Journal of Neuroimmune Pharmacology (2019) 14:642–648
https://doi.org/10.1007/s11481-019-09871-0

BRIEF REPORT

Changes of Serum IgG Dimer Levels after Treatment with IVIg in Guillain-Barré Syndrome

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Received: 21 February 2019 / Accepted: 28 July 2019 / Published online: 12 September 2019

Abstract

Intravenous immunoglobulins (IVIg) are standard treatment for Guillain-Barré syndrome (GBS). Their exact mechanisms of action are versatile and not fully understood. One possible mechanism is neutralization of circulating autoantibodies via binding to anti- idiotypic antibodies forming idiotype-anti-idiotype dimeric IgG immune complexes. To examine the role of immune complex formation as mechanism of action for IVIg in GBS, 34 C57Bl/6 mice were either treated with anti-ganglioside antibodies and IVIg or IVIg and PBS alone, whereas eight additional mice were treated either with anti-ganglioside autoantibodies and IVIg or anti-ganglioside autoantibodies alone. Subsequently IgG dimer formation was assessed by high performance liquid chromatography (HPLC) and enzyme- linked immunosorbent assay (ELISA). In addition, IgG dimer formation was measured in sera of eight GBS patients who were treated with IVIg. In mice, a significant increase of dimeric IgG after administration of anti-ganglioside antibodies and IVIg could be observed. Re-monomerized IgG dimers showed immunoreactivity against gangliosides and serum immunoreactivity was significantly reduced after IVIg infusion. Likewise also in GBS patients, IgG dimer formation could be detected after IVIg treatment. Our data indicate that dimeric IgG immune complexes contain anti-idiotypic antibodies and provide proof of concept that IVIg treatment in GBS results in measurable amounts of IgG dimers. Larger patient cohorts are needed to evaluate serum IgG dimer increase as a possible marker for treatment response in GBS.

Keywords Guillain-Barré syndrome · Intravenous immunoglobulins · IgG dimers · Anti-ganglioside antibodies

Introduction

Intravenous immunoglobulins (IVIg) are commonly used as immunomodulatory treatment for Guillain-Barré syndrome (GBS) (Gilardin et al. 2015; Lehmann and Hartung 2011; van Doorn et al. 2010). It is assumed that mechanisms of action of IVIg include inhibition of phagocytotic activity of monocytes/macrophages (Jungi et al. 1990), modulation of B- and T-lymphocytes (Brem et al. 2019; Andersson et al. 1996; Hillion et al. 2013), and inhibition of complement deposition (Zhang et al. 2004). Moreover IVIg contain also anti-idiotypic antibodies (Kaveri 2012), that are able to target autoantibodies within the blood stream (Ritter et al. 2015; Zhang et al. 2004).

In a previous study we could demonstrate the feasibility to measure IgG dimer formation subsequent to IVIg application in patients with CIDP. Serum IgG dimer levels correlated with clinical outcome suggesting that anti-idiotypic-idiotypic IgG may play a role as mechanism of action of IVIg in immune mediated neuropathies (Ritter et al. 2015).

Antibodies against gangliosides GD1a, GD1b and GT1b are considered to be pathogenic in axonal subtypes of GBS and are associated with severe motor disability (Kaida et al. 2007; Kaida et al. 2008). Benefiting from the availability of monoclonal anti-ganglioside antibodies, we explored here the kinetics and nature of IgG dimer formation in vivo by administering monoclonal anti-ganglioside antibodies (anti-GD1a/GT1b, anti-GD1b) and IVIg to mice. Furthermore we assessed IgG dimer formation after IVIg infusion in a cohort of patients with GBS.

Martin K. R. Svačina and Philip Röth contributed equally to this work.

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Methods

Mouse Experiments

42 female 6- month old C57Bl/6 wild type mice were used for the experiments. Animals were housed on sawdust bedding in plastic cages and maintained on a 12 h light/dark cycle with food and water provided ad libitum. All animal experiments were carried out according to German Laws for Animal Protection and were approved by the local animal care committee and local governmental authorities. Mice were divided into three groups: The first group ($n = 14$) received intraperitoneally mouse anti-GD1b monoclonal antibody (2.5 mg/kg BW, Seigaku) and IVIg (Privigen®, 1 g/kg BW). Mice were anaesthetized with ketamine/xylazine, and sacrificed via terminal blood withdrawal of 1 ml right ventricular blood 24 h ($n = 5$), 48 h (n = 5) and 1 week ($n = 4$) post injection. The second group ($n = 13$) received IVIg (Privigen®, 1 g/kg BW) and 0.2 ml PBS intraperitoneally, and was sacrificed 24 h (n = 5), 48 h (n = 4) and 1 week (n = 4) after injection. The third group ($n = 7$) was injected with mouse anti- GD1a/GT1b bivalent antibody (5 mg/kg BW, provided by the research group of K. Sheikh, Houston, TX, USA) and IVIg (Privigen®, 1 g/kg BW). As a significant difference in IgG dimer formation was observed 48 h post injection in the first two groups, all mice in the third group were sacrificed 48 h post injection via terminal blood withdrawal.

To compare titers of circulating anti- GD1a/GT1b antibodies after co-injection with IVIg and without, $n = 4$ mice were injected with IVIg and anti- GD1a/GT1b monoclonal antibody, whereas n = 4 only received anti- GD1a/GT1b monoclonal antibody. Mice were all sacrificed 48 h post injection, whole blood sera underwent ELISA analysis separately from afore mentioned samples without undergoing previous high performance liquid chromatography (HPLC).

Patient Characteristics

Serum samples of eight GBS patients were obtained before (pre- IVIg) and 24 h after first IVIg infusion (0.4 g/kg BW; post IVIg). Patients had a medium age of 42 ± 18.3 years, with a gender ratio (female/male) of 3/5. For evaluation of clinical course, patients were distributed into three groups: the worsening group showed an increase of Hughes GBS disability score ≥ 1 (Table 1) or MRC Sum Score deterioration of ≥2 points subsequent to IVIg treatment. The improving group showed a decrease ≥1 point in Hughes GBS disability score or an amelioration of 2 points in MRC Sum Score. The stable group remained within the boundaries of the before mentioned range.

High Performance Liquid Chromatography (HPLC)

Murine blood samples were diluted to a total volume of 2 ml using low salt buffer solution (100 mM HEPES and 10 mM NaCl at pH 7.5), then centrifuged at 14000 rpm for 15 min. After centrifugation, diluted serum was collected and stored at +4 °C.

Human serum samples were obtained after centrifugation of full blood (3000 rpm over 10 min) and stored at +4 °C in a sample volume of 2 ml.

For high performance liquid chromatography (HPLC), the AKTA FPLC System (GE Healthcare, UK) and Unicorn® 4.0 analytical software (GE Healthcare, UK) were used. Whole serum IgG was collected using High Trap Q® 5 ml and High Trap SP HP® 1 ml ion exchange chromatography columns (GE Healthcare, UK) in series connection. After equilibration with low salt buffer, a sample volume of 2 ml was injected into the columns. Serum IgG was dissolved from the High Trap SP HP® column using high salt buffer (100 mM HEPES and 500 mM NaCl at pH 7.5) with a defined gradient and flow rate over 20 min. Whole serum IgG was divided into its subfractions (polymers, dimers and monomers) regarding their molecular weight profiles, using the GE Healthcare Superdex200® chromatography column and standard gel filtration buffer at a flow rate of 0.5 ml/min. Dimeric and monomeric IgG fractions were collected in 2 ml tubes and stored at +4 °C for further analysis. To test for preformed IgG dimers within the IVIg solution, 2 ml of pure Privigen® were injected directly into the chromatography column and analyzed as described.

Percentage of dimeric IgG on whole IgG content was analyzed using ImageJ® 1.51 (Wayne Rasband, NIH) software on Unicorn® graphs. Areas under the curve (AUC) of dimeric IgG and whole IgG were measured before their ratio was calculated. Whole IgG content was measured analyzing the peak milli absorbance unit on chromatography graphs.

Anti-Ganglioside-Antibody ELISA

Dimeric IgG fractions were first re- monomerized using acetic acid (10 mM at pH 4.0) for 24 h, before being stored at −20 °C for further use. 96-well plates were coated with 200 ng of gangliosides GD1b or GT1b (Merck Millipore, USA) in 100% ethanol per well and incubated overnight. After evaporation of ethanol, wells were washed three times with PBS for 10 min. Monomerized dimeric IgG fractions, monomeric IgG fractions and negative controls (PBS) were added (100 μl per well) and incubated over 24 h. After washing three times with PBS, sheep peroxidase-linked anti-mouse IgG monoclonal secondary antibody (GE Healthcare, UK; 1:500 in PBS, 100 μl per well) was incubated for 90 min at +4 °C. After washing, substrate solution (15 mg o-phenylenediamine in 0.01 mM citrate buffer and 30% H_2O_2 at pH 6.0) was added, and peroxidase reaction was blocked with 3 M H_2SO_4 after

10 min. Plates were read at 490 nm using a Multiscan plate reader (Thermo Electron, USA). All ELISA assays were performed in duplicate. Optical densities exceeding 0.2 were considered positive at the dilutions used.

Statistical Analysis

Statistical testing was performed using GraphPad Prism® 6 software. Unpaired t-tests were used for calculation of significance when analyzing two groups, multiple group analysis was performed using one-way ANOVA. A p- value of <0.05 was considered statistically significant. To compare optical density values derived from ELISA assays, data were normalized calculating the arithmetic mean of samples treated with anti-ganglioside antibodies and controls before statistical analysis.

Results

IgG Dimer Formation in Mice Treated with IVIg and Anti-Ganglioside Antibodies

Mice treated with IVIg and monoclonal anti-ganglioside antibodies (either anti-GD1b or anti-GD1a/GT1b) revealed significantly higher IgG dimer levels compared to control animals that were treated with IVIg and PBS, with a significant

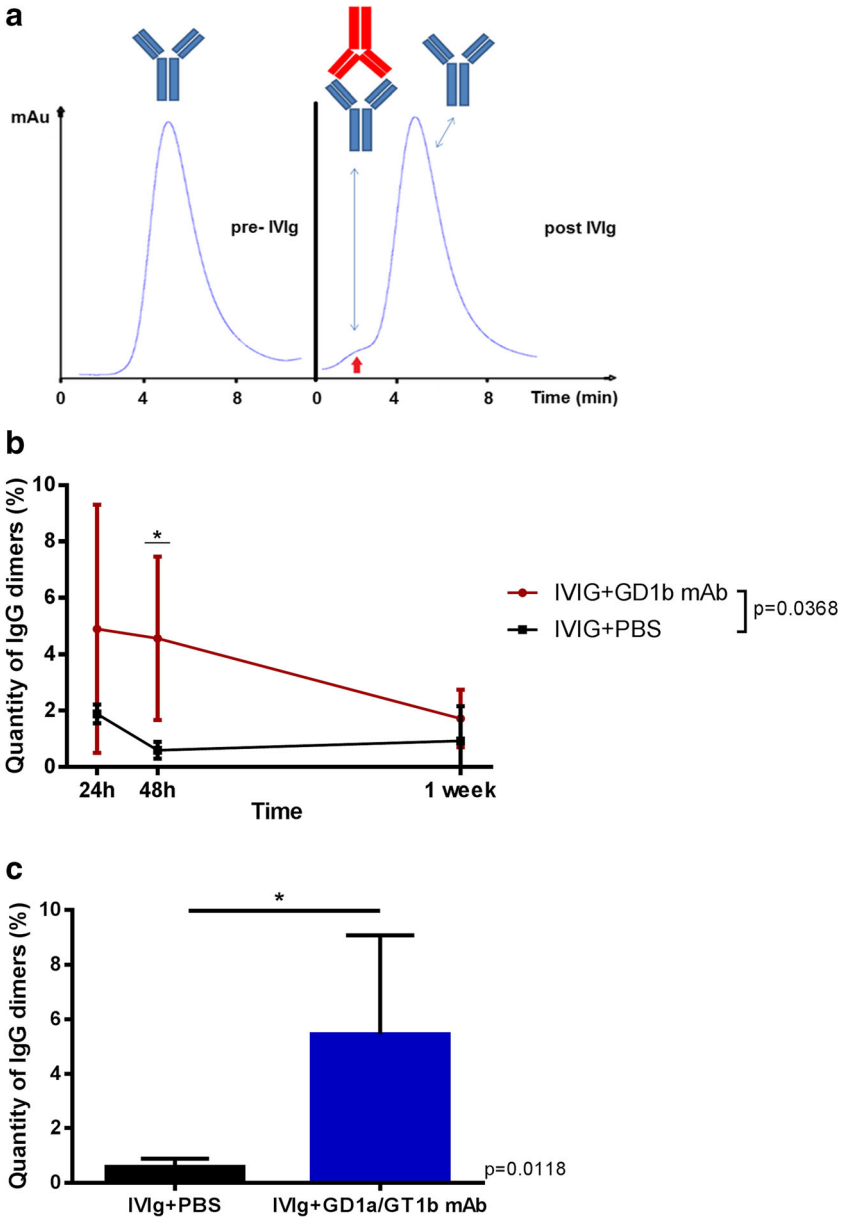

Fig. 1 **a** HPLC graph showing serum IgG content before treatment with IVIg (pre-IVIg) and after IVIg infusion (post IVIg). The left graph only shows IgG monomers, whereas the right graph includes a second, smaller peak as a correlate of IgG dimer formation (red arrow). **b** Relative quantity of IgG dimers in mice treated with IVIg and anti-GD1b-antibody or IVIg and PBS**. C:** IgG dimer levels in animals treated with IVIg and anti- GD1a/GT1b antibody or IVIg and PBS 48 h after injection ($p = 0.0118$)

increase 48 h post injection (4.6 ± 1.3% [IVIg + anti-GD1b] vs. 5.2 ± 1.6% [IVIg + anti-GD1a/GT1b] vs. 0.6 ± 0.15% [IVIg + PBS]; Fig. 1a- c). Control animals showed maximum IgG dimer levels 24 h post injection (1.9 ± 0.15%). After one week, IgG dimer levels decreased to 1.7 ± 0.5% in mice treated with IVIg and anti-GD1b antibody (Fig. 1b).

Dimeric IgG Contain Anti-Ganglioside Antibodies

ELISA assays on re-monomerized dimeric IgG fractions and monomeric IgG fractions derived from mice treated with IVIg and anti-GD1a/GT1b monoclonal antibody revealed reactivity of these fractions against ganglioside GT1b, indicating that these fractions contain anti-GD1a/GT1b antibody (Fig. 2a). Normalized optical density was slightly but significantly higher in dimeric and monomeric IgG fractions derived from mice that were treated with IVIg and anti-ganglioside antibodies compared to those collected from control mice (1.0496 vs. 0.9633, $p = 0.0389$ in dimeric IgG fractions; 1.0487 vs. 0.9307, $p = 0.0187$ in monomeric IgG fractions, Fig. 2a). Anti-GD1b-antibody ELISA did not reveal significant reactivity of mouse sera treated with IVIg and anti-GD1b-antibody compared to controls (data not shown).

IVIg Reduces Serum Titers of Circulating Anti-Ganglioside Antibodies in Mice

Serum antibody titers in mice treated with anti-GD1a/GT1b monoclonal antibody exceeded 1:5000 (Fig. 2b). Co-treatment of IVIg and anti-GD1a/GT1b monoclonal antibody resulted in measurable anti-ganglioside antibody reactivity at a dilution of 1:500 and 1:1000 but not at a dilution of 1:5000 (Fig. 2b), indicating lower titers of anti-ganglioside antibodies in these mice.

IVIg Treatment in GBS Patients Results in IgG Dimer Formation

IVIg infusion in GBS patients led to a significant increase of whole serum IgG content post infusion (Milli absorbance units: 36.93 ± 6.729 [post IVIg] vs. 12.28 ±

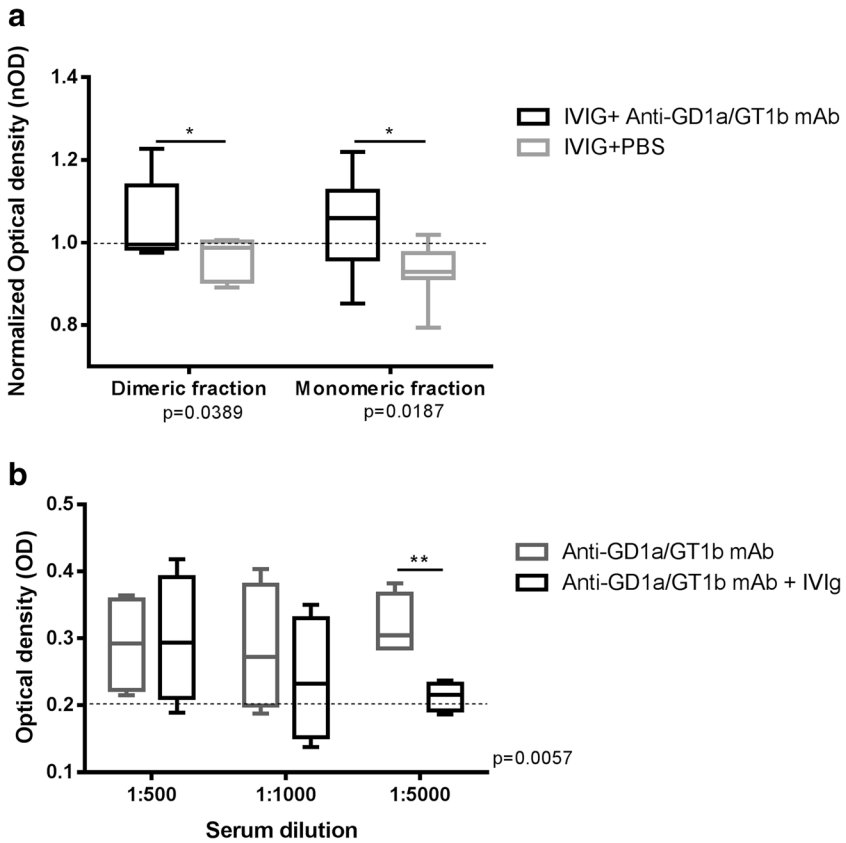

Fig. 2 **a** ELISA assay of re-monomerized dimeric and monomeric serum IgG fractions. Mice receiving anti-GD1a/GT1b monoclonal antibody and IVIg reveal reactivity against their target ganglioside GT1b in both dimeric and monomeric IgG fractions, whereas controls receiving IVIg and PBS show significantly lower normalized optical density values (1.0496 vs. 0.9633, $p = 0.0389$ in dimeric IgG fractions; 1.0487 vs. 0.9307, $p = 0.0187$ in monomeric IgG fractions). **b** ELISA assay of blood sera from mice receiving anti-GD1a/GT1b monoclonal antibody alone or in combination with IVIg, to determine titers of circulating anti-GD1a/GT1b antibodies. Anti-GD1a/GT1b titers exceed values of 1:5000 and are significantly reduced after IVIg co-administration at a dilution of 1:5000 (mean optical density values: 0.3186 [anti- GD1a/GT1b alone] vs. 0.2135 [IVIg + anti- GD1a/GT1b]; $p = 0.0057$)

1.745 [pre-IVIg], $p = 0.0077$; Fig. 3a). Furthermore, IVIg infusion led to a significant increase of serum IgG dimers in all eight GBS patients (2.4 ± 0.4% [post IVIg] vs. 0.3 ± 0.1% [pre-IVIg], $p = 0.0008$; Fig. 3b). Pure IVIg revealed a dimer content of 5.25%, when directly administered to the chromatography column. Correlation of IgG dimer content and clinical outcome revealed no statistical differences in patients that improved compared to patients with stable or worsening clinical course (Fig. 3c).

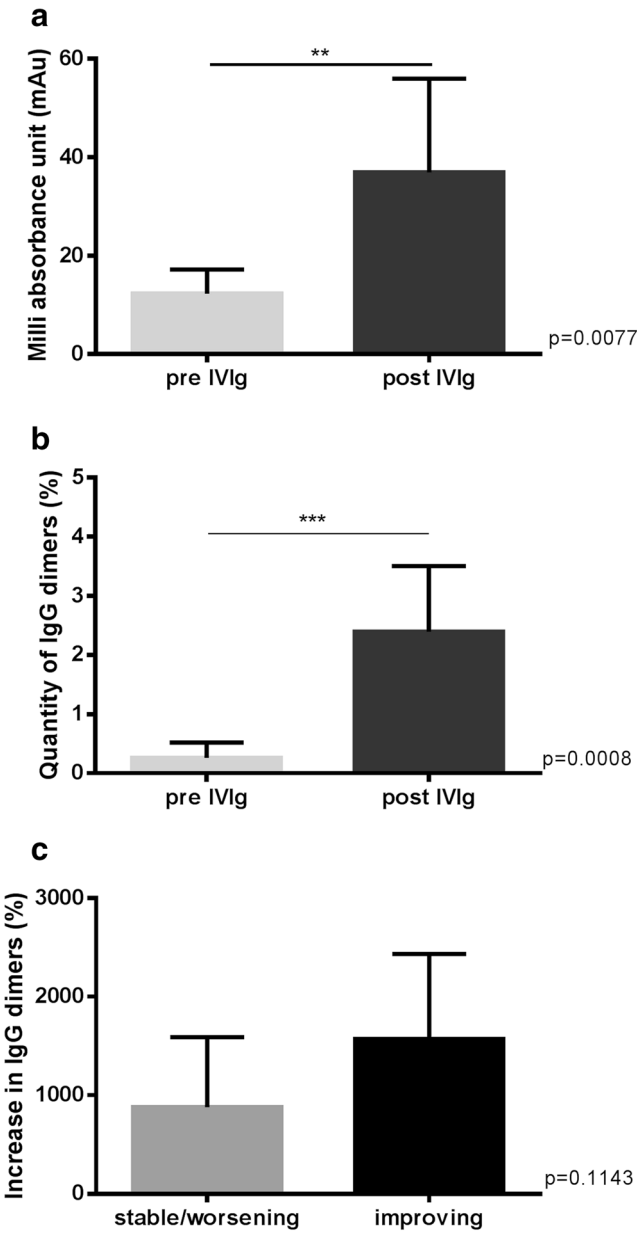

Fig. 3 **a** Whole IgG levels before and after IVIg infusion in $n = 8$ GBS patients. Whole IgG content is significantly increased after IVIg infusion (Milli absorbance units: 36.93 ± 6.729 [post IVIg] vs. 12.28 ± 1.745 [pre-IVIg], $p = 0.0077$). **b** IgG dimer content before and after IVIg infusion in $n = 8$ GBS patients. IgG dimer levels are significantly increased subsequent to IVIg infusion (2.39 ± 0.39% [post IVIg] vs. 0.26 ± 0.09% [pre-IVIg], $p = 0.0008$). **c** Relative increase in IgG dimer levels subsequent to IVIg infusion related to clinical course of $n = 8$ GBS patients

Discussion

Our results demonstrate that IVIg treatment can induce formation of IgG dimers that contain immune complexes of antibodies and their anti-idiotypes. For our study we used monoclonal anti-ganglioside antibodies, which do not occur under normal conditions in mice and which can be detected by antibody ELISA (Lunn et al. 2000). Co-administration of these autoantibodies with IVIg led to a significant increase of dimeric IgG after 48 h and a subsequent decrease of IgG dimers after one week. There are only a few studies that addressed formation of IgG dimers in mice. Roux and Tankersley reported that dimerization of IgG occurs to an amount of about 2% in serum pooled from 49 mice (Roux and Tankersley 1990). In ex vivo experiments, Tremblay and colleagues reported formation of dimeric IgG by application of IVIg in different doses (5-20 mg) to mouse serum (Tremblay et al. 2013). Commercial IVIg preparations contain a priori 5 to 15% IgG dimers, depending on age, storage, formulation, and presence of chemical stabilizers (Bleeker et al. 2000; Bolli et al. 2010; Teeling et al. 2001). In our study, we used a commercial IVIg preparation that contained 5.25% of IgG dimers. After administration of IVIg only, we observed small amounts of IgG dimers with a maximum after 24 h, which most likely represent these preexisting dimeric IgG in commercial IVIg preparation. In contrast, our finding that the amount of IgG dimers is more as seven fold as high in mice that were co-treated with additional autoantibodies and twice as high as the levels reported by Roux and Tankersley indicates a significantly higher propensity of IgG dimerization in our experimental paradigm possibly mediated via idiotypic-anti-idiotypic interactions. The decrease of IgG dimers after one week most likely reflects the kinetics of circulating antibodies and immunoglobulins that are subsequently metabolized. Our observations that I) re-monomerized IgG from the dimer fraction bind to ganglioside GT1b and II) mice treated with IVIg revealed lower anti-ganglioside serum reactivity further support the notion that IgG dimers may contain to some extent anti-idiotypic antibodies against anti-ganglioside-antibodies. However the absence of immunoreactivity against GD1b and the narrow difference in optical densities in monomerized IgG fractions when tested against GT1b also indicate that the amount and / or affinity of anti-idiotypic antibodies against monoclonal anti-ganglioside antibodies in IVIg is probably very low, which is in line with previous in vitro observations that IVIg-mediated decrease in binding to ganglioside antigens is much less or even entirely absent (Zhang et al. 2004).

Like in CIDP (Ritter et al. 2015), also in patients with GBS, application of IVIg resulted in an increase of IgG dimer serum levels after treatment in most of our patients. We conclude that also in GBS, IVIg infusion results in the spontaneous formation of novel immune complexes in vivo.

In summary our study provides proof-of-concept that IVIg contain anti-idiotypic antibodies that contribute to the dimer formation subsequent to IVIg administration. Larger patient samples are warranted to address the question if serum IgG dimer formation may serve as surrogate marker for treatment response in GBS.

Compliance with Ethical Standards

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For animal experiments, all applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed Consent Written informed consent was obtained from all subjects participating in this study.

Conflict of Interest The authors declare no conflict of interest.

Appendix

Table 1 Hughes GBS disability score (modified after van Koningsveld et al. 2007, and Hughes et al. 1978)

Score	Disability
0	A healthy state
1	Minor symptoms and capable of running
2	Able to walk 10 m or more without assistance but unable to run
3	Able to walk 10 m across an open space with help
4	Bedridden or chairbound
5	Requiring assisted ventilation for at least part of the day
6	Dead

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