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EVALUATING THE MURINE ANTI-HUMAN ANTIBODY RESPONSE AND ASSESSMENT OF GENERAL ACTIVITY AND COGNITION AFTER TREATMENT WITH HUMAN INTRAVENOUS IMMUNOGLOBULINS IN HEALTHY ADULT C57/B6J MICE

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Human intravenous immunoglobulin (IVIG) is a fractionated blood product that is used for the treatment of several autoimmune and immunodeficiency disorders. Recently, IVIG has been suggested for the treatment of Alzheimer's disease (AD). However, the molecular mode of action is still largely unknown. Therefore, preclinical assessment of the therapeutic efficacy of IVIG in animals may provide valuable information of the function of IVIG *in vivo*. However, it is recommended to determine the murine-anti-human antibody (MAHA) response in those animals before starting immunotherapy and subsequent assessment of the therapeutic efficacy in animal models for AD. After weekly administration of 400 µg IVIG in C57/B6J mice for the duration of twelve weeks, we found a significant increase of MAHA response against human IgG. Even after increased MAHA levels starting from week nine after treatment, there was no significant change in basic exploratory behavior, anxiety, and cognition. Therefore, it is suitable to study pharmacological and immunological activity, therapeutic efficacy, as well as mode of action of IVIG in animal models only for a short duration to avoid interference with IVIG treatment and neutralize possible therapeutic effects.

Injection of human immunoglobulins initiate an immune reaction in mice, which subsequently leads to the generation of murine anti-human antibodies (MAHA). MAHA response in mice develops within days to weeks after application of human proteins. The magnitude of the MAHA response depends on treatment protocol (for instance, dose regimen, application schedule, and prior exposure to human immunoglobulins) and is comparable to human anti-mouse antibody (HAMA) responses seen in clinical settings in which patients with a variety of medical conditions are treated with murine, chimeric, and humanized immunoglobulins (1, 2). Although MAHA response can probably interfere with human antibodies tested in experimental animal models for a variety of diseases and neutralize possible therapeutic effects, little preclinical data is available concerning MAHA response (3, 4), despite numerous studies using polyclonal human immunoglobulin (IVIG) in experimental mice models (3, 5-7).

Key words: intravenous immunoglobulin, Alzheimer's disease, MAHA, immunization, behavior, cognition, murine anti-human antibodies, immunotherapy

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Recently, we investigated the therapeutic efficacy of specific naturally occurring polyclonal human antibodies against beta-Amyloid (nAbs-Aβ), extracted from IVIG, in a mouse model of AD (8, 9). Systemic injection of 400 μg nAbs-Aβ once a week over two months evoked an anticipated but non-significant MAHA response in those mice (8). This raised several questions regarding the acceptable and tolerable magnitude of an MAHA response, to avoid interference with ongoing therapeutic application and neutralization of human immunoglobulins in experimental animal models. Therefore, further preclinical assessment of function of its components *in vivo* is pivotal, especially in terms of a potential MAHA response, which could interfere with putative beneficial outcomes in animal models of medical conditions. This view is supported by findings showing that an antibody response can affect cognition in an animal model for lupus (10).

In this study, we investigated the effect of a twelve-week IVIG treatment on the development of MAHA response, and possible subsequent effects on exploratory activity, anxiety, and cognition-based behavior in healthy adult C57/B6J mice, which is a widely used background strain for modeling medical conditions in animals, and appropriate for the investigation of basic interactions between IVIG and a potential MAHA response. The rising therapeutic use of human immunoglobulins in experimental mice models for neuropsychiatric and neurodegenerative diseases (e.g. AD) provides the rationale for examining the possible effects of the MAHA response on animal behavior, since it is a significant primary endpoint in most preclinical models of the aforementioned diseases. To the best of our knowledge, this study is the first to investigate MAHA response and cognition in mice.

MATERIALS AND METHODS

Animals

A total of 110 adult female C57/B6J mice (R. Janvier Breeding Center, Le Genest St. Isle, France), four months of age, were used for this experiment. Since neurodegenerative and immunodeficiency disorders often appear in advanced age, the models in research use are full-grown and not juvenile mice (11).

All mice were housed in groups of five under standard laboratory conditions in polycarbonate macrolon cages (type III; size of the area 820 cm^2) on a twelve hour light-dark schedule (lights on 06:00-18:00) in a constant temperature (21±2°C) and humidity (48-52%). Drug treatment and behavioral testing were carried out during the light phase. The mice had free access to tap water and were fed *ad libitum*. C57/B6J mice weighed between 20-25 g at beginning of the experiments.

All experiments were performed in strict adherence to national laws and international recommendations for the use and care of experimental animals and were approved by the animal care committee of the Philipps-University Marburg.

Experimental design

For the twelve-week treatment study, 110 adult female C57/B6J mice were assigned to 22 groups. For the outcome variables we assumed a very large effect in the study and a statistical power of >60%. Based on the work by Crawley, we calculated a number of ~5 mice in each group (12). The mice were grouped into groups of five in order to conduct all the tests in the day period, so there were a cohort of eleven groups of five mice for IVIG and eleven groups of five mice as control. In accordance with several other preclinical human IVIG studies, we injected 400 μg of IVIG to the test cohort i.p. (11, 13). We administered IVIG and control solution once a week on day one (n = 11 per cohort in week 1).

On day seven, we conducted the behavioral test and mice were sacrificed and tissue was taken to investigate the MAHA response against human IgG in one group of IVIG-therapy (n = 5) and control group (n = 5). The following day (day one of week two), the rest of the groups (n = 10 per cohort) were administered the second injection, on day seven of week two another two groups (n = 5 each cohort) underwent behavioral tests and tissue removal. So the first group (n = 5) received one injection i.p. of 400 μg of IVIG (Octagam 5%, donated by Octapharma, Austria). The second group (n = 5) received weekly injections i.p. for a period of two weeks. The third group (n = 5) received weekly injections for a period of three weeks. The fourth group (n = 5) received weekly injections for a period of four weeks. Treatment design for the first month was adapted to the next eight weeks in the same manner. Age-matched C57/B6J mice receiving an equal volume phosphate-buffered saline (PBS) served as controls and were assigned to eleven groups (n = 5 for every group). To ensure comparability between groups and due to time constraints, behavioral testing and subsequent blood and brain collection were tightly scheduled on identical time points during the same day, one week after the last treatment with IVIG.

Behavioral experiments

The experimenter was blind to the treatment status of the animals. An open field test was conducted first,

followed by the Y-maze test. The inter-test interval between both behavioral tasks was 30 min.

Open field test

The effects of IVIG on exploratory behavior and anxiety of the mice were investigated using the open field. Hypothesis of the open field test is that exploratory behavior is affected by internal factors such as anxiety. Motor activity in the open field was recorded six days after the last IVIG injection during the light phase. Two open field cages (40 x 40 cm), with 40 cm walls made of grey PVC placed inside a rearing indicator frame with infrared-light barriers were used for detection of rearing movement. Recording and analysis of the horizontal and vertical locomotion of the mice in the open field were carried out with the computerized Videomot2 system (TSE System, Bad Homburg, Germany). At the beginning of the test, the mice were placed in the center of the open field and monitored for 20 min. Open field cages were cleaned with 70% ethanol solution after each mouse.

Y-maze

Continuous spontaneous alternation in the Y-maze is a valuable tool to investigate short-term memory in mice (14, 15). Cognition in the Y-maze was assessed six days after the last IVIG treatment during the light phase. A Y-maze made of grey PVC with three goal arms (A, B and C) in the shape of a Y was used. Each arm was 30 cm long, 8.5 cm wide and 15 cm high. The arms converged at an equilateral triangle central area. The mice were placed at this central area and were allowed to move freely through the Y-maze for the duration of 10 minutes. The sequence of arm choices was recorded and analyzed using the PC-based Videomot2 system (TSE System, Bad Homburg, Germany). Continuous spontaneous alternation behavior was assessed by calculating the ratio of actual alternation to possible alternation (i.e. total number of entries minus two). The Y-maze was cleaned with 70% ethanol solution after each mouse.

Murine anti-human antibody response (MAHA)

After administration of IVIG and subsequent assessment of behavior after the last injection, mice were sacrificed and tissue was taken to investigate MAHA response against human IgG.

Firstly, after anaesthetizing, the bodyweight of each mouse was measured. Blood was immediately collected by sterile cardiac puncture of the right ventricle (approximately 5 ml per mouse). Then the spleen was removed and left untreated. Bodyweight and weight of the spleen were measured to control for physiological effects. The whole brains were isolated and frozen in fluid nitrogen and stored at -80°C. For ELISA experiments, they were mechanically crushed in a T-PER (Pierce®, Thermo Scientific) and Complete solution (100:1; Roche Applied Science, Mannheim) and centrifuged at 100.000 g for 10 min at 4°C. ELISA of brain samples were performed with equivalent protein concentration determined with a BCA Protein Assay Kit (Pierce®, Thermo Scientific).

Blood samples were centrifuged at 2,000 g for 15 min. ELISA of tissue samples was performed as followed: plates were coated overnight at 4°C with 5 µg/ml hIgG1 (Octagam, Octapharma, Germany), diluted in sodium bicarbonate buffer (pH 9.6). After washing four times with 1x PBS 0.05%-Tween 20 (pH 7.4), plates were blocked for 3 h with SuperBlock® (Thermo Scientific #37535). After washing four times with 1x PBS 0.05%-Tween 20 (pH 7.4), the serum samples were diluted 1:100 and added to the wells for overnight incubation at 4°C in triple determination. Wells were then washed four times with 1xPBS 0.05%-Tween 20 (pH 7.4), and subsequently 0.1 µg/ml goat anti-mouse IgG horseradish peroxidase (Calbiochem, Germany) was added, and plates were incubated for one hour at room temperature. Plates were then washed four times with 1x PBS 0.05%-Tween 20 (pH 7.4), and 100 µl of tetramethylbenzidine (Calbiochem) substrate were added to each well. The plates were incubated for 10 min until the desired blue color was reached; then the reaction was stopped by the addition of 25 µl/well of 2 N H_2SO_4. The plates were then read at 450 nm with a 570-nm reference filter. The standard curve reagent used for this assay was obtained by performing these measurements on serial dilutions of mouse anti-human IgG1 (BD Pharmingen) in concentrations between 1 and 1.000 ng/ml in two-fold increments.

Coating and blocking of brain ELISA were performed analog to serum ELISA. The brain tissue was applied in equivalent protein concentration diluted in TPER and Complete 100:1 in triple determination. For the sake of consistency, the standard mouse-anti-human-Immunoglobulin (BD Biosciences Pharmingen) was also diluted in TPER + Complete. The further steps of the experiment were performed as outlined above.

Statistical analysis

All analyses were performed using the software packages SigmaStat (Statcon, Germany). Data were analyzed using analysis of variance (ANOVA) with treatment (IVIG *vs* PBS) and time (week 1-12) as dependent variables. For pair-wise multiple comparisons, Tukey's *t*-test was used with *p*-values of <0.05 (*) and <0.01 (**) as the criterion for significance.

RESULTS

MAHA response

To investigate the occurrence and development of a murine antibody response against human IgG in

plasma and brain, healthy adult mice were treated up to twelve weeks, including groups of mice receiving one injection, two injections weekly for two weeks, three injections weekly for three weeks, and so on for the next eight weeks. A small but detectable increase of murine antibodies against human IgG in plasma was observed in the first week, which remained on a stable level over the first eight-week period. No significant differences were found between IVIG- and PBS-treated mice in weeks 1–8. In comparison to week one, in week nine (after nine injections), there was an abrupt increase and a measurable trend in MAHA response (week 1 *vs* week 9, t (8) = -1.929, p = 0.09), as well stack up against the control group after nine injections. Beginning in week ten, serum MAHA concentration significantly increased after treatment with IVIG (week 10 *vs* week 1, t (7) = 2.94, p <0.05; week 11 *vs* week 1, t (8) = 1.07, p < 0.05; Fig. 1C). In the control groups of mice, no similar reaction was detectable. Investigation of the MAHA response in the brain of untreated and IVIG-treated mice did not identify a detectable MAHA response in the brains of either group over the 12-week period (data not shown).

To identify possible physiological effects in mice after treatment with human IgG, bodyweight and spleen weight were measured. As expected, bodyweight (F (1.10) = 4.4, p < 0.01) and spleen weight F (1,10) = 3.1, p < 0.01) of both treatment groups increased over the course of the study. No significant differences were found between IVIG- and PBS-treated groups (Fig. 1, A and B).

Open field

To study treatment effects on basic behavior, we measured motor function, exploratory activity, and anxiety in the open field six days after the last injection of IVIG of the respective treatment group (Fig. 2). Independent of the treatment status, mice of both groups showed significant differences regarding the distance travelled in the open field over the 12-week period (F (1,10) = 9.24, p < 0.01 (**); Fig. 3A). IVIG treatment and week of testing had no effect on the distance travelled (Fig. 2A), time spent in the center (Fig. 2B), and the number of rearings (Fig. 2C) in the open field.

Y-maze

Spatial learning and memory in the Y-maze was

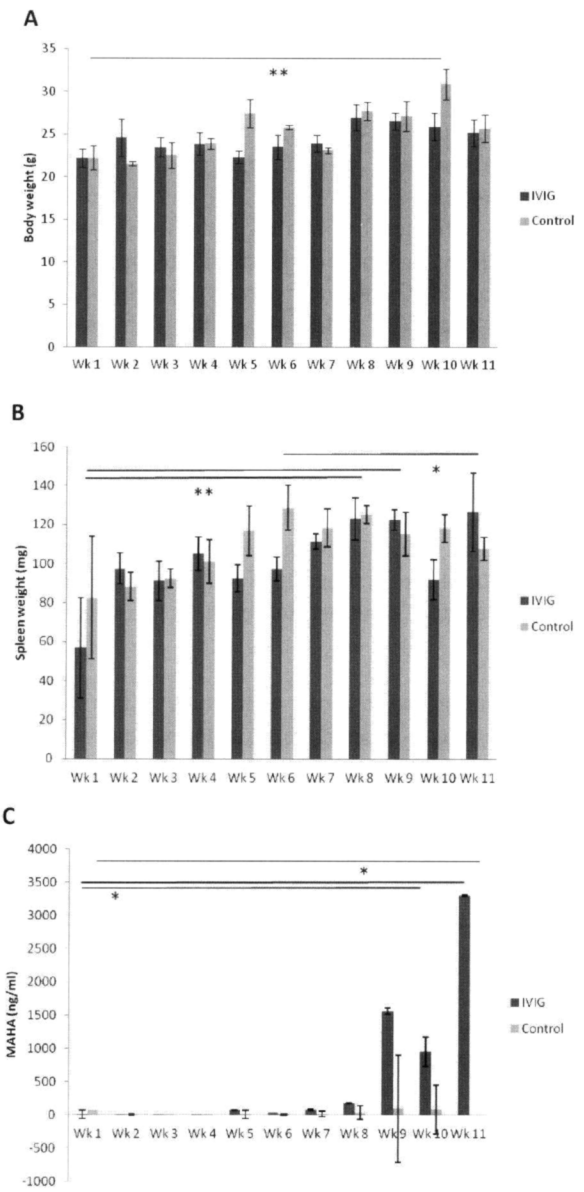


Fig. 1. *Body weight, spleen weight, and serum MAHA concentration of IVIG- and PBS-treated adult female C57/B6J mice are shown. No significant differences were found regarding bodyweight (**A**) and spleen weight (**B**) between the two groups. There is a significant increasing of body and spleen weight over the 12-week period. Exemplarily shown the comparison of week 1 vs 10 and week 1 vs 8, 9 [p<0.01 (**)], week 6 vs 11 [p<0,05 (*)]. Treatment with IVIG led to a significantly increased MAHA concentration in mice in week 9. Week 1 vs 10 [t (7) = 2.94, p<0.05]; Week 1 vs. 11 [t (7) = 2.92, p< 0.05; (*)]. The difference in the control group is shown in week 1 vs 11 [t (7) = 2.62, p<0.05 (*)]. Results are presented for every other week as means ± S.E.M. Please refer to the text for statistical analysis.*

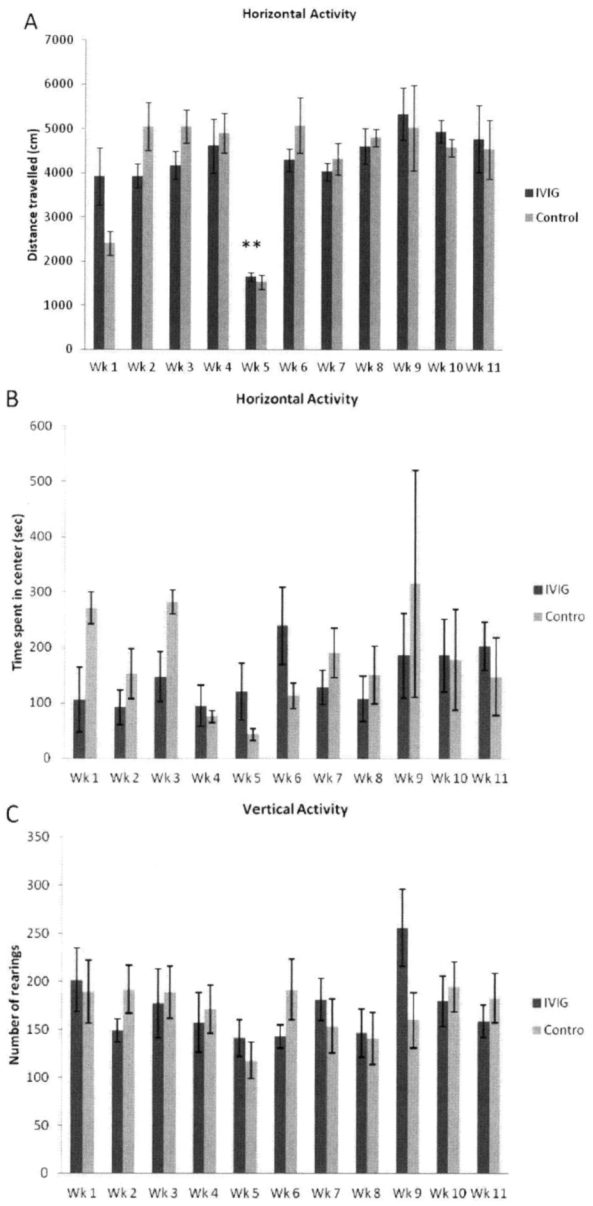


Fig. 2. *Exploratory behavior was assessed in the open field analyzing horizontal and vertical activity in C57/B6J mice one week after the last IVIG treatment. Total distance travelled (**A**), time spent in the center (**B**), and total number of rearings (**C**) is shown for groups treated with IVIG and PBS (Control). Data are presented for every other week as means ± S.E.M. Please refer to the text for statistical analysis.*

tested using the continuous spontaneous alternation task (Fig. 3). Treatment with IVIG had no effect on spontaneous alternation (Fig. 3A), distance travelled (Fig. 3D), number of arm entries (Fig. 3B), and time spent in arms (Fig. 3C) of the Y-maze. Significant differences were found concerning the week of the travelled distance ($F(1, 10) = 2.03$, $p < 0.05$) and total number of arm entries ($F(1,10) = 2.2$, $p < 0.05$) in the Y-maze (Fig. 3 D, B). Spontaneous alternation and time spent in arms of the Y-maze did not change over the time course (Fig. 3. A, C).

DISCUSSION

This study investigated the effect of a 12-week treatment with human intravenous immunoglobulins in adult C57/B6J mice, which are the most frequently used inbred strain for animal models for a variety of human diseases. As expected, all mice developed murine anti-human antibodies against human IgG1, which is the most dominant IgG subclass of IVIG. However, for the first eight weeks, detectable antibody titers were negligible following the treatment with 400 µg IVIG. Therefore, treatment with human immunoglobulins weekly for two months, in the course of a preclinical assessment of pharmacological and immunological activity, therapeutic efficacy as well as mode of action, is scientifically and ethically acceptable. Because there was no measurable MAHA response in brain tissue of treated mice, a contamination can be debarred.

Treatment with IVIG did not influence basic exploratory behavior and cognition in mice over the twelve-week period. Nevertheless, it should be mentioned that differences between groups and time points during the treatment period occurred, which is related to the decreased distance travelled, number of rearings and time spent in the center of the open field, as well as reduced distance travelled, total number of arm entries, and slightly increased time spent in arms of the Y-maze, especially in week five. Behavior of mice of both groups in week five was quite unusual compared to behavior in other weeks. Unfortunately, during the fifth week, construction work on the exhaust air filtration system inside the animal facility took place, which is probably the most likely explanation for the divergent results, reflecting heightened anxiety of the mice. Thus, these differences are more relevant in a statistical rather than biological point of view, reflecting normal variability in animal response to unspecific effects

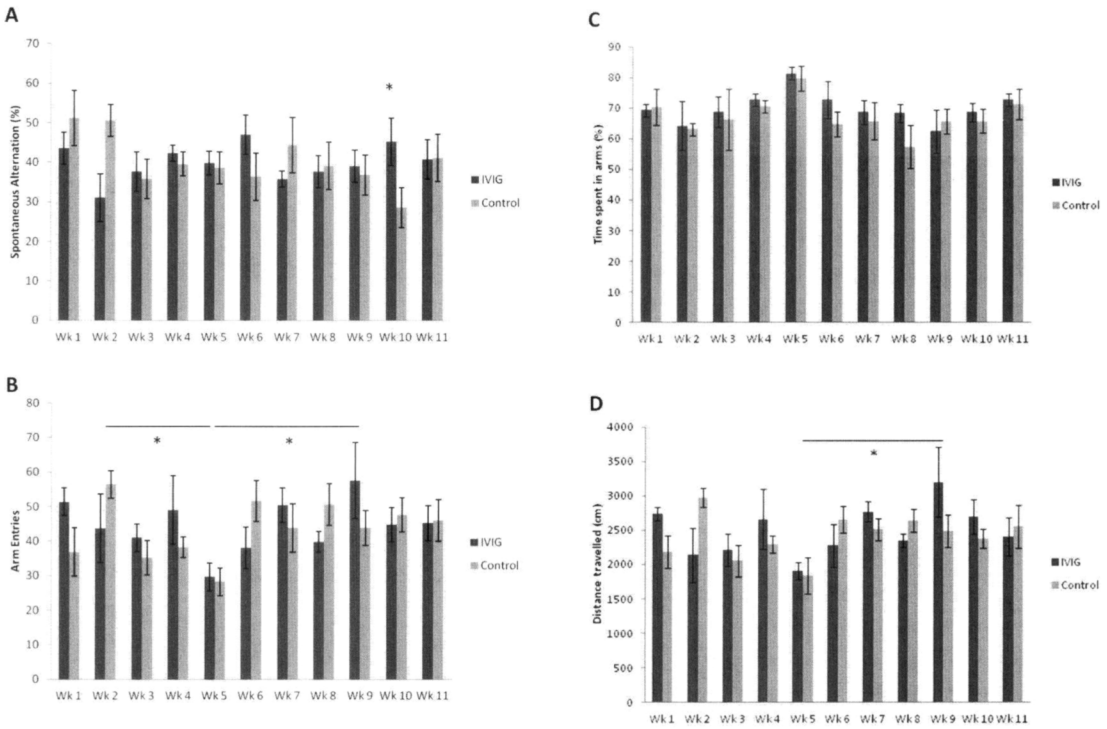


Fig. 3. *Short-term spatial memory was assessed in the Y-maze using the continuous spontaneous alternation task one week after the last IVIG administration in C57/B6J mice. Spontaneous alternation, total distance travelled, arm entries, and time spent in arms is shown for groups treated with IVIG and PBS (Control). No significant differences were observed between IVIG and PBS treatment on spontaneous alternation (**A**), distance travelled (**B**), number of arm entries (**C**) and time spent in arms (**D**). A) In week 10 there is a significant difference between treatment groups [F (1, 10)=2,08, p<0,05 (*)] in spontaneous alternation. B) There is a significant difference detectable in week 5 vs 9 [F (1,10) = 2.03, p<0.05 (*)]. C) Furthermore there is a difference measurable in week 2 vs 5 and also week 5 vs 9 [F (10) = 2.15, p <0.05 (*)] independent of treatment in distance travelled. D) Total distance travelled. There is a significant difference detectable in week 5 vs 9. Results are presented for every other week as means ± S.E.M. Please refer to the text for statistical analysis.*

of external events and subsequent behavior. This observation is strengthened by absent significant differences between motor activity in the open field and spontaneous alternation in the Y-maze in the first and last week of treatment.

Due to the experimental design and technical limitations of the model, the behavioral investigation focused on the more basic components of mouse behavior, namely exploratory activity and anxiety (time spent in the center and periphery) in the open field, and continuous spontaneous alternation, a simple short-term memory task in the Y-maze. It would be interesting to investigate more complex behavior of different domains of spatial and non-spatial learning and memory and emotional learning and memory, to name a few.

IVIG is used as an immunomodulatory drug for the treatment of a variety of health conditions, mostly in patients with immune and autoimmune defects and neurological disorders. More recently, the use of IVIG was investigated in neurodegenerative disorders like AD. The rationale behind the use of IVIG in AD patients is based on the detection of naturally occurring autoantibodies against amyloid-β in the sera of healthy human and AD patients as well as in commercially available IVIG preparations. These findings make IVIG a promising treatment option for AD, and human IVIG preparations are already in clinical phase II and phase III trials (16-19). To date, little preclinical data on the general functioning of human IVIG in rodents and the subsequent MAHA response is available or, as in the case for mice

models for AD, do not exist. This is quite surprising, since a large quantity of studies of the therapeutic use of IVIG in experimental mice models has been published, for instance for experimental autoimmune encephalomyelitis (5), mycobacterium tuberculosis infection (6), adenocarcinoma (3), Chagas disease (7), systemic lupus (20), thrombocytopenia (21, 22), Kawasaki disease (23), pneumococcal infection (24), arterial injury (25), myocarditis (26), to name only a few. But in most of the studies, controlling for an MAHA response is absent or has not been published, with exceptional cases like a study on antitumor effects of immune-cytokine therapy in murine adenocarcinoma (3). A recent work published by Loeffler et al. (2012) investigated the effect of repeated IVIG administration on a variety of clinical, physiological, and histopathological parameters. Behavioral measurements, however, were not included in this elaborated study. They found a strong humoral immune response and hematological deficits after a six-week treatment with 1g/kg IVIG administered i.p. weekly. No clinical or histological abnormalities were found (4).

Generation of an MAHA response in experimental animal models as a result of the administration of foreign proteins like human IVIG is likely and could interfere with IVIG treatment and neutralize possible therapeutic effects. Therefore, to avoid possible inhibitory effects of the administered human antibodies in mice, the treatment period needs to be limited or the total amount administered should be restricted.

To the best of our knowledge, this is the first report evaluating the general effect of foreign human antibodies given over a twelve-week period in healthy C57/B6J wild type mice with subsequent animal behavior testing. Since the treatment does not evoke a significant MAHA response in these mice within the first nine weeks, this finding leads to the conclusion that treating C57/B6J mice with human IVIG over a period of eight weeks is suitable for studying pharmacological and immunological activity, therapeutic efficacy and mode of action of IVIG in C57/B6J mice (a widely used background inbred strain for modeling a variety of medical conditions in animals). Of course, it is conceivable that the murine anti-human antibodies are able to reduce the therapeutic efficacy of IVIG, but this impairment is more likely to increase during prolonged human antibody treatment. As we showed here, the MAHA response does not play a detrimental role within the first two months of IVIG treatment. However, it may become an increasing issue as the boost immunization continues. For that reason, it would be interesting and necessary to investigate the development and generation of the MAHA response caused by a prolonged administration of IVIG in more detail, and maybe to determine the maximum length of treatment possible prior to affecting therapeutic efficacy due to neutralizing and clearing foreign antibody proteins from the circulation.

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Conflict of interest statement. RD holds several patents on the role of naturally occurring autoantibodies for the treatment of patients with neurodegenerative diseases. There are no other conflicts of interest regarding the content of the manuscript. All other authors declare that there are no conflicts of interest.

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