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Journal of Neuroimmunology 194 (2008) 89–96

Journal of Neuroimmunology

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A disease-specific fraction isolated from IVIG is essential for the immunosuppressive effect of IVIG in experimental autoimmune myasthenia gravis

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Received 16 October 2007; received in revised form 27 November 2007; accepted 28 November 2007

Abstract

Intravenous immunoglobulin (IVIG) treatment is beneficially used in autoimmune disorders including myasthenia gravis (MG) although its mode of action and active components are still not fully identified. In an attempt to isolate from IVIG a disease-specific suppressive fraction, IVIG was passed on columns of IgG from rats with experimental autoimmune MG (EAMG) or from MG patients. These chromatographies resulted in depletion of the suppressive activity of IVIG on rat EAMG whereas the minute amounts of IgG fractions eluted from the EAMG- or MG-specific columns retained the immunosuppressive activity of IVIG. These results demonstrate that a minor disease-specific immunoglobulin fraction present in IVIG is essential for its suppressive activity.

Keywords: IVIG (intravenous immunoglobulin) therapy; Disease-specific anti-antibodies; Myasthenia gravis (MG); Experimental autoimmune myasthenia gravis (EAMG)

1. Introduction

Intravenous immunoglobulin (IVIG) administration has been beneficially used in recent years for the treatment of a variety of autoimmune diseases (Kazatchkine and Kaveri, 2001; Dalakas, 2004; Lemieux et al., 2005; Bussel and Hilgartner, 1984; Durelli and Isoardo, 2002; Ferrero and Durelli, 2002; Hughes, 2002; Pyne et al., 2002; Rutter and Luger, 2002). However, the mechanism of action of IVIG treatment and the identification of the fraction(s) within the IVIG preparation that is responsible for its therapeutic effect in autoimmune diseases are still not established. Multiple mechanisms, of action, not necessarily mutually exclusive, have been proposed for explaining the therapeutic effect of IVIG (Bayry et al., 2007; Gold et al., 2007). Some of the mechanisms depend on the interaction between the Fc fraction of IVIG and Fc receptors on target cells and others rely on the variable regions of IgG antibodies in the IVIG preparations. The various mechanisms proposed include modulation of expression and function of Fc receptors, interference with activation of complement and/or the cytokine network, regulation of cell growth, alteration of cellular adhesion processes, effects on the activation, differentiation and effector functions of T and B cells and of antigen-presenting cells as well as modulation of idiotype networks (Bayary et al., 2006; Bayry et al, 2007). In addition, steric hinderance directly interfering with the binding of anti-AChR antibodies to the receptor and not acting through immunoregulatory networks has also been suggested (Buchwald et al., 2002).

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doi:10.1016/j.jneuroim.2007.11.020

Myasthenia gravis (MG) and experimental autoimmune myasthenia gravis (EAMG) are T cell-dependent, antibody-mediated autoimmune diseases in which the acetylcholine receptor (AChR) at the neuromuscular junction is the main autoantigen. IVIG has been employed to treat MG for about two decades (Fateh-Moghadam et al., 1984; Gajdos et al., 1984; Edan and Landgraf, 1994; Gajdos et al., 1997). In a SCID mouse model of human MG it has been demonstrated that IVIG and pooled normal human IgM (IVIgM) decreased the production of anti-AChR antibodies in the serum and diminished the loss of endplate AChR in the diaphragms of myasthenic SCID mice (Vassilev et al., 1999).

We have been studying the therapeutic effect of IVIG administration in EAMG in rats, the most suitable model for human MG, in order to test its effectiveness and delineate its mechanism of action. In a recent study from our lab (Zhu et al., 2006) we have demonstrated the efficacy of IVIG treatment in preventing the induction of EAMG and in immunosuppressing an ongoing disease both at its acute and chronic stages. The optimal conditions for immunosuppression of EAMG by IVIG have been established and immunological analyses suggested that IVIG modulates EAMG by suppression of Th1 and B cell proliferation but probably not by generation of regulatory T cells (Zhu et al., 2006).

The drawbacks of IVIG treatment include its high cost and the fact that patients are given huge amounts of protein of which only a minor fraction may be responsible for the therapeutic effect. In an attempt to isolate a specific fraction from pooled normal human immunoglobulins and to analyze its immunosuppressive activity in an autoimmune disease we have chosen to employ the rat EAMG model. We demonstrate here that chromatography of pooled human IVIG on immobilized immunoglobulins, isolated from either EAMG rats or from MG patients, results in a complete depletion of the suppressive activity of the IVIG preparation. Moreover, reconstitution of the activity-depleted IVIG with the eluted minute IVIG fractions that had been adsorbed onto the EAMG- or MG-specific columns, recovers the depleted immunosuppressive activity. This study demonstrates that a disease-specific anti-immunoglobulin fraction present in IVIG preparations is essential for the suppressive effect of IVIG and raises the possibility that such disease-specific fractions can be isolated from IVIG and considered as improved reagents for therapeutic purposes.

2. Materials and methods

2.1. Induction and clinical evaluation of EAMG

Experiments in animals were approved by our Institutional Animal Care and Use Committee (IACUC). AChR was purified from the electroplax tissue of Torpedo californica, as previously described (Aharonov et al., 1977). EAMG was induced in rats as previously described (Im et al., 1999) by immunization with Torpedo AChR (40 μg/rat) emulsified in CFA and containing additional 0.5 mg/rat Mycobacterium tuberculosis (Difco, Detroit, Michigan). Clinical severity of EAMG was scored on a scale of 0–4 according to Lennon et al. (1991) as follows: grade 0, normal; grade 1, mildly decreased activity, weak grip, with fatigability; grade 2, weakness, hunched posture at rest, loss of body weight and shaking; grade 3, severe generalized weakness, obvious loss of body weight, moribund; grade 4, dead. Animals were evaluated for clinical score and body weight by blinded observers twice a week, throughout the experiment.

2.2. Immunoglobulin adsorbents

2.2.1. Rat immunoglobulin adsorbents

IgG fractions were prepared from EAMG rats or from control, CFA-immunized rats. Sera were collected and pooled from 10–15 rats with EAMG exhibiting high anti-AChR antibody titers, with a clinical score of ≥ 2, 6–8 weeks following disease induction by immunization with Torpedo AChR (EAMG-specific), and from 10–15 control rats immunized with CFA, collected also 6–8 weeks following immunization. Immunoglobulins were prepared from the sera by ammonium sulfate precipitation and were then subjected to IgG purification on a Protein-G column.

For preparation of the adsorbents, 25 mg of EAMG-specific IgG, prepared from rats immunized with Torpedo AChR or of control IgG, prepared from rats immunized with CFA were mixed each with 2.5 ml of resin slurry (Toyopearl 650 M formyl, TOSOH Corporation, Japan). Sodium cyanoborohydride was added to 50 mM in order to stabilize the binding. The mixture was mixed gently on a roller at RT overnight. The supernatant was removed and fresh sodium cyanoborohydride was added, followed by addition of 0.5 M glycine to block the remaining binding sites on the resin. After an additional hour the supernatant was removed and the resin was washed by copious amounts of distilled water, 1 M NaCl and 1 M NaCl + 20% EtOH and stored in the latter solution. About 30% of the IgG added to the resin, were absorbed to the resins under these conditions.

2.2.2. Human immunoglobulin adsorbents

IgG fractions were prepared from pooled plasma from plasma exchange filtrates of five unidentified MG patients with anti-AChR antibody titers in the range of 5–20 pmol/ml or from pooled plasma of five healthy controls. Preparation of human IgG and adsorbents was performed as described for the rat adsorbents with some modifications. For preparation of the human IgG adsorbents 100 mg of IgG from pooled plasma of MG patients or of healthy controls were mixed each with 5 ml of the resin.

2.3. Fractionation of IVIG

2.3.1. Fractionation on rat IgG columns

For fractionation on the rat IgG adsorbents IVIG was fractionated either by sequential chromatography on the control and then on the EAMG-specific column or by chromatography on the control column alone. One ml of packed resin was used for each column. For the depletion of IVIG from the EAMG-specific activity, IVIG was passed through both columns serially with the control column preceding the EAMG-specific column to remove non-specific IgG (see Fig. 1). For each run, 0.5 l of

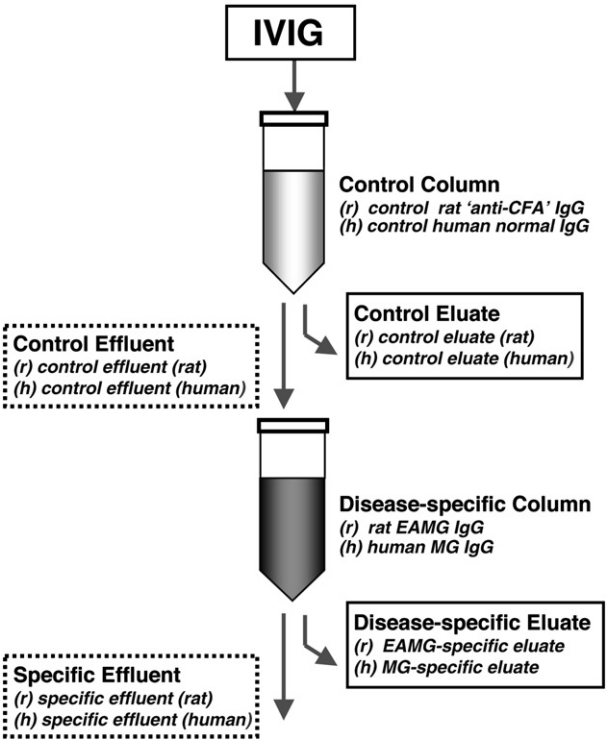

Fig. 1. Schematic presentation of IVIG fractionation on rat (r) and human (h) IgG columns.

5% IVIG (Omrigam, Omrix Biopharmaceuticals, Nes Ziona, Israel was mixed with 0.5 l of x2 column buffer (i.e. 40 mM sodium citrate/240 mM NaCl). The run was conducted at 4 °C with an average flow rate of 0.7 ml/min). The first 500 ml of the effluent from the two serially connected columns were collected and marked as 'specific effluent (rat)'. The run was continued until the entire liter passed through the columns and the last 500 ml of the effluent were discarded. For preparation of the 'control effluent (rat)', chromatography was performed as described above, using only the control rat 'anti-CFA' column. Two different lots of IVIG have been used in the five independent experiments performed.

Following chromatography, each column was washed separately with the column buffer and eluted with 0.1 M Gly–HCl buffer at pH 2.7. Eluted fractions were collected into tubes containing Tris–HCl pH 9 for pH neutralization. The eluates were labeled as 'control eluate (rat)' and 'EAMG-specific eluate', respectively. The columns were washed with column buffer and 20% EtOH/1 M NaCl and stored for further use in the latter buffer at 4 °C.

A schematic presentation of the fractionation procedure of IVIG on rat and human IgG columns and the various fractions collected are depicted in Fig. 1.

2.3.2. Fractionation on human IgG columns

Fractionation on human-derived adsorbents was performed as described above for the rat adsorbents with some modifications (see Fig. 1). Three ml of packed resin was used for each column. For the depletion of IVIG from the MG-specific activity, to obtain the 'specific effluent (human)', IVIG was passed serially through the control human column and the MG-specific column with the control column (human normal IgG) preceding the MG-specific column (see Fig. 1). For preparation of the control depleted IVIG, chromatography was performed by passing IVIG twice through the control human IgG column .

2.4. Polyacrylamid gel electrophoresis and Western blot analysis

Analysis of IVIG and of the fractions eluted from the IgG columns (1 μg/lane) was performed by polyacrylamid gel electrophoresis followed by protein staining with Coomassie blue and by Western blot with peroxidase-conjugated mouse anti-human IgG, Fcγ fragment-specific (Jackson, ImmunoResearch, Pennsylvania, USA) or with peroxidase-conjugated goat anti-human IgA or goat anti-human IgM , Zymed, California, USA).

2.5. Administration of IVIG or of fractions derived from it

IVIG or fractions derived from it (50 mg in 2 ml) as specified were intravenously administered daily. Rats in the control groups were administered with the vehicle (20 mM sodium citrate/120 mM NaCl, 5% maltose) alone. Two five-day courses were given with a one week interval, starting 8 days following EAMG induction. In cases in which the experiment extended over 8 weeks, a third five-day-course was sometimes given, spaced by 4 weeks from the second course.

3. Results

In order to find out whether a component isolated from pooled human IVIG by chromatography on a disease-specific IgG column, contributes to its immunomodulatory therapeutic activity, we have fractionated IVIG on rat EAMG-specific or on human MG-specific columns, and studied the immunosuppressive activity of the resulting fractions in EAMG.

3.1. Fractionation of IVIG on rat immunoglobulin columns

Fractionation of IVIG on rat IgG columns was performed as described in Materials and methods and depicted in Fig. 1. IVIG was first passed through a rat control column consisting of immobilized IgG of rats immunized with CFA in order to remove non-specific human anti-rat antibodies. The effluent from this column was then passed on an EAMG-specific column. For control, IVIG was passed on the rat control column alone. The adsorbed proteins from each of these columns were eluted as described and designated 'EAMG-specific eluate' and 'control eluate (rat)', respectively (see Fig. 1). Chromatography of 1 l of IVIG containing 25 g of pooled normal human immunoglobulins, yielded about 250 μg and 400 μg, respectively of EAMG-specific eluate and control eluate in various runs. The ratio between the amounts of IVIG loaded onto the column (25 g) and the amount eluted from the EAMG-specific column (250 μg) represents a 100,000 fold enrichment of the disease-specific fraction. It is quite likely that the first control column did not deplete all the non-specific anti-rat

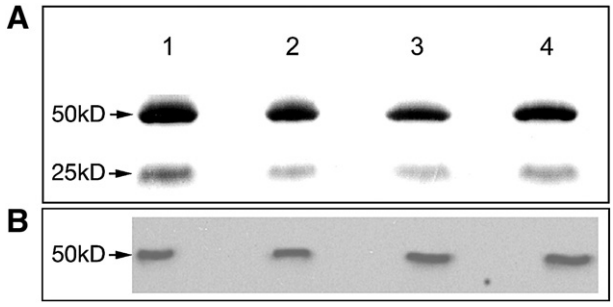

Fig. 2. Gel electrophoresis and Western blot analysis of IVIG and IVIG-derived fractions. Samples of 1) IVIG, 2) specific effluent (rat), 3) control eluate (rat) and 4) EAMG-specific eluate fractions (1 μg/lane) were analyzed by protein staining with Coomassie blue (A) and by Western blot with peroxidase-conjugated, Fcγ fragment-specific mouse anti-human IgG (B).

immunoglobulins that are not associated with EAMG and therefore the EAMG-specific eluate probably represents just an enriched fraction and in fact the amount of the disease-specific fraction is even smaller. The eluted material from both columns was identified as immunoglobulins by gel electrophoresis (Fig. 2A) and verified as IgG by its reactivity in Western blot analysis with antibodies to human IgG (Fig. 2B). No signal was detected in any of the samples with anti-IgM or anti-IgA antibodies (data not shown). The eluates had identical profiles to those of the unfractionated IVIG and of the effluent from the EAMG-specific column (Fig. 2).

The therapeutic effect of the various fractions was tested in rats in which EAMG had been induced by immunization with AChR. Treatments were initiated one week following disease induction, when signs of acute EAMG are usually observed. The employed treatment protocol was based on our previous study (Zhu et al., 2006) in which we have shown that two five-day courses of IVIG (0.4 g/kg/rat /day), initiated at the acute phase of EAMG and spaced by a 7–10 day interval, resulted in an effective suppression of EAMG, which was maintained for at least four more weeks. An additional five-day course of IVIG was sometimes given at this point (at four weeks following the second course) and was found to alleviate some of the symptoms (Fig. 3) as was also previously shown (Zhu et al., 2006).

Groups of eight AChR-immunized rats were treated with either IVIG or with a fraction derived from it. In a typical experiment (out of five similar independent experiments) presented in Fig. 3, rats were treated as described in Materials and methods with the following reagents: Rats in the first group were treated with unfractionated IVIG (0.4 g/kg/day/rat). Rats in the second group were administered with 0.4 g/kg/day/rat of the effluent from the rat control column ('control effluent (rat)'; see Fig. 1). The third group was administered with 0.4 g/kg/day /rat of a fraction obtained by chromatography first through the control and then through the EAMG-specific column ('specific effluent (rat)', depleted from both anti-'anti-CFA' IgG and from anti-EAMG IgG). The forth group was administered with a mixture of the effluent from the EAMG column ('specific effluent (rat)', 0.4 g/kg/day/rat) reconstituted with the eluate from the EAMG-specific column ('EAMG-specific eluate', 40 μg/kg/day/rat). Rats in the fifth group served as a positive control for EAMG and were administered with the vehicle only. Rats were followed daily for disease development and their clinical score and body weight were recorded twice a week by blinded observers. The mean clinical scores and mean body weights are shown in Fig. 3A and B, respectively.

As previously shown by us (Zhu et al., 2006), IVIG treatment had a suppressive effect on the clinical score of EAMG. There was a clear difference between the effects of IVIG that was depleted by passing through the non-specific control column and IVIG that was depleted by passing first through the non-specific and then through the EAMG-specific column. IVIG that had been chromatographed on the rat control column only and was presumably depleted of non-specific human anti-rat immunoglobulins ('control effluent (rat)'; see Fig. 1) did not lose its therapeutic effect and had a similar suppressive effect on the clinical score and on body weight changes as that of unfractionated IVIG (Fig. 3). The mean clinical score for the

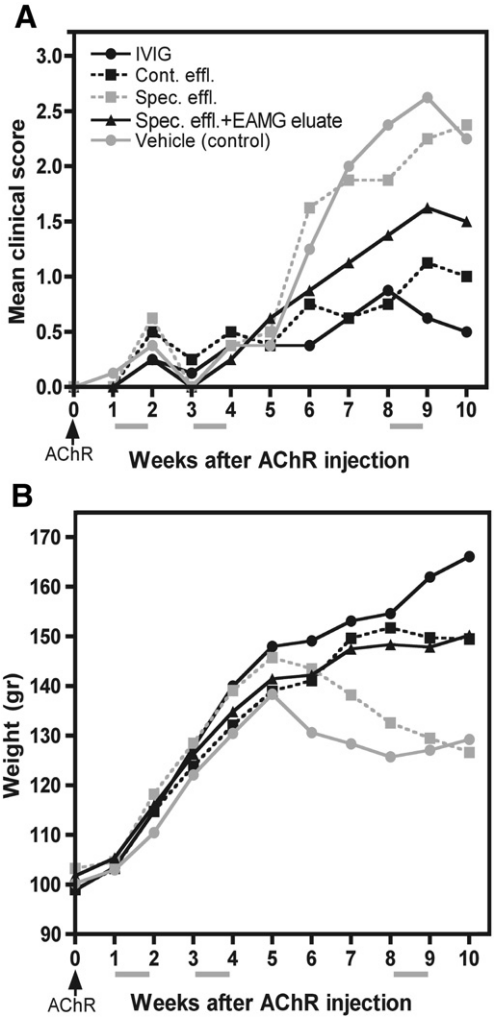

Fig. 3. Fractionation and reconstitution of the suppressive activity of IVIG on EAMG following chromatography on rat IgG columns. Mean clinical score (A) and body weight (B) of AChR-immunized rats ($n=8$ for each group) following treatment with IVIG, 'control effluent (rat)', 'specific effluent (rat)', reconstituted ('specific effluent (rat)'+'EAMG-specific eluate') and vehicle (control). Representative of five similar independent experiments. Bars at the bottom represent each a five-day treatment.

IVIG-treated group and the 'control effluent (rat)'-treated group was 0.9 and 0.8, respectively, at 8 weeks after disease induction and following two courses of treatment. At 10 weeks and following an additional third course of treatment, given eight weeks following disease induction, the mean clinical score was 0.5 and 1.0 for the IVIG-treated group and for the 'control effluent (rat)'-treated group, respectively. On the other hand, IVIG that was passed through both columns, namely the control and EAMG-specific columns ('specific effluent (rat)'), lost its therapeutic effect and rats treated with it exhibited similar clinical scores and body weight patterns to those of the vehicle-treated control group (Fig. 3). The mean clinical scores for the 'specific effluent (rat)'-treated and the control, vehicle-treated group were 1.9 and 2.3 respectively, when monitored 8 weeks after disease induction (after two courses of treatment) and 2.3 for both, 10 weeks following disease induction (after three courses of treatment).

To find out whether the suppressive activity in the IVIG preparation was retained by the EAMG-specific column, the effluent from this column ('specific effluent (rat)') that has been devoid of the suppressive activity of IVIG, was reconstituted with the eluate from this column ('EAMG-specific eluate') and the resulting reconstituted mixture was applied for treatment. The therapeutic effect that was lost in the group treated with 'specific effluent (rat)' was indeed partially recovered in the group treated by the reconstituted 'specific Effluent (rat)' + 'EAMG-specific eluate' (Fig. 3A) as reflected also in the rise in the mean body weight, (Fig. 3B). It should be noted that reconstitution of the non-suppressive depleted IVIG fraction ('specific effluent (rat)') with the 'control eluate (rat)' fraction, did not recover any of the therapeutic activity (data not shown).

3.2. Fractionation of IVIG on human-derived immunoglobulin columns

After establishing the fractionation of IVIG on *rat* immunoglobulin columns and in order to further test the notion that the suppressive effect of IVIG involves a minor anti-antibody disease-specific fraction present in IVIG, we examined a similar fractionation procedure on human-derived immunoglobulins. IVIG was fractionated using a column of *human* immunoglobulin isolated from pooled plasma of five MG patients and for control, on a column of immunoglobulins from five healthy controls as described in Materials and methods and in Fig. 1.

Chromatography of 1 l of IVIG containing 25 g of pooled normal human immunoglobulins, yielded about 290 μg and 440 μg, respectively of MG-specific eluate and human control eluate in various runs. The ratio between the amount of IVIG loaded onto (25 g) and eluted from the MG-specific column (290 μg) represents a 86,000 fold enrichment of the disease-specific fraction. The eluted fractions were identified as immunoglobulin by gel electrophoresis and verified as IgG (data not shown).

The suppressive activity of the various fractions on EAMG was tested by their administration to AChR-immunized rats each consisting of eight animals. Rats were treated with the following reagents: Rats in the first group were treated with unfractionated IVIG (0.4 g/kg/day/rat). Rats in the second group were treated with the effluent from the human control column ('control effluent (human)', 0.4 g/kg/day/rat) and the third group received the effluent fraction that was first chromatographed through the human control column and then through the MG-specific column ('specific effluent (human)', 0.4 g/kg/day/rat). Rats in group 4 were treated with a mixture of the above described effluent from the MG-column ('specific effluent (human)', (0.4 g/kg/day/rat) reconstituted with the eluate from the MG-specific column ('MG-specific eluate', 40 μg/kg/day/rat). Rats in the fifth group served as control and were administered with the vehicle only. The mean clinical scores and mean body weights are shown in Fig. 4.

Generally, the results of fractionation on the *human* IgG columns yielded similar results to those obtained following fractionation on the rat-derived IgG columns and where even more impressive. As with the fractionation on the rat-derived immunoglobulin columns there was a clear difference between the suppressive effects of the human control effluent (depleted of anti-normal human IgG) and the effluent from the

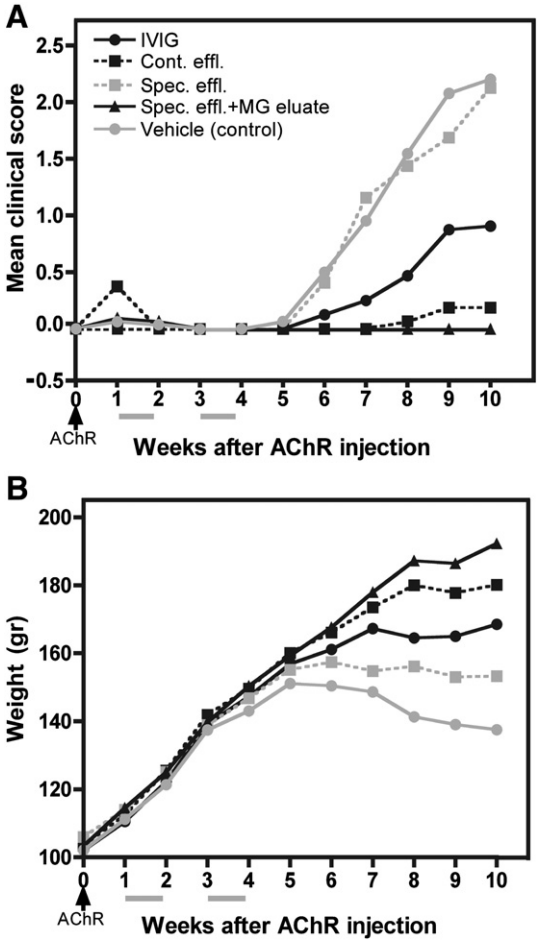

Fig. 4. Fractionation and reconstitution of the suppressive activity of IVIG on EAMG following chromatography on human IgG columns. Mean clinical score (A) and mean body weight (B) of AChR-immunized rats ($n=8$ for each group) following treatment with IVIG, 'control effluent (human)', 'specific effluent (human)', reconstituted ('specific effluent (human)' + 'MG-specific eluate') and vehicle (control). Representative of three similar experiments. Bars at the bottom represent each a five-day treatment.

MG-specific column (depleted of anti-MG IgG). As demonstrated in Fig. 4 the effluent obtained following chromatography of IVIG first through the human control column and then through the MG-specific column (designated 'specific effluent (human)'), completely lost its therapeutic effect. In contrast, the control effluent, obtained following two subsequent chromatographies through the control human column retained its full suppressive effect on EAMG and was in this experiment even more effective than the non-fractionated IVIG in suppressing disease symptoms. The mean clinical score for rats treated by the 'specific effluent (human)' fraction was 2.1 at 10 weeks following disease induction, in comparison with a mean clinical score of 2.2 in the vehicle-treated group and 0.18 in the group treated with the 'control effluent (human)' fraction (Fig. 4A).

To find out whether the lost suppressive activity in the effluent from the MG-specific column (presumably depleted of antibodies to MG IgG) could be recovered by the addition of the eluate from the MG-specific column that presumably contains antibodies to MG IgG, these two fractions were combined and applied for treatment. A marked suppressive effect, greater than that of unfractionated IVIG, was observed in the group of rats treated with a mixture of the 'specific effluent (human)' fraction (0.4 g/kg/day/rat) reconstituted with the MG-specific eluate' fraction (40 μg/kg/day/rat). All eight rats in this group remained completely healthy throughout the experiment (Fig. 4A) as reflected also by their constant gain of weight, higher in its extent than that of all other treatment groups in the experiment (Fig. 4B). The marked suppression by the mixture consisting of the 'specific effluent (human)' fraction and the 'MG-specific eluate' fraction could be due to the use of the eluate at a large excess compared to the IVIG it was derived from, to compensate for losses during the fractionation procedures.

4. Discussion

IVIG treatment is being used beneficially for the treatment of several autoimmune diseases. Although patients tolerate this treatment quite well, the amounts of human IgG administered are very high (tens of grams daily). Thus, IVIG treatment causes sometimes undesirable side effects and is also rather costly. Although the mechanisms involved in the therapeutic effect of IVIG are complex and not fully understood, it is believed that only a minor fraction(s) of the preparation employed, participates in and contributes to its suppressive effect. In this study we have attempted to isolate and identify such a disease-specific fraction from pooled human IVIG.

Similarly to other reported studies on the application of allogeneic pooled *human* immunoglobulin (IVIG) for treatment of experimental autoimmune diseases in non-human species, such as *mice* and *rats* (Achiron et al., 2000; Jia and Pollock, 2000; Jorgensen and Sorensen, 2005; Pashov et al., 1998; Samuelsson et al., 2001; Saoudi et al., 1993; Shoenfeld et al., 2002; Wada et al., 2001; Weishaupt et al., 2002; Zhu et al., 2006), we have also employed *human* IVIG and its fractions for investigating the therapeutic effect on *rat* EAMG. The constraint of using *human* immunoglobulins in an allogeneic system stems from the technical difficulty of obtaining *rat* (autologous) immunoglobulins in the amounts needed for the treatment experiments. The effectiveness of experiments performed in murines (rats or mice) with human IVIG implies that there is an interspecies cross-reactivity in the active components of IVIG. We have demonstrated in our study that IVIG could be depleted of a suppressive, EAMG-specific active component(s) by passing through either EAMG or MG IgG columns, suggesting that idiotype–anti-idiotype networks are involved. Indeed, cross-species idiotypes in the anti-AChR response have been previously reported by us (Souroujon et al., 1985; Souroujon and Fuchs, 1986) and anti-idiotypes were shown to have a broad suppressive effect on the overall anti-AChR titer. We assume that pooled *human* immunoglobulins, obtained from thousands of healthy donors contain a vast repertoire of anti-antibodies (anti-idiotypes) some of which may have interspecies cross-reactivity. It should also be noted that the EAMG and MG IgG columns may contain, in addition to anti-AChR antibodies, also antibodies to other muscle-specific proteins that might be involved in the pathogenesis of the disease. Hence, these columns may also deplete IVIG of anti-idiotypes to idiotypes other than anti-AChR antibodies.

It should be noted that although the disease-specific fractions are anti-immunoglobulins by definition, we could not demonstrate in vitro direct binding of IVIG or of the disease-specific fraction isolated from it, to EAMG or MG-derived immunoglobulins, or their ability to inhibit the binding of EAMG- or MG-derived immunoglobulins to AChR. Such a discrepancy between the functional suppressive effect of IVIG and of the fractions derived from it, and their ex vivo binding properties could be due, among others, to technical limitations in the assays resulting from high background of normal IgG or from low affinity of the anti-antibodies that can also be related to their specificity variations (see Buchwald et al., 2002, 2005 and references therein).

The most striking observation in this study is the ability to specifically eliminate the therapeutic activity on EAMG from the IVIG preparation by its chromatography through either an EAMG- or an MG-specific column. This type of chromatography resulted in an adsorption of a very small amount of IgG from the IVIG preparation and eliminated its immunosuppressive activity on EAMG. On the other hand, chromatography of the IVIG through the non-specific control columns (IgG from CFA-immunized *rats* and IgG from normal *human controls*, respectively) did not reduce its therapeutic capability (Figs. 3, 4). This is a strong indication that only the disease-specific columns were able to adsorb and remove the disease-specific immunosuppressive fraction from the IVIG preparation. These observations indicate that the fraction removed from IVIG by the EAMG- or MG-specific column is *essential* for the therapeutic activity of the IVIG. Whether it may also be *sufficient* is still not clear.

As we have examined the therapeutic activity of the disease-specific eluates from the EAMG- or MG-specific columns in the presence of the non-active effluents from these columns, respectively (Figs. 3, 4), we cannot exclude the possibility that additional components in these non-active IVIG effluent

fractions are required for reconstituting the full suppressive activity. Such components could include factors that have been proposed to account for some of the clinical effects of IVIG, e.g., factors modulating the expression and function of Fc receptor and of complement activation, immunoglobulin molecules interacting with the specific anti-antibodies to form dimers and other factors (Dalakas, 2004; Bayary et al., 2006; Bayry et al., 2007; Kaneko et al., 2006). Although needed for the immunosuppressive activity of IVIG, such components that are not necessarily disease-specific may require the presence of the disease-specific anti-antibodies (anti-idiotypes) that are *essential* for the observed clinical effect on EAMG. Experiments to elucidate this issue are now underway.

The IVIG preparations are prepared from plasma pools of thousands of donors. Thus they may represent the entire repertoire of antigen-combining variable regions of antibodies that would be present in normal serum. It is assumed that the specificity of the isolated anti-antibody active fraction is determined by the disease-specific immunoglobulin employed to fractionate the IVIG preparation. In our case it is only the anti-antibody fraction isolated on immunoglobulin columns from rats with EAMG or from MG patients and not on immunoglobulin columns from control animals, immunized with CFA or from normal patients that had a therapeutic clinical effect on EAMG. Anti-antibodies with specificity for other autoimmune diseases can possibly be isolated by their selective binding to the respective disease-specific IgG. In another reported study DNA-specific antibodies from SLE patients were employed to isolate from IVIG, a fraction of anti-antibodies (anti-idiotypes) that suppressed experimental SLE (Shoenfeld et al., 2002). It is possible that the same IVIG preparation could serve for several serial fractionations of anti-antibodies *each* specific for a different disease and could thus make this therapeutic approach more specific and possibly less expensive. Finally, as IVIG is a treatment applied in diseases that are usually of a chronic nature, a personalized fractionation of IVIG on the patient's own autoreactive immunoglobulins might in the future be considered as the treatment of choice.

Acknowledgements

This research was supported by grants from The Muscular Dystrophy Association of America (MDA), The European Commission (EC, No QLG1-CT-2001-10918, QLRT-2001-00225 and LSHN-CT-2006-037833) and The Wood–Byer Foundation.

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