

Antibody concentrations to A β 1-42 monomer and soluble oligomers in untreated and antibody-antigen-dissociated intravenous immunoglobulin preparations

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Abstract

Cognitive improvement in Alzheimer's disease (AD) patients treated with intravenous immunoglobulin (IvIg) has been attributed to its antibodies to amyloid beta ($A\beta$). We compared the concentrations of specific antibodies to soluble $A\beta$ 1-42 conformations, namely $A\beta$ 1-42 monomer and $A\beta$ 1-42 soluble oligomers, between three IvIg preparations, Gamunex, Gammagard, and Flebogamma. To determine specific antibody concentrations to these $A\beta$ 1-42 conformations, nonspecific binding of the IvIg preparations to the $A\beta$ reverse sequence, $A\beta$ 42-1, was subtracted. These antibodies were measured in untreated IvIg preparations and also after they were treated to dissociate antibody-antigen complexes, because this procedure has been reported to increase the detectable levels of serum anti- $A\beta$ antibodies. Antibody levels to $A\beta$ 1-42 monomer were significantly higher in untreated Gamunex than in the other two IvIg preparations, and antibody-antigen dissociation increased the measured anti- $A\beta$ monomer concentrations in Gamunex and Gammagard. Dissociated Gamunex and Gammagard had higher anti- $A\beta$ monomer levels than Flebogamma. Generally similar results were found for antibodies to soluble $A\beta$ 1-42 oligomers, with the exception that after antibody-antigen dissociation, only Gammagard had significantly higher antibody levels than Flebogamma. These differences in antibody concentrations to $A\beta$ 1-42 conformations (particularly to $A\beta$ 1-42 soluble oligomers, thought to be the most neurotoxic conformation of soluble $A\beta$) and the increased availability of these antibodies after antibody-antigen complex dissociation have important implications for IvIg treatment of AD patients. (**Keywords:** Alzheimer's disease; Amyloid beta; Anti-amyloid beta antibodies; ELISA; Intravenous immunoglobulin; Oligomers)

1. Introduction

Amyloid beta ($A\beta$) is the major protein in senile plaques in the Alzheimer's disease (AD) brain. In a transgenic mouse model of AD, systemic vaccination with $A\beta$ prevented plaque deposition and cognitive loss in young mice and reduced plaque counts and cognitive deficits in older animals [1]. These findings were extended in subsequent studies [2-4]. Similar results were also achieved with systemic administration of anti- $A\beta$ antibodies [5-8], suggesting that antibodies to $A\beta$ may be able to promote plaque clearance from the brain in AD patients. This was confirmed by the finding of reduced brain $A\beta$ content in eight AD patients who were vaccinated with $A\beta$ in a phase I trial, although, surprisingly, there was no difference in the time required for patients to progress to severe dementia between vaccinated and placebo patients in this trial [9]. This result differed from another study which suggested that $A\beta$ vaccination may slow cognitive decline in AD patients [10]. Whether systemic antibodies to $A\beta$ facilitate its removal by entering the brain, or via a "peripheral sink" mechanism [6] without entering the brain, is unclear.

Intravenous immunoglobulin (IvIg) is currently being investigated as a treatment for AD. IvIg is composed of purified immunoglobulins (more than 95% is IgG, with only trace amounts of IgA or IgM [11]) pooled from thousands of clinically normal donors, and has long been used for treatment of selected autoimmune and immunodeficiency disorders [12,13]. It is currently approved by the Food and Drug Administration for six conditions [14], and is also commonly used in many "off-label" applications. Improved cognitive scores were obtained in AD patients treated with IvIg in two short-term, open-label trials [15,16]. A recent retrospective study also suggested that individuals who

receive IvIg have a reduced risk of developing AD [17]. While developing enzyme-linked immunosorbent assays (ELISAs) to measure IvIg's antibodies to the two soluble conformations of A β 1-42, namely A β monomer and A β soluble oligomers, we found increased optical density (OD) readings not only when diluted IvIg preparations were incubated in wells previously coated with A β 1-42, but also when they were incubated in wells coated with irrelevant proteins (i.e., the reverse A β sequence A β 42-1 or bovine serum albumin [BSA]) or even buffer alone, compared to the standard negative control in which buffer was substituted for IvIg. Although anti-A β antibodies in IvIg have been measured previously by ELISA [18-20], no controls for the specificity of antibody binding to A β were described in these studies; thus, the extent to which specific antibodies to A β 1-42 were measured is unclear. The objective of the present study, therefore, was to compare the concentrations of specific IgG to A β 1-42 monomer and soluble oligomers between different IvIg preparations. Because low pH dissociation of antibody-antigen complexes has been reported to increase the detectable levels of serum anti-A β antibodies [21], these experiments were performed with dissociated as well as untreated IvIg preparations.

2. Materials and methods

2.1. Production of A β monomer and soluble oligomers

A β 1-42 (0.5 mg; AnaSpec, San Jose, CA) was disaggregated by suspending in 0.25 ml trifluoroacetic acid (hereafter, TFA; reagent grade TFA, Sigma-Aldrich, Inc., St. Louis, MO) followed by an equal volume of hexafluoro-2-propanol (Sigma-Aldrich). After water bath sonication for 1 hr, it was aliquotted into 0.6 ml eppitubes (20 μ l/tube), dried

with N₂ gas for 90 min, and stored at -20° C. To produce A β monomer, 20 μ g of the disaggregated A β 1-42 was resuspended by vortexing for 3 min in 0.6 ml HPLC-grade water, adjusted to pH 3.0 with TFA (hereafter, “TFA water”). This was repeated twice more, yielding 1.8 ml of resuspended A β . 21.8 mg of Tris base (Trizma base, Sigma) was then added with vortexing to bring the Tris concentration to 100 mM. The pH of this solution was adjusted to 8.8 by adding 12.1 N HCl. This preparation, whose protein concentration was measured as 6 μ g/ml with the Bio-Rad protein assay (data not shown), was centrifuged (12,000 rpm [11,752 x g] x 5 min, room temperature), passed through a 0.2 μ m filter (GHP Acrodisc 13 mm Syringe Filter with 0.2 μ m GHP Membrane, Pall Life Sciences, East Hills, NY), and used immediately. The A β reverse sequence, A β 42-1 (AnaSpec), was prepared in a similar manner. A β oligomers were produced as described by Kaye et al. [22] with slight modifications. 60 μ g of previously disaggregated A β 1-42 (and, as a negative control, A β 42-1) was resuspended in 4.8 μ l of 1% NH₄OH, yielding a concentration of 2.8 mM. This was sonicated in a water bath for 4 min, incubated at room temperature for 1 hr, and then diluted in phosphate buffered saline (PBS; 10 mM, pH 7.4, with 0.02% azide) to a final A β concentration of 45 μ M. It was used immediately or stored at 4° C for up to one week.

2.2. Evaluation of A β conformations by Western blot

A β preparations were electrophoresed under reducing conditions through 4-20% Tris-HCl Ready Gels (Bio-Rad Laboratories, Hercules, CA). 20 μ l of the 6 μ g/ml monomer preparation (0.12 μ g) was mixed with an equal volume of Laemmli Sample Buffer (Bio-Rad), and then loaded into appropriate lanes; for the oligomer preparation, 10 μ l of the 45

μM (203 $\mu\text{g}/\text{ml}$) preparation (2.03 μg) was mixed with an equal volume of Laemmli sample buffer and then loaded onto the gel. After electrophoresis, the proteins were transferred to Westran S PVDF membranes (Whatman International Ltd., Maidstone, UK). The membranes were then blocked with 10% non-fat dry milk in 0.01M PBS, pH 7.4, filtered through qualitative filter paper (Whatman), for 1 hr at room temperature with agitation. Membranes were incubated overnight at 4° C with agitation in mouse monoclonal anti-A β (1-16) 6E10 (Covance Research Laboratories, Berkeley, CA; 1:5,000 dilution). After incubation in horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA; 1:10,000 dilution) for 1 hr at room temperature with agitation, membranes were developed in SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL). Bands were detected on CL-XPosure film (Thermo Scientific).

2.3. IvIg preparations

Three IvIg preparations were evaluated: Gamunex Immune Globulin Intravenous (Human), 10% (Talecris Biotherapeutics, Inc., Research Triangle Park, NC), Gammagard Liquid [Immune Globulin Intravenous (Human)] 10% (Baxter Healthcare Corp., Westlake Village, CA), and Immune Globulin Intravenous (Human) Flebogamma 5% DIF 2.5 g (Grifols Biologicals Inc., Los Angeles, CA).

2.4. Dissociation of antibody-antigen complexes in IvIg preparations

The procedure described by Li et al. [21] for antibody-antigen dissociation (hereafter, “dissociation”) was followed with slight modifications. 40 μl of each IvIg preparation

was diluted 1:100 by adding 3,960 μ l of dissociation buffer (0.01 M PBS, pH 7.2, with 1.5% BSA and 0.2 M glycine, adjusted to pH 3.5 with glacial acetic acid). After 20 min at room temperature, it was centrifuged (3,000 x g, 90 min) through a YM-30 filter (Amicon Ultra-4 Ultracel-30k, Millipore Corp., Billerica, MA). Tris buffer (1 M, pH 9.0) was added to bring the retentate pH to 7.0, and PBS with 0.1% Tween-20 (Sigma) and 1% BSA (hereafter, PBS-T-BSA) was added to bring the final volume of the retentate to 4 ml. This was stored at 4° C for up to one week.

2.5. ELISA measurement of antibodies to A β monomer and soluble oligomers in IvIg preparations

Specific antibody concentrations to A β 1-42 monomer and soluble oligomers were measured by ELISA in four to five experiments for each IvIg preparation, separately for the two A β conformations; in all, 27 experiments were performed. IvIg preparations were randomized as to the order in which these experiments were performed. A β monomer and oligomers were generated as described above, subjecting the A β reverse sequence A β 42-1 to the same procedures in order to generate a negative control. In experiments in which anti-A β monomer antibodies were measured, the monomer preparation and its reverse sequence, A β 42-1, were incubated at a concentration of 6 μ g/ml in Tris buffer (0.1 M, pH 8.8) overnight at 4° C on a 96-well Nunc Maxisorp plate. In separate experiments in which IvIg's anti-A β oligomer antibodies were measured, the oligomer preparation and reverse A β sequence were incubated on the plates overnight at 0.9 μ g/ml. (In these latter experiments, additional wells were also incubated with an equal concentration of the monomer preparation. Because densitometric analysis

indicated that approximately 30% of the total band intensity in the oligomer preparation was due to A β monomer [data not shown], after calculating the mean anti-monomer antibody concentration, 30% of this was subtracted from the specific antibodies to the oligomer preparation.) Wells were then treated with SuperBlock (SuperBlock Blocking Buffer in PBS, Thermo Scientific) as per the manufacturer's instructions, followed by untreated or dissociated IvIg preparations. Untreated and dissociated Gamunex were diluted 1:1,000 in PBS-T-BSA, while Gammagard and Flebogamma were diluted 1:100. (These dilutions were chosen on the basis of preliminary experiments to determine which dilutions of each IvIg would result in similar OD readings in the linear portion of the standard curve.) Three to six wells were incubated for each condition. Four-fold dilutions of mouse monoclonal 6E10 anti-A β antibody (1:4,000 [250 ng/ml], 1:16,000 [62.5 ng/ml], 1:64,000 [15.6 ng/ml], and 1:256,000 [3.9 ng/ml]) were included for the standard curve on each plate. Secondary antisera were biotinylated goat anti-human IgG for wells previously incubated with IvIg preparations and biotinylated goat anti-mouse IgG for wells receiving mouse antibodies (both from Vector; dilution was 1:1,000 in PBS-T-BSA). After incubation with streptavidin-alkaline phosphatase (Zymed Laboratories, Invitrogen, Carlsbad, CA; 1:1,000 in PBS-T), para-nitrophenol phosphate (Sigma) was added (5 mg in 40 ml of 1 M diethanolamine buffer, pH 9.8) and the plate was read at 405 nm with a Vmax kinetic microplate reader (Molecular Devices Corp., Sunnyvale, CA) until the standard curve OD reached 1.0. Softmax Pro software version 3.0 (Molecular Devices) was used to generate the best-fit plot of the standard curve, using the log-logit option. To calculate specific anti-monomer antibody concentrations, the mean antibody concentration measured when each IvIg preparation was incubated on

wells previously coated with A β 42-1 was subtracted from antibody concentrations measured on wells coated with an equal concentration of monomeric A β 1-42. Calculation of specific antibodies to A β oligomers was performed as described above.

2.6. Statistics

Data were analyzed from 27 experiments: five experiments for anti-A β monomer antibodies, and four experiments for anti-A β oligomer antibodies, for each of the three IvIg products. Data from each experiment were adjusted for interassay variation by multiplying them by a normalization factor. This factor was derived by first determining the observed (calculated) concentration, in each experiment, of the 1:64,000 dilution (= 15.6 ng/ml) of mouse monoclonal anti-A β antibody in the standard curve. (This dilution was chosen because its OD value most closely approximated that of the diluted IvIg samples.) The mean concentration (\pm SEM) of this dilution of the antibody for all 27 assays was 14.995 ± 0.182 μ g/ml, and the coefficient of variation was 6.3%. To calculate the normalization factor for each plate, this mean value was divided by the observed concentration of this dilution on the plate. Observations that were identified as outliers on graphical and statistical inspection were excluded from analysis. The distribution of each data set was first analyzed to determine if it met the assumptions of the statistical tests proposed to analyze it. Based on this assessment, either Student's t-test (two-tailed), Wilcoxon Two-Sample Test using t-approximation, Fixed Effects ANOVA with Least Squares analysis, or Kruskal-Wallis test was used to compare the mean concentrations or median scores of anti-monomer and anti-oligomer antibodies between untreated IvIg preparations, between dissociated IvIg preparations, and between

the untreated and dissociated forms of each IvIg preparation. Post hoc pairwise comparisons were performed, when necessary, with Hochberg's adjustment [23]. P-values less than an alpha of 0.05 (probability of type I error) were considered statistically significant for all tests. Statistical analysis was performed using SAS System for Windows version 9.2.

3. Results

3.1. Production of A β monomer and soluble oligomers

A β is supplied commercially as a lyophilized, trifluoroacetic acid salt. When PBS was added to this preparation, A β did not go into solution, in agreement with Burdick et al. [24] that A β 1-42 is relatively insoluble at pH 7.4 and in aqueous media. When A β previously disaggregated in our laboratory was first dissolved in TFA water (pH 3.0) and the pH was then adjusted to 7.0, only one band was generated in SDS PAGE gels (Fig. 1, lane A) and native gels (not shown). This was assumed to represent monomeric A β (molecular weight = 4.5 kDa) because its molecular weight on the SDS PAGE gel was less than the lowest molecular weight standard, 7 kDa. When disaggregated A β was resuspended in NH₄OH and incubated for 1 hr before adjusting the pH to 7.4, multiple oligomeric bands were visualized on the SDS PAGE gel, although some monomer was also present (Fig. 1, lanes B and C). Lower molecular weight bands were also evident in this preparation, probably representing A β fragments. Our efforts to remove A β monomer from this latter preparation by selective molecular weight filtration, centrifugation, or treatment with insulin-degrading enzyme [25] were unsuccessful.

3.2. Specific antibodies in IvIg preparations to A β 1-42 monomer

The ELISA standard curve for detection of specific antibodies to A β 1-42 monomer is shown in Fig. 2. The curve was generated with four-fold dilutions of mouse monoclonal antibody 6E10 (anti-A β 1-16), and the lower limit of sensitivity of the assay was approximately 4 ng/ml. The mean concentrations of specific antibodies in the three IvIg preparations, both untreated and after antibody-antigen dissociation, to A β 1-42 monomer are shown in Fig. 3. In untreated IvIg preparations, the highest concentration of these antibodies was detected in Gamunex ($p = 0.0172$ vs. both Flebogamma and Gammagard). Flebogamma's anti-monomer antibodies were also higher than those in Gammagard. After dissociation of antibody-antigen complexes, Gamunex still contained the highest concentration of these antibodies, followed by Gammagard and then Flebogamma ($p < 0.05$ only for dissociated Gamunex vs. dissociated Flebogamma). Dissociation significantly increased the detectable anti-monomer levels in Gamunex and Gammagard (both $p = 0.0172$ vs. their untreated forms), while decreasing it in Flebogamma ($p = 0.0014$). The mean OD values for binding of the IvIg preparations to A β 1-42 monomer and to the A β reverse sequence A β 42-1 are shown in Fig. 4, indicating the extensive nonspecific binding that was subtracted in order to calculate the specific anti-A β 1-42 antibody concentrations.

3.3. Specific antibodies in IvIg preparations to A β 1-42 soluble oligomers

The mean concentrations of anti-A β 1-42 oligomer antibodies in IvIg preparations are shown in Fig. 5. Results were generally similar to those for anti-monomer antibodies. Untreated Gamunex had the highest concentration of anti-oligomer antibodies

($p = 0.0172$ vs. both Flebogamma and Gammagard). Antibody-antigen dissociation significantly increased these antibodies in all three IvIg preparations, and dissociated Gammagard had significantly higher anti-oligomer antibodies than dissociated Flebogamma ($p = 0.0081$). The mean OD values for IvIg binding to A β 1-42 oligomers and the A β reverse sequence are shown in Fig. 6; the percentage of binding to A β 1-42 oligomers that was specific tended to be greater, particularly for Flebogamma and Gammagard, than their binding to A β 1-42 monomers.

4. Discussion

The encouraging results in the trials in which IvIg was administered to AD patients [15,16] suggest that IvIg may be of value for treatment of AD. The investigators in these trials suggested that anti-A β antibodies might be responsible for IvIg's benefits in AD patients. However, other explanations are also possible; for example, IvIg has marked anti-inflammatory actions [12] which could have contributed to improvement in the AD patients. In the present study, untreated Gammagard's specific antibody levels to A β 1-42 monomer was lower than the other two IvIg preparations examined, and its specific antibody level to A β 1-42 soluble oligomers was intermediate between Gamunex and Flebogamma. The relatively low anti-A β levels in Gammagard suggest that its benefits in the AD trial in which it was administered [16] may not have been due solely to anti-A β antibodies.

Various approaches have been used to produce soluble A β oligomers [22,25-28]. Our results underscore the difficulties in producing a pure oligomer preparation. In agreement with previous findings by Teplow [27], we were able to consistently produce

oligomers by initially disaggregating A β and then resuspending it in alkaline pH. The oligomer preparation contained A β monomer as well. Our finding that all three IvIg preparations contained measurable levels of antibodies to A β oligomers even after subtracting out anti-monomer antibodies suggests that these preparations may contain some antibodies which recognize A β oligomers but not A β monomer, in agreement with the conclusions from a recent immunocytochemical study [29].

The surprisingly high levels of nonspecific binding of the IvIg preparations to the reverse sequence A β 42-1, compared to their binding to A β 1-42 monomer and oligomers, are shown in Figs. 4 and 6, respectively. This nonspecific binding was typically almost as high as the specific binding to A β 1-42, and in some experiments it equaled it. Further investigation of the nature of this nonspecific binding, and how to reduce or prevent it, is beyond the scope of this study. It should be noted, however, that there is extensive nonspecific binding of IvIg's immunoglobulins when antibodies to A β are measured in the indirect ELISA, and this binding must be taken into account when calculating IvIg's specific anti-A β antibody concentration.

The presence of antibodies to A β in IvIg preparations reflects the presence of these antibodies in serum and plasma in clinically normal individuals. Numerous studies have compared the levels of these antibodies between non-cognitively impaired subjects and those with AD, but whether these antibodies increase, decrease, or remain unchanged in AD subjects is unclear [30-37]. Anti-A β antibodies in human serum are present in IgM as well as IgG, and the anti-A β titer for these antibodies has been reported to be higher in IgM than in IgG [20]. In the present study antibodies to A β were measured in IgG but not in IgM because IvIg contains only trace amounts of IgM [11].

In conclusion, this study revealed differences in specific antibody levels to soluble A β 1-42 conformations between IvIg preparations, as well as within IvIg preparations after antibody-antigen complex dissociation. These findings could have important implications for treatment of AD patients with IvIg. Among the untreated IvIg products, Gamunex had the highest concentration of anti-monomer antibodies, in agreement with findings presented at the ICAD 2006 meeting by Talecris Biotherapeutics, the manufacturer of Gamunex [38] (our measurements of anti-A β monomer antibodies were completed before we learned of these results), and also the highest level of anti-oligomer antibodies. The relatively low specific antibody levels in Gammagard to both A β conformations were surprising in view of its success in the previously-mentioned AD clinical trial [16]. Our finding that antibody-antigen complex dissociation tended to increase the available levels of anti-A β antibodies in IvIg preparations suggests that this procedure might increase the ability of IvIg preparations to reduce brain A β and/or neutralize its neurotoxic actions, particularly those of A β soluble oligomers [39].

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Figure legends

Fig. 1: Western blots of A β 1-42 preparations generated in various conditions. Lane A: monomeric A β (MW 4.5 kDa) generated by resuspending lyophilized A β 1-42 in TFA and hexafluoro-2-propanol to disaggregate it, followed by drying it with N₂ gas, dissolving it in TFA water (pH 3.0), and adding Tris base and HCl to adjust the pH to 7.0. Lanes B and C: disaggregated A β , after resuspending in NH₄OH, brief sonication, incubating for one hr at room temperature, and then adjusting to neutral pH. (Total protein loaded: lane A, 0.2 μ g; lane B, 1.0 μ g; lane C, 2.0 μ g.)

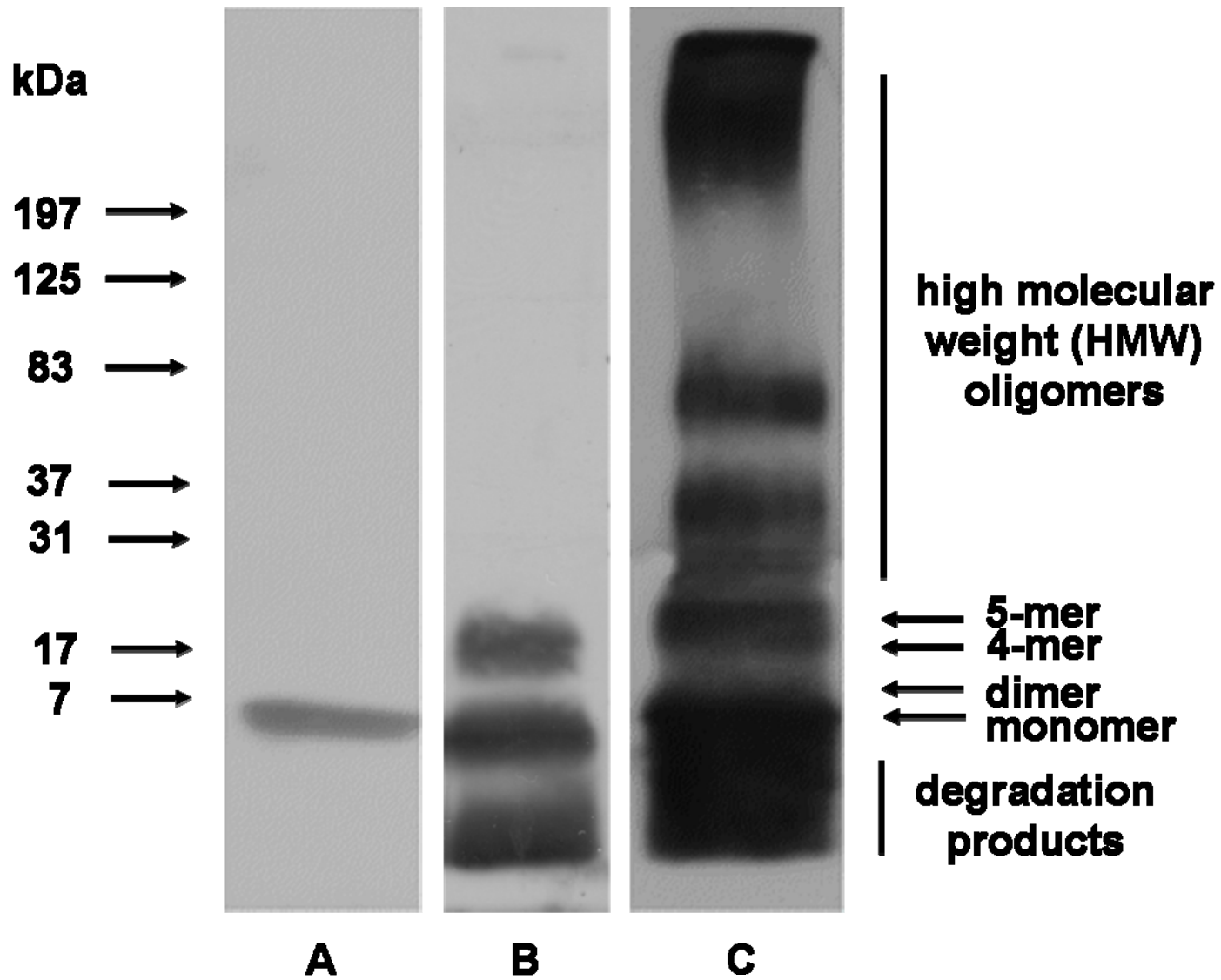
Fig. 2: ELISA standard curve with four-fold dilutions of mouse monoclonal antibody 6E10 (anti-A β 1-16) placed in wells previously coated with A β 1-42 monomer. The lower limit of sensitivity of the assay was approximately 4 ng/ml. A similar standard curve was generated for binding of the antibody to the A β 1-42 oligomer preparation.

Fig. 3: Mean concentrations of specific antibodies to A β 1-42 monomer in each IvIg preparation. Data shown are means \pm SEM from pooled data from five experiments (5-6 wells per condition in each experiment) for each IvIg preparation. (GX = Gamunex, FG = Flebogamma, GG = Gammagard Liquid; ^a p < 0.05 vs. untreated Flebogamma; ^b p < 0.05 vs. untreated Gammagard; ^c p < 0.05 vs. dissociated Gamunex; ^d p < 0.05 vs. dissociated Flebogamma)

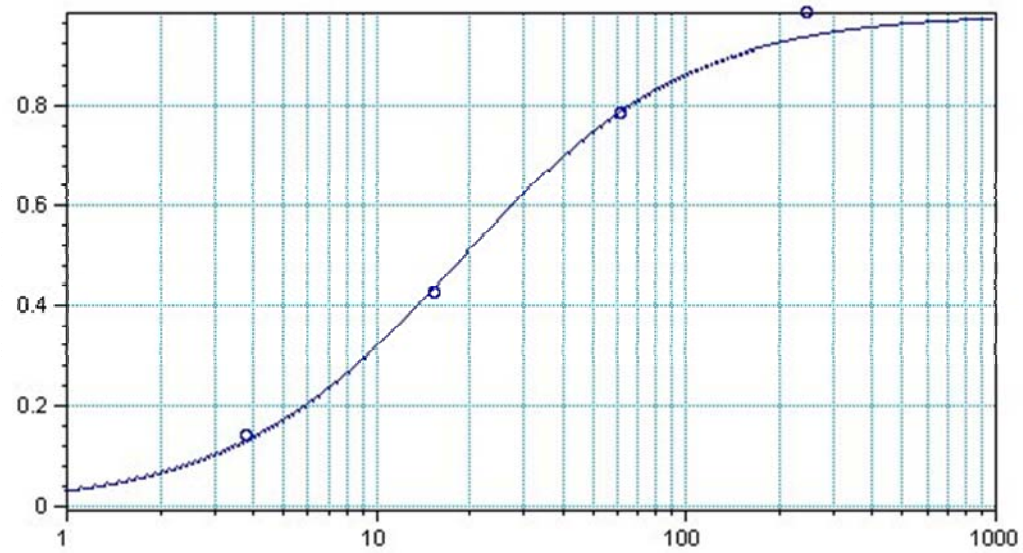
Fig. 4: Mean optical density (OD) values for binding of untreated and antibody-antigen-dissociated IvIg preparations to A β 1-42 monomer vs. the A β reverse sequence, A β 42-1. The dilutions of the IvIg preparations were: Gamunex, 1:1,000; Gammagard, 1:100; and Flebogamma, 1:100. These dilutions were chosen to provide similar OD readings, in the linear part of the standard curve, for the untreated IvIg products. The extensive binding to A β 42-1 indicates that only a small percentage of IvIg's binding to A β 1-42 was actually specific.

Fig. 5: Mean concentrations of specific antibodies to A β 1-42 oligomers in each IvIg preparation. Data shown are means \pm SEM from pooled data from four experiments (3-6 wells per condition in each experiment) for each IvIg preparation. (GX = Gamunex, FG = Flebogamma, GG = Gammagard Liquid; ^a p < 0.05 vs. untreated Flebogamma; ^b p < 0.05 vs. untreated Gammagard; ^c p < 0.05 vs. untreated Gamunex; ^d p < 0.05 vs. dissociated Flebogamma)

Fig. 6: Mean optical density (OD) values for binding of untreated and antibody-antigen-dissociated IvIg preparations to A β 1-42 oligomers vs. the A β reverse sequence, A β 42-1. The percentage of binding to A β 1-42 oligomers that was specific tended to be greater for Flebogamma and Gammagard than for their binding to A β 1-42 monomers (Fig. 4).



**Mean Value
(Optical
Density at
405 nm)**



Mouse monoclonal 6E10 anti-Aβ1-16, ng/mL

