----	Feb 08
IgG3 Part 1	-----	-----	-----	4.23	25.06	70.71	Jan 09
	0.60	9.00	-----	27.09	15.30	48.02	Feb 08
IgG3 Part 2 - enriched	1.07	-----	97.81	-----	-----	1.12	Jan 09
	0.24	0.71	-----	98.27	-----	0.77	Feb 08
LE 12 G 030							
10% IGIV	0.19	9.47	-----	89.83	-----	0.51	Jan 09
IgG3	11.90	-----	74.92	-----	1.86	11.31	Jan 09
	5.58	-----	82.96	-----	1.30	10.17	Nov 07

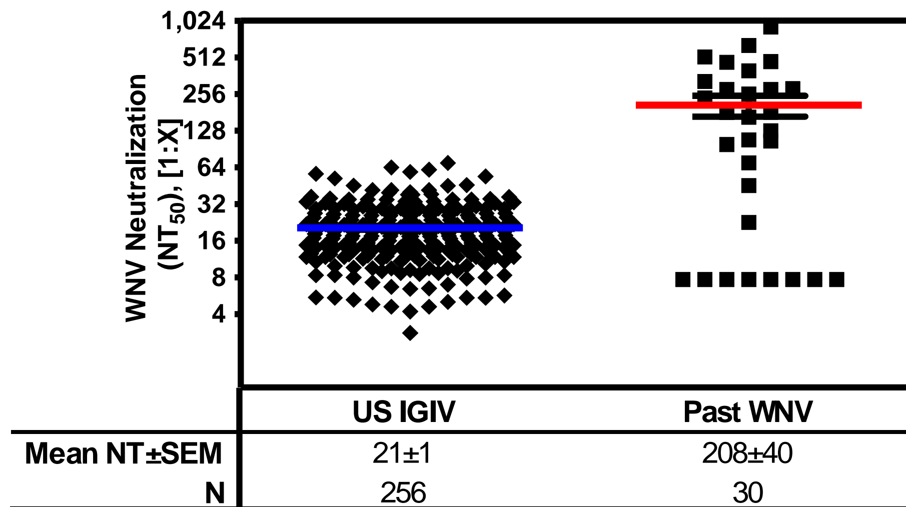
Table 8 HPLC-data of IGIV and subsequent IgG subclasses
IgG subclass HPLC-data of two different 10% parent IGIV final container lots (LE 12 G 031, LE 12 G 030). Given is the content of IgG [%] in the different size categories given as peak 1 - 6, representing kDa measured by UV-detection [280nm]. In addition the date of analysis is given.

3.4. WNV micro neutralization assay

3.4.1. IGIV lots released in 2008

IGIV lots were tested undiluted, whereas sera were diluted 1:10. WNV neutralization titers were calculated as NT_{50} and are expressed as mean ± Standard Error of the Mean (SEM). Titer differences between groups were evaluated by an unpaired t-test.

Based on an extrapolation from the WNV screening of the US blood supply (Busch et al., 2006)the annual number of WNV infections in the US was calculated. Briefly, the number of neuroinvasive cases reported for the year to the US Centers for Disease Control and Prevention (CDC) through ArboNET was multiplied by 256, i.e. the factor between all WNV infections and neuroinvasive cases. The cumulative infection rate for every year was then calculated by dividing the infections that had occurred up to that year by the US population for that year, according to the US Census Bureau (accessed at http://www.census.gov/popest/states/NST-ann-est.html). Plasma obtained from people after WNV infection showed a dramatically higher titer, with a mean ± SEM titer of 1:208±40 for 30 individuals available for testing. When corrected for the IgG concentration in plasma as compared to the 10% IGIV preparations, the mean neutralization titer of the patient samples was almost exactly 100-fold higher than that of the IGIV lots tested (1:2,080 versus 1:21).



	US IGIV	Past WNV
Mean NT±SEM	21±1	208±40
N	256	30

Figure 6 *In-vitro* WNV NT determined for IGIV final container lots released in the year 2008 and for plasma from donors past WNV infection
WNV neutralization titers determined for 10% IGIV final container lots and for Plasma past WNV infection, which were provided by the American Red Cross (ARC) and from Department of Health (Minnesota). WNV neutralization titers were calculated as NT_{50}, i.e. the test article dilution that resulted in 50% virus neutralization, with detection limits of 1:$\leq$0.8 for undiluted IGIVs and 1:$\leq$7.7 for pre-diluted sera, and are expressed as mean$\pm$SEM.

3.4.2. ***In vitro*** Neutralization capacity of parent IGIV and derived IgG subclasses

WNV neutralization titers were determined for 5% IGIV, 10% IGIV lots as well as the respective IgG subclass fractions.

The samples were tested at least as duplicates. The test was performed on different days and performed by different operators.

In comparison to the NTs of the parent 10% IGIV final container lot with the subsequent fractionated IgG subclasses reveals that almost the entire ***in vitro*** neutralization capacity is conferred exclusively by the IgG1 subclass. (Table 8 Panel A)

At identical protein concentration the IgG1 (31±5; 7) enriched fraction has a higher NT than the parent 10% IGIV final container lot (28±5; 7) (Table 5). Compared with the other IgG subclasses, IgG1 subclass has an approximately 15-fold higher NT than IgG2 subclass and an approximately 10-fold higher NT than the IgG3 subclass fraction (Table 8), respectively.

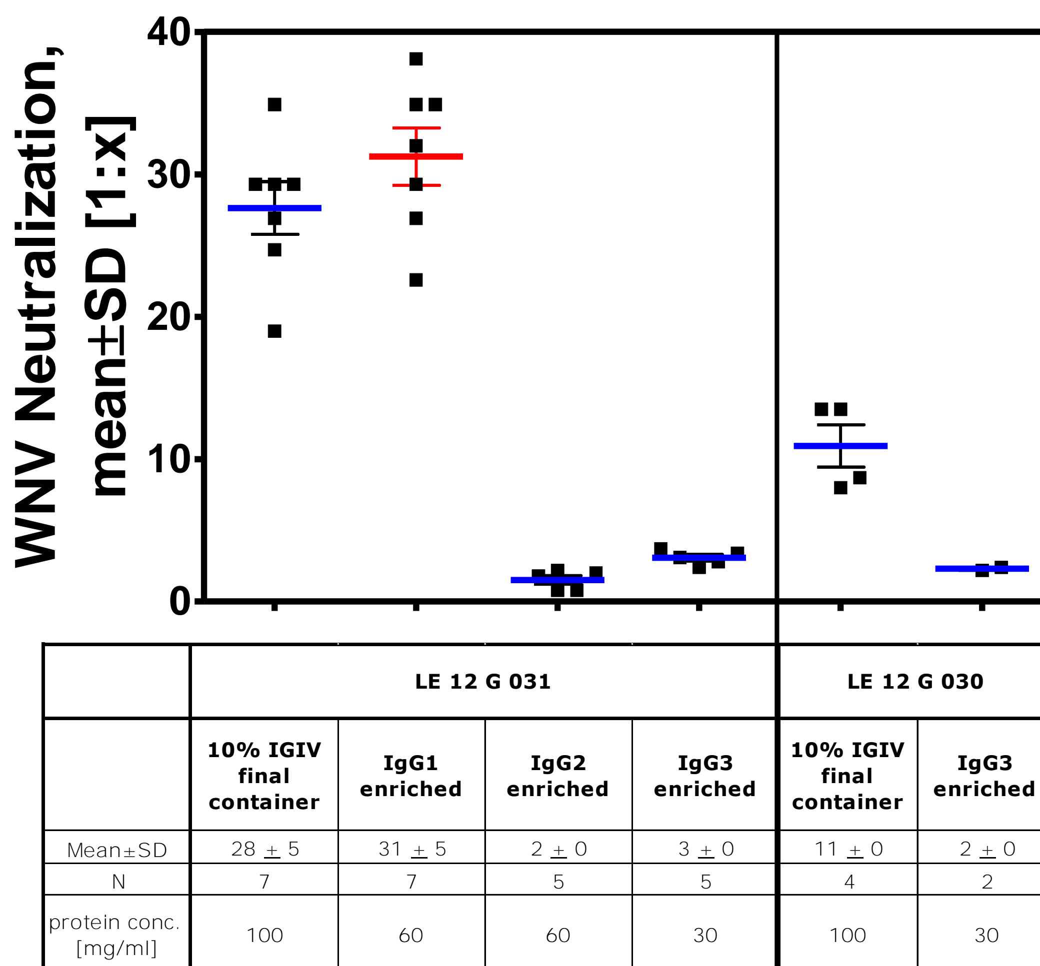
The 10% IGIV lots (LE 12 G 031, LE 12 G 030) analyzed, have a different neutralization capacity against WNV. The LE 12 G 031 lot has about 3 times higher neutralization capacity than the LE 12 G 030 lot.

IgG3 subclass fractions of both analyzed parent 10% IGIV final container lots have a similar, low NT. Compared to the parent 10% IGIV final container lots, IgG3 subclass fraction has a 10-fold lower NT than LE 12 G 031 and a 5-fold lower NT than LE 12 G 030. (Table 8, Panel A/B)

The relatively low IgG3 neutralization titer is somewhat surprisingly as IgG1 and IgG3 are generally described as the major subclass contributors to viral clearance (Skvaril, 1986).

Another 5% final container IGIV product, Endobulin, has a rather low NT capacity (9±2; 7) against WNV, i.e. 3 times lower than IgG1 subclass fraction (LE 12 G 031). Due to a different manufacturing process, IgG3 is virtually absent in this product (Audet et al., 2006).

5% Endobulin was used at a protein concentration of 50mg/ml, IgG1 and IgG2 diluted to a protein concentration of approximately 60mg/ml, i.e. roughly comparable protein concentrations.



	LE 12 G 031				LE 12 G 030	
	10% IGIV final container	**IgG1 enriched**	**IgG2 enriched**	**IgG3 enriched**	**10% IGIV final container**	**IgG3 enriched**
Mean±SD	28 ± 5	31 ± 5	2 ± 0	3 ± 0	11 ± 0	2 ± 0
N	7	7	5	5	4	2
protein conc. [mg/ml]	100	60	60	30	100	30

Figure 7 *In-vitro* WNV NT determined for IGIV final container and subsequent IgG subclasses
WNV neutralization titers determined for two 10% IGIV final container lots and the subsequent IgG subclass fractions (IgG1, IgG2, IgG3). The calculated neutralization titer is given as mean±SD and the protein concentration is given as [mg/ml].

	Test article (LOT number)	Dilution (1:V)	Count of negative wells (N_{neg})	NT_{50}	Mean NT_{50} (1:X)	SD	N
A	10% IGIV	2	37	34.9	27.6	5	7
		2	33	24.7			
		2	30	19.0			
		2	35	29.3			
		2	35	29.3			
		2	34	26.9			
		2	35	29.3			
	IgG1 enriched	2	32	22.6	31.2	5	7
		2	36	32.0			
		2	38	38.1			
		2	34	26.9			
		2	35	29.3			
		2	37	34.9			
		2	37	34.9			
	IgG2 enriched	2	0	≤0.8	2.0	0	5
		2	0	≤0.8			
		2	5	2.2			
		2	3	1.8			
		2	4	2.0			
	IgG3 enriched	2	8	2.8	3.1	1	5
		2	9	3.1			
		2	10	3.4			
		2	6	2.4			
		2	11	3.7			
B	10% IGIV	2	21	8.7	10.9	3	4
		2	20	8.0			
		2	26	13.5			
		2	26	13.5			
	IgG3 enriched	2	6	2.4	2.3	0	2
		2	5	2.2			
C	5% IGIV	2	19	7.3	9.4	2	7
		2	20	8.0			
		2	21	8.7			
		2	20	8.0			
		2	24	11.3			
		2	25	12.3			
		2	23	10.4			

Table 9 *In-vitro* WNV NT determined for IGIV final container and subsequent IgG subclasses
See Figure 8
Given is the test article, the first dilution used in the test setup and the number of negative wells.

3.5. *In vivo* protection against a WNV challenge

3.5.1. *In vivo* protection against a WNV challenge - IGIV and the respective IgG subclasses diluted to **identical protein concentration**

In a mostly lethal WNV challenge model the protection offered by IGIV and the respective IgG subclasses diluted to certain identical protein concentration was investigated. Therefore all test items, i.e. 5% and 10% IGIV final container and IgG subclasses were diluted in PBS to contain 60 and/or 30mg/ml.
WNV neutralization titers were determined for IGIV lots as well as the respective IgG subclass fractions as described earlier. (see 3.4.2 High titer IGIV lot and subsequent IgG subclasses)

Significantly higher survival rates as well as symptom free survival was observed when mice were passively protected by prophylaxis, i.e. IGIV and subsequent IgG subclasses diluted to contain 60 and/or 30mg/ml, two hours before WNV challenge compared to the challenge control group.

When all test items were diluted to contain 60mg/ml, IgG1 contains over proportional WNV neutralization capacity compared to other subclasses ***in vitro***.
100% protection for mice treated with IgG1 subclass ($p=0.0126$ compared to challenge control), whereas 80% of mice protected with IgG2 survived ($p=0.0448$ compared to challenge control).

IgG3, only available in a protein concentration of 30mg/ml was not used in the described experiment.
Symptom free survival is measured as mice, which survived without showing any symptoms of illness. It can be seen that the symptom free survival for 10% parent IGIV final container lot and subsequent subclasses is equivalent to the observed symptom rates ($p=0.0031$ compared to challenge control group). The WNV challenge control of $TCID_{50}$ confirms that all mice suffered from WNV infection and symptoms were present by day 8.

When test items were diluted to contain 30mg/ml, 100% of the 10% parent IGIV final container, IgG1 subclass and IgG2 subclass treated mice survived ($p=0.0126$) the otherwise mostly lethal WNV challenge. In contrast, only 40% of mice protected with IgG3 subclass survived ($p=0.2739$ compared to challenge control group), which is not significantly higher than the survival observed in the WNV challenge control group (see Figure 9). This IgG3 subclass fraction was determined to be functionally impaired, as HPLC analysis revealed 48.02% fragmentation (see 3.3 HPLC, Table 8).

Symptom free survival for the 10% parent IGIV final container ($p=0.0031$ compared to challenge control group) and IgG3 subclass ($p=0.029$ compared to challenge control group) treated mice was worse than the survival, e.g. all mice protected by IgG3 suffered from WNV symptoms. In addition the WNV challenge control of $TCID_{50}$ shows that all mice suffered from WNV infection by day 7.

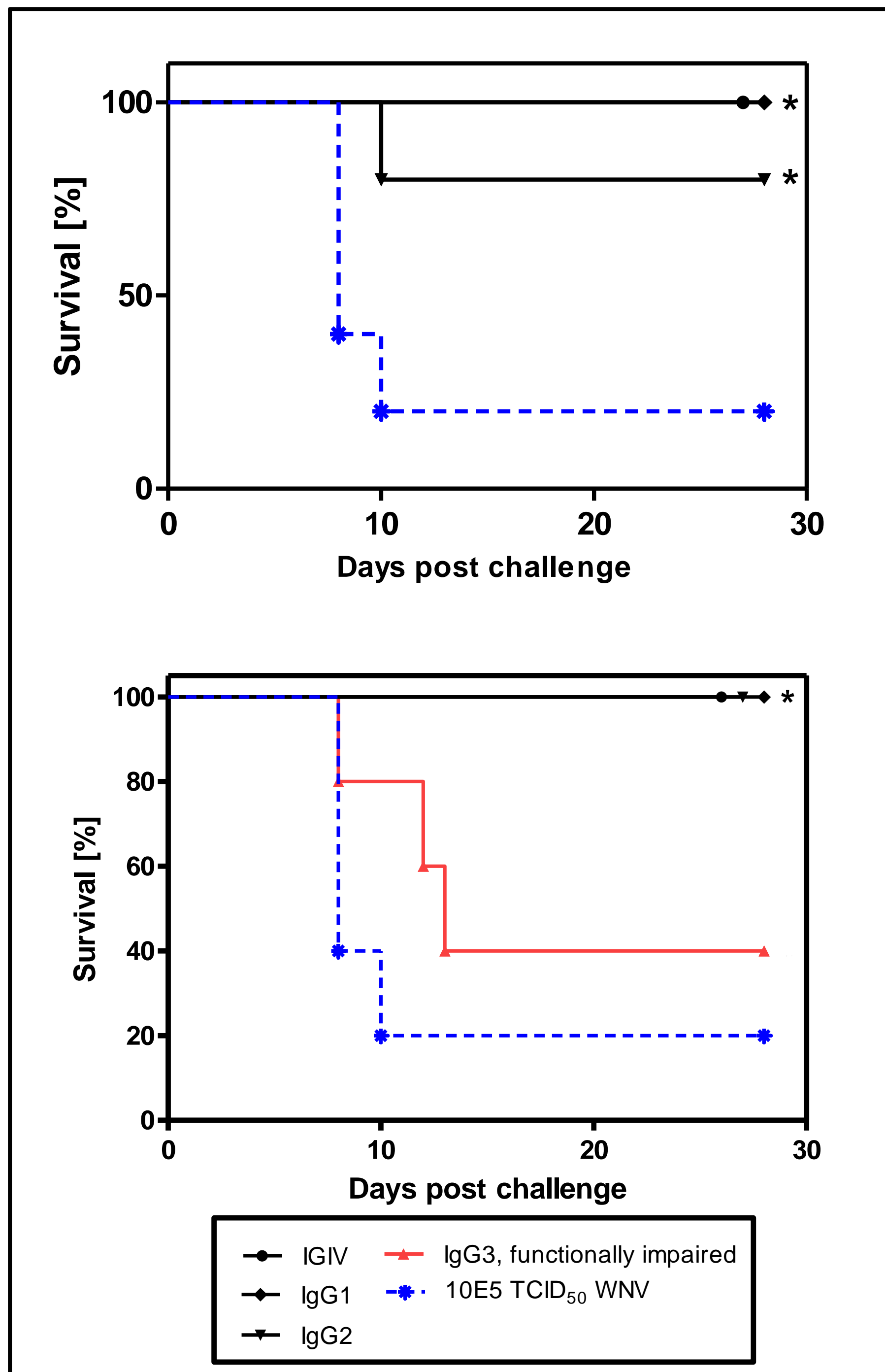


Figure 8 *In vivo* protection by US-IGIV and IgG subclasses at 60mg/ml (A) or 30mg/ml (B) against a WNV challenge (10E5TCID$_{50}$;s.c.)
Survival at a protein concentration of 60mg/ml (Panel A) and mice protected by a protein concentration of 30mg/ml (Panel B) was monitored for 28 days. Groups of 5 mice were treated with 5% or 10% parent IGIV and subsequent IgG subclasses. Asterisks indicate significant differences between mice protected with 10% IGIV/subsequent IgG subclasses and WNV challenge group.
(p***: <0.001,p**: 0.001 to 0.01, p*: 0.01 to 0.05)

3.5.2. *In vivo* protection against a WNV challenge - IGIV and the respective IgG subclasses diluted to **identical WNV neutralization capacity**

In vivo protection of 10% parent IGIV final container lots (LE 12 G 030, LE 12 G 031), as well as the diluted respective IgG subclass fractions were determined.
Two hours before WNV challenge, mice received a sc injection of 0.2 ml of 10% IGIV final container/ IgG1 subclass/ IgG2 subclass/ IgG3 subclass all diluted to a NT of 2 and 0.2 respectively. This time IgG3 subclass fraction fully functionally intact as HPLC analysis revealed approximately 88% intact (see 3.3 HPLC, Table 8).

When test items are diluted with PBS to a NT of 2, mice treated with IgG1 subclass showed 100% survival ($p<0.0001$ compare to challenge control group), whereas protection with the IgG3 subclass ($p=0.0005$ compared to challenge control group) and IgG2 subclass ($p=0.0002$ compared to challenge control group) resulted in 90% survival.
At the same NT determined *in vitro*, IgG1 subclass confers a higher protection than the 10% parent IGIV final container (100% vs. 95%).
Mice treated with IgG2 subclass showed 80% symptom free survival ($p=0.0001$ compared to challenge control group), whereas mice treated with 10% parent IGIV final container (LE 12 G 031) and IgG3 subclass showed approximately 90% symptom free survival. 100% symptom free survival was obtained by IgG1 subclass (see Figure10)
In addition 100% of the WNV challenge control displayed symptoms by day 8 post infection.

The results show, that the IgG1 subclass is the most protective subclass against a WNV infection *in vivo*.

Also at the NT of 0.2 the IgG1 subclass is the subclass that confers highest protection (70%), even higher than 10% parent IGIV final container lot ($p=0.0123$ compared to challenge control).

The IgG1 subclass conferred survival in 60% of the mice, whereas only 30% of mice treated with IgG3 subclass survived the WNV challenge (p=0.057 compared to challenge control).
The IgG2 subclass (p=0.3329 compared to challenge control) shows a significant overall lesser protection than IgG1.
In addition 90% of the WNV challenge control displayed symptoms by day 12 post infection.

Symptoms from the WNV challenge were present in mice treated either with the 10% parent IGIV final container lot, IgG1 subclass, IgG2 subclass and IgG3 subclass.
In addition 100% of the WNV challenge control displayed symptoms by day 8 post infection.

IgG2 and IgG3 both inferior to IgG1 in affording protection from lethal infection and from WNV disease, respectively.

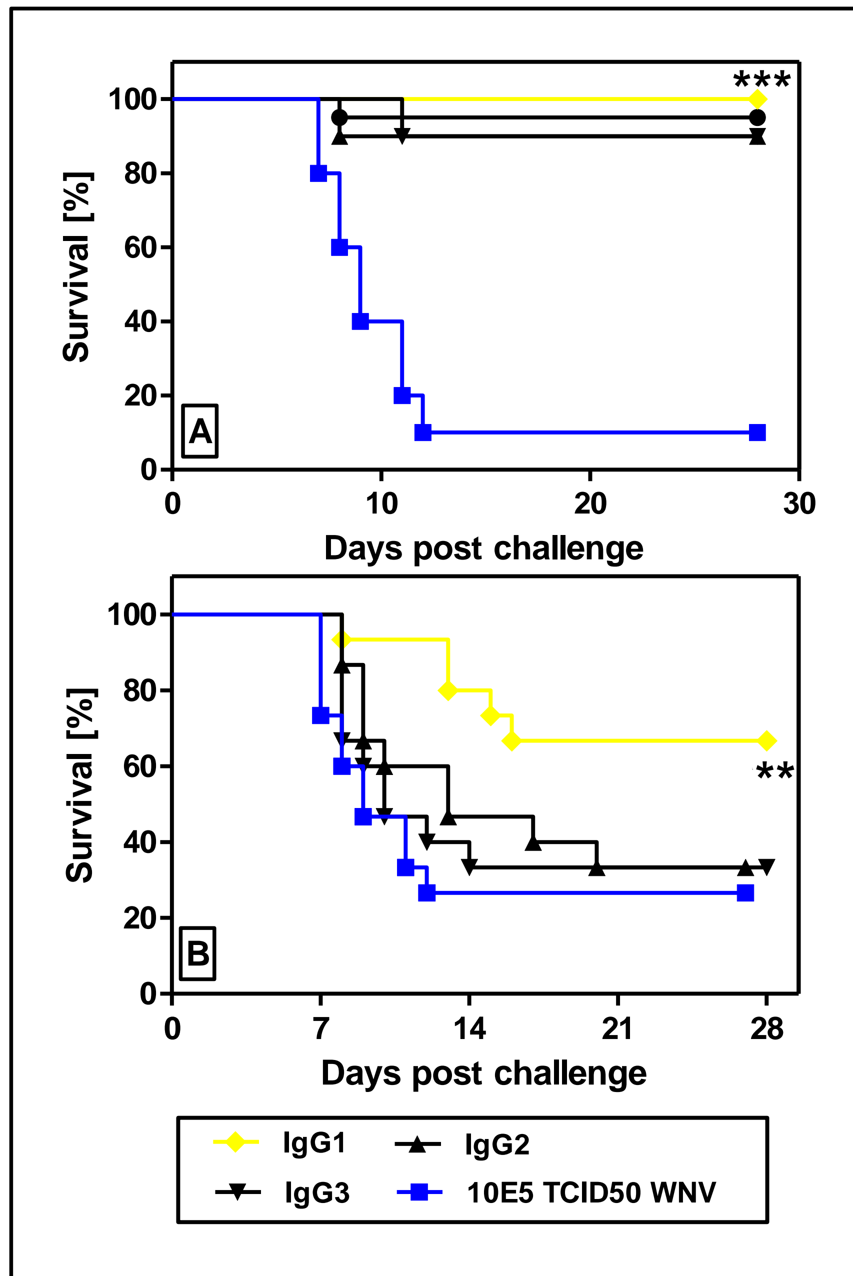


Figure 9 *In vivo* protection by US-IGIV and IgG subclasses at NT 2 (A) or NT 0.2 (B) against a WNV challenge (10E5TCID$_{50}$;s.c.)
Survival at a NT of 2 (Panel A) Survival at a NT of 0.2 (Panel B) was monitored for 28 days.
3 independent experiments (NT 0.2) were performed, and 5 mice per group/experiment were used.2 independent experiments (NT 0.2) were performed, and 5 mice per group/experiment were used.
Asterisks indicate significant differences between mice protected with 10% IGIV/subsequent IgG subclasses and WNV challenge group.
(p***: <0.001,p**: 0.001 to 0.01, p*: 0.01 to 0.05)

Conclusion of ***in vitro*** neutralization and ***in vivo*** protection

IgG1 is over proportional induced after WNV infection. At identical protein concentration, i.e. 30mg/ml, IgG1 subclass contains significantly higher levels of WNV neutralizing antibodies compared to the 10% parent IGIV final container lot, the IgG2 subclass and the IgG3 subclass. Asterisks indicate differences that were statistically significant compared to the 10% parent IGIV final container lot (****** p=0.0023 compared to challenge control)

To evaluate the level of protection afforded by IGIV a mostly lethal WNV challenge model was investigated ***in vivo***.

At identical WNV neutralization titers, superior protection can be seen by IgG1 subclass in a lethal WNV challenge model. At suboptimal neutralization capacity, i.e. NT_{50} 0.2, prophylaxis with the IgG1 subclass resulted in significantly increased survival rates compared to the 10% parent IGIV final container lot, the IgG2 subclass, the IgG3 subclass and the control. The survival outcomes of the IgG2 and IgG3 subclass treated groups did not differ from the WNV challenge control group. Asterisks indicate differences that were statistically significant compared to the control group (****** p=0.0057 compared to challenge control)

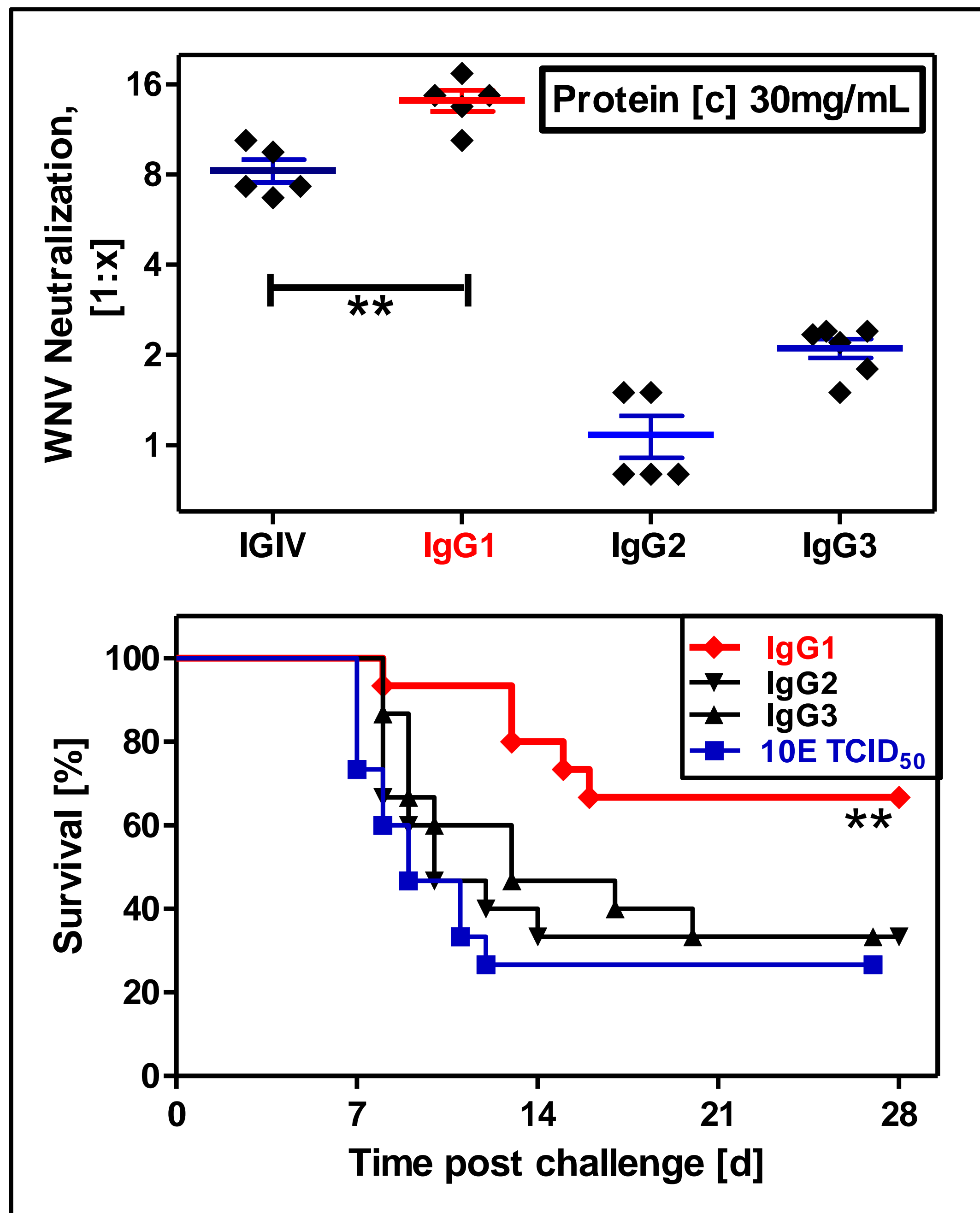


Figure 10 *In vitro* neutralization at same protein concentration, i.e. 30mg/ml, and *in vivo* protection at suboptimal neutralization capacity, i.e. NT 0.2

WNV neutralization titers (Panel A) determined for 10% IGIV final container lot (LE 12 G 031), the subsequent IgG subclass fractions (IgG1, IgG2; IgG3). The calculated neutralization titer is given as mean±SD.

Survival (Panel B) was monitored for 28 days. Three independent experiments with groups of N=5 mice were treated with 10% parent IGIV and subsequent IgG subclasses.

Differences in WNV neutralization titer between individual test articles were evaluated by unpaired t test.

4. Discussion

WNV, an emerging virus in the US, is responsible for infections in approximately 1% of a formerly naïve population since its introduction ten years ago.
Consequently, IGIV produced from the plasma of thousands of healthy US donors contain WNV neutralizing antibodies with titers ranging between non-reactive to highly protective, as shown by pre-exposure treatment protective in a mostly lethal WNV challenge mouse model (Planitzer et al., 2007).

The IgG subclass separation was performed using an IGIV at high WNV neutralization capacity by a pH gradient elution from a rProtein A-Sepharose, as previously described (Scharf et al., 2001).

Due to the high protease sensitivity of the IgG3 subclass, the IgG3 subclass of the first separated lot (LE 12 G 031) was degraded. For another IGIV lot (LE 12 G 030), the structural integrity of the IgG3 fraction was confirmed by HPLC analysis and this fraction was further used in the second ***in vivo*** experiment, where test items were diluted to contain the same WNV neutralization capacity.
The HPLC data confirmed the structural integrity and ELISA data of two correlated laboratories proved the purity of the IgG subclass fractions.
To make the IgG subclasses comparable among each other single subclass fractions have been concentrated.

IgG1 contains a significantly higher neutralization capacity than any other IgG subclass or even the parent IGIV, compared at equivalent protein concentration, i.e. 30 mg/ml, as shown on Figure 1 and Table 3.
IgG1 has a 15-fold higher neutralization capacity than IgG2 and a 10-fold higher than IgG3.

This means IgG1 subclass rather than IgG3 subclass is over proportional induced after WNV infection and is also the most potent IgG subclass in WNV clearance.
However, there are controversial data on the importance of IgG subclasses in viral clearance.
IgG3 subclass from polyclonal HIVIG has enhanced anti-HIV-1 activity compared to that of IgG1 and IgG2 subclass as described earlier (Scharf et al., 2001).
It was suggested that the more flexible hinge region of IgG3 subclass could lead to better virus neutralization activity.
Other previously published studies describe IgG1 subclass as most effective for neutralization and antibody response to envelope antigens has been reported to be primarily limited to IgG1 (Cavacini et al., 2003).

5% IGIV, Endobulin, produced by using an earlier manufacturing process, the IgG3 subclass is impaired due to protease treatment.
Measles neutralization capacity of 5% IGIV, with low IgG3 antibody levels, was much lower than with 10% IGIV as previously shown (Audet et al., 2006).
However, the WNV neutralization capacity of the 5% IGIV product was in the range of the 10% parent IGIV lot.
So, from our results it looks like there is little if any contribution of IgG3 subclass in WNV neutralization.

Neutralizing antibodies elicited after human WNV infection, are predominantly of the IgG1 subclass. When adjusted to identical neutralization titers, IgG1 is also most protective, which might be based on the more effective adaptor functions of IgG1 with other parts of the immune system (Nimmerjahn and Ravetch, 2008).
The IgG2 and IgG3 subclasses show lower protection as the control group does.
There is only data of mouse IgG subclasses in an in vivo test set up available since now (Wilson et al., 2000).

All of the completely protective antibodies were of the IgG2a subclass in the presence of complement C1q, whereas IgG1 and IgG3 subclass of the mouse were not protective. IgG2a subclass binds C1q more effectively than IgG1 and IgG3 subclass of the mouse and varies in its affinity for different Fc receptors as described before (Wilson et al., 2000).
Mouse IgG2a subclass is claimed to be comparable to human IgG1 and IgG3 subclass (Nimmerjahn and Ravetch, 2008), which makes it more difficult to differentiate between the subclasses.
huFcγRI is centrally involved in antibody-dependant cellular cytotoxicity (ADCC). The ran**king of binding to the huFcγ**RI is IgG1 > IgG3 > IgG4 ≥ IgG2 (Nimmerjahn and Ravetch, 2008; Unkeless et al., 1988). Based on the varying affinity and specifici**ty of IgG subclasses to different FcγR receptors,** their ability to mediate effector responses differ substantially (Nimmerjahn and Ravetch, 2005).
Both, human IgG1 and IgG3, which are considered to be the most proinflammatory IgG subclasses and dominate antiviral immunity bind to the activating hu**FcγRI** (Nimmerjahn and Ravetch, 2008).
The underlying molecular basis (i.e. effector functions) of the protection afforded by IgG subclasses is so far poorly understood. Therefore the utility of approach is the necessary basis to understand the molecular differences of the IgG subclasses.

In general, despite the capacity of different IgG subclasses to bind to **several FcγR receptors with varying specificity and affinity** ***in vitro***, this does not necessarily imply that all of these receptors are involved in mediating the effector functions of a specific IgG subclass ***in vivo*** (Nimmerjahn and Ravetch, 2008).
Especially for the development of a WNV vaccine, it might be desirable to (also) effectively induce WNV neutralizing antibodies of the IgG1 subclass.

Reference List

Audet S, Virata-Theimer ML, Beeler JA, Scott DE, Frazier DJ, Mikolajczyk MG, Eller N, Chen FM, Yu MY (2006) Measles-virus-neutralizing antibodies in intravenous immunoglobulins. J Infect Dis 194:781-789.

Ben-Nathan D, Gershoni-Yahalom O, Samina I, Khinich Y, Nur I, Laub O, Gottreich A, Simanov M, Porgador A, Rager-Zisman B, Or N (2009) Using high titer West Nile intravenous immunoglobulin from selected Israeli donors for treatment of West Nile virus infection. BMC Infect Dis 9:18.

Busch MP, Wright DJ, Custer B, Tobler LH, Stramer SL, Kleinman SH, Prince HE, Bianco C, Foster G, Petersen LR, Nemo G, Glynn SA (2006) West Nile virus infections projected from blood donor screening data, United States, 2003. Emerg Infect Dis 12:395-402.

Campbell GL, Marfin AA, Lanciotti RS, Gubler DJ (2002) West Nile virus. Lancet Infect Dis 2:519-529.

Cavacini LA, Kuhrt D, Duval M, Mayer K, Posner MR (2003) Binding and neutralization activity of human IgG1 and IgG3 from serum of HIV-infected individuals. AIDS Res Hum Retroviruses 19:785-792.

Chowers MY, Lang R, Nassar F, Ben-David D, Giladi M, Rubinshtein E, Itzhaki A, Mishal J, Siegman-Igra Y, Kitzes R, Pick N, Landau Z, Wolf D, Bin H, Mendelson E, Pitlik SD, Weinberger M (2001) Clinical characteristics of the West Nile fever outbreak, Israel, 2000. Emerg Infect Dis 7:675-678.

Kreil TR, Berting A, Kistner O, Kindermann J (2003) West Nile virus and the safety of plasma derivatives: verification of high safety margins, and the validity of predictions based on model virus data. Transfusion 43:1023-1028.

Lanciotti RS, Ebel GD, Deubel V, Kerst AJ, Murri S, Meyer R, Bowen M, McKinney N, Morrill WE, Crabtree MB, Kramer LD, Roehrig JT (2002) Complete genome sequences and phylogenetic analysis of West Nile virus strains isolated from the United States, Europe, and the Middle East. Virology 298:96-105.

MacKenzie JS, Gubler DJ, Petersen LR (2004) Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat Med 10:S98-109.

Marfin AA, Gubler DJ (2001) West Nile encephalitis: an emerging disease in the United States. Clin Infect Dis 33:1713-1719.

Mehlhop E, nsarah-Sobrinho C, Johnson S, Engle M, Fremont DH, Pierson TC, Diamond MS (2007) Complement protein C1q inhibits antibody-dependent enhancement of flavivirus infection in an IgG subclass-specific manner. Cell Host Microbe 2:417-426.

Mukhopadhyay S, Kim BS, Chipman PR, Rossmann MG, Kuhn RJ (2003) Structure of West Nile virus. Science 302:248.

Nimmerjahn F, Ravetch JV (2005) Divergent immunoglobulin g subclass activity through selective Fc receptor binding. Science 310:1510-1512.

Nimmerjahn F, Ravetch JV (2008) Fcgamma receptors as regulators of immune responses. Nat Rev Immunol 8:34-47.

Petersen LR, Marfin AA (2002) West Nile virus: a primer for the clinician. Ann Intern Med 137:173-179.

Petersen LR, Roehrig JT (2001) West Nile Virus: A Reemerging Global Pathogen. Emerg Infect Dis 7:611-614.

Planitzer CB, Modrof J, Kreil TR (2007) West Nile virus neutralization by US plasma-derived immunoglobulin products. J Infect Dis 196:435-440.

Samuel MA, Diamond MS (2006) Pathogenesis of West Nile Virus infection: a balance between virulence, innate and adaptive immunity, and viral evasion. J Virol 80:9349-9360.

Scharf O, Golding H, King LR, Eller N, Frazier D, Golding B, Scott DE (2001) Immunoglobulin G3 from polyclonal human immunodeficiency virus (HIV) immune globulin is more potent than other subclasses in neutralizing HIV type 1. J Virol 75:6558-6565.

Sejvar JJ, Haddad MB, Tierney BC, Campbell GL, Marfin AA, Van Gerpen JA, Fleischauer A, Leis AA, Stokic DS, Petersen LR (2003) Neurologic manifestations and outcome of West Nile virus infection. JAMA 290:511-515.

Skvaril F (1986) IgG subclasses in viral infections. Monogr Allergy 19:134-143.

Unkeless JC, Scigliano E, Freedman VH (1988) Structure and function of human and murine receptors for IgG. Annu Rev Immunol 6:251-281.

Wilson JA, Hevey M, Bakken R, Guest S, Bray M, Schmaljohn AL, Hart MK (2000) Epitopes involved in antibody-mediated protection from Ebola virus. Science 287:1664-1666.

Curriculum Vitae

Name	Yvonne Hofmeister
Adress	2230 Gänserndorf / Austria, Joh. Marschallstraße 22/1/9
E-Mail	yvonne.hofmeister@gmx.at
Nationality	Austria
Date of birth	31.01.1987

Work experience	
Position	Trainee (07/2008 - 05/2009)
Employer	Baxter BioScience Department Global Pathogen Safety (GPS) 1221 Vienna / Austria, Benatzkygasse 2-6 Tel.: 0043 (1) 20 100 www.baxter.com
Education	
Date	2005 - 2009
Name of organization	FH Campus Wien Biotechnologie 1030 Wien, Viehmarktgasse 2A Campus Vienna BioCenter 2 www.fh-campuswien.ac.at
Date	1997 - 2005
Name of organization	Konrad Lorenz Secondary School 2230 Gänserndorf / Austria, Gärtnergasse 5-7 www.klg.or.at

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