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ELISA measurement of specific antibodies to phosphorylated tau in intravenous immunoglobulin products

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ABSTRACT

The therapeutic effects of intravenous immunoglobulin (IVIG) products were recently studied in Alzheimer's disease (AD) patients. Pilot studies produced encouraging results but phase II and III trials gave disappointing results; a further study is in progress. IVIG products contain antibodies to tau protein, the main component of neurofibrillary tangles (NFTs). The tau used to detect IVIG's anti-tau antibodies in previous studies was non-phosphorylated recombinant human tau-441, but NFT-associated tau is extensively phosphorylated. The objective of this study was to determine if various IVIG products contain specific antibodies to phosphorylated tau (anti-pTau antibodies). ELISAs were used to evaluate binding of six IVIG products to a 12 amino acid peptide, tau 196–207, which was phosphorylated ("pTau peptide") or non-phosphorylated ("non-pTau peptide") at Serine-199 and Serine-202. Both amino acid residues are phosphorylated in AD NFTs. Each IVIG's "anti-pTau antibody ratio" was calculated by dividing its binding to the pTau peptide by its binding to the non-pTau peptide. Seven experiments were performed and data were pooled, with each experiment contributing one data point from each IVIG product. Mean anti-pTau antibody ratios greater than 1.0, suggesting specific antibodies to phosphorylated tau, were found for three IVIG products. Because administration of antibodies to phosphorylated tau has been found to reduce tau-associated pathology in transgenic mouse models of tauopathy, increasing the levels of anti-pTau antibodies, together with other selected antibodies such as anti-Aβ, in IVIG might increase its ability to slow AD's progression.

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1. Introduction

Tau protein-containing neurofibrillary tangles (NFTs) and Aβ-containing senile plaques are the main pathological findings in the Alzheimer's disease (AD) brain. In healthy neurons, tau binds to tubulin, promoting its assembly into microtubules. This microtubule stabilization is necessary for neurite development and maintenance. The extent of tau's phosphorylation is thought to control its microtubule binding [1]. Phosphorylation in normal tau is limited to 2–3 amino acids but this number increases to more than 39 in the AD brain [2]. This "hyperphosphorylation" decreases tau's binding to microtubules, resulting in neuronal injury due to decreased axonal transport [3]. Both hyperphosphorylation and aggregation of tau are believed to be necessary for NFT formation.

Therapies to reduce Aβ including β- and γ-secretase inhibitors and monoclonal anti-Aβ antibodies have thus far failed to slow AD's progression, increasing interest in targeting its tau pathology. Treatment with a tau aggregation inhibitor slowed AD's progression in a phase II trial [4] and a trial with a next-generation tau aggregation inhibitor is in progress. An inhibitor of glycogen synthase kinase-3β (GSK-3β), an enzyme which phosphorylates tau, produced "positive trends" in tests of cognitive function in AD patients but statistical significance was not achieved [5]. A phase I tau vaccine trial with AD patients (ClinicalTrials.gov Identifier: NCT0203119) is also in progress. In transgenic mouse models of tauopathy, administration of monoclonal antibodies to phosphorylated tau [6,7] was found to reduce tau-associated pathology, suggesting possible therapeutic applications for these antibodies.

The effects of intravenous immunoglobulin (IVIG) products were recently studied in clinical trials with AD patients. Pilot studies with Octagam (Octapharma) and Gammagard (Baxter Healthcare) produced encouraging results but phase II and III trials, respectively, with these products gave disappointing results [8,9]. In the Gammagard study, positive trends in cognitive assessments were noted in individuals with moderate AD and in AD patients carrying the ApoE4 allele, but the study was not powered to detect differences for these subgroups. A phase II/III AD trial (ClinicalTrials.gov Identifier: NCT01561053) combining plasmapheresis with administration of Flebogamma (Grifols Biologicals) and albumin is in progress. We previously reported that IVIG products contain specific antibodies to tau [10]. However, the tau used for detection of anti-tau antibodies in that study was recombinant human tau-441, which is non-phosphorylated. The extent to which IVIG products may contain antibodies specific for phosphorylated tau

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is unclear. A study published as an abstract [11] indicated that CSL Behring's IVIG Privigen contains antibodies to phosphorylated tau, but no details of the ELISA used to detect these antibodies were given. The objective of the present study was therefore to determine the specificity of binding of six IVIG products, including Privigen, to phosphorylated tau.

2. Methods

2.1. IVIG preparations

The six IVIG preparations evaluated in this study were Gamunex-C (Grifols Therapeutics Inc., Research Triangle Park, NC), Gammagard Liquid (Baxter Healthcare Corp., Westlake Village, CA), Gammaked (Talecris Biotherapeutics, Inc., Research Triangle Park, NC), Flebogamma (Grifols), Privigen (CSL Behring AG, Bern, Switzerland), and Octagam (Octapharma AB, Stockholm, Sweden).

2.2. ELISA measurement of specific anti-phospho-tau antibodies

Specific binding of one IVIG product, Gammagard, to phosphorylated tau was examined in preliminary experiments by comparing its binding between phosphorylated and non-phosphorylated recombinant human tau-441. Tau was phosphorylated by incubating it with GSK-3β and ATP as described previously [12]. These experiments suggested that IVIG's extensive binding to multiple regions of non-phosphorylated tau, as we reported earlier [13] could complicate detection of its specific binding, if any, to phosphorylated tau. Therefore subsequent experiments examined binding of IVIG products to a 12 amino acid tau peptide, tau 196–207, which was either non-phosphorylated ("non-pTau peptide") or phosphorylated ("pTau peptide") at Serine-199 and Serine-202. Both of these tau amino acid residues are phosphorylated in the AD brain [14]. These peptides were purchased from US Biological, Salem, MA (cat. # T1040-09, lot # L11031419). Seven independent ELISAs were performed, and data were pooled with each experiment contributing one data point from each IVIG product.

Non-pTau peptide, pTau peptide, and bovine serum albumin (BSA, Sigma-Aldrich Co., St. Louis, MO) were diluted in Tris buffer (0.1 M, pH 8.8) to 50 μg/mL and aliquoted into wells (volume = 100 μL/well for this and subsequent steps) of a 96-well Nunc Maxisorp plate. BSA was included to permit subtraction of polyvalent binding of the IVIG products to ELISA plates [15]. After incubation overnight at 4 ° C, wells were treated with SuperBlock (Thermo Scientific, Rockford, IL) followed by the IVIG products. Each IVIG was diluted 1:100 in phosphate-buffered saline (PBS; 0.01 M, pH 7.2) with 1% BSA and 0.5% Tween-20 (hereafter, PBS-T-BSA). The diluted IVIG products were then added in duplicate to wells previously coated with non-pTau peptide, pTau peptide, or BSA. Additional wells received PBS-T-BSA or positive control antibodies: rabbit polyclonal anti-Tau 194–214 antibody (AnaSpec Inc., Fremont, CA; diluted 1:160 [1250 ng/mL] in PBS-T-BSA) or rabbit monoclonal anti-tau pS199 (Invitrogen/Life Technologies, Carlsbad, CA; diluted 1:100 [5000 ng/mL] in PBS-T-BSA). After 4 ° C incubation overnight, biotinylated goat anti-human IgG (Fcγ specific; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) was added to wells that had received IVIG products or PBS-T-BSA, and biotinylated anti-rabbit IgG (heavy and light chain specific; Vector Laboratories, Burlingame, CA; 1:1000 in PBS-T-BSA 1%) was added to wells that had received rabbit anti-tau antibodies. After incubation at 37 ° C for 1 h, streptavidin–alkaline phosphatase (Zymed Laboratories, Invitrogen, Carlsbad, CA; 1:1000 in PBS-T) was added, followed by incubation at 37 ° C for 1 h. Para-nitrophenol phosphate (Sigma) was then added (5 mg in 40 mL of 1 M diethanolamine buffer, pH 9.8). The plate was read at 405 nm with a Vmax kinetic microplate reader (Molecular Devices Corp., Sunnyvale, CA) until mean optical density (OD) values reached 1.0 for the wells in which rabbit anti-Tau 194–214 antibody had been incubated on the non-pTau peptide. OD values for wells in which PBS-T-BSA was incubated on non-pTau peptide, pTau peptide, or BSA were subtracted from the OD values for wells in which IVIG products were incubated on the peptides or BSA. Specific binding of IVIG products to non-pTau and pTau peptides was calculated as follows:

$$\text{Specific binding to non-pTau peptide} : \frac{\text{OD for binding to non-pTau peptide}}{\text{OD for binding to BSA}}$$

$$\text{Specific binding to pTau peptide} : \frac{\text{OD for binding to pTau peptide} - \text{OD for binding to BSA}}{\text{OD for binding to non-pTau peptide} - \text{OD for binding to BSA}}$$

These ratios, which will be referred to as "anti-nonpTau antibody ratios" and "anti-pTau antibody ratios," were calculated separately for each IVIG product in each of the seven experiments. An anti-pTau antibody ratio greater than 1.0 suggested the possibility of specific antibody binding to phosphorylated tau for the IVIG product in that experiment.

2.3. Statistics

The use of standard mixed models with ELISA as a random effect and IVIG product as a fixed effect was first evaluated for the two anti-tau antibody ratios. Model diagnostics were examined to assess the reasonableness of the assumptions for this model (normality and common variance). Based on this examination, for analysis of anti-nonpTau antibody ratios, Gammagard was assigned its own variance and the five other IVIG products were assigned a smaller common variance. A similar model, but assigning one variance for Flebogamma and a common smaller variance for the other IVIG products, was used to analyze the anti-pTau antibody ratios. Both an overall test (F test for fixed effects) and Tukey–Kramer multiple comparison procedures (with adjusted p-values and 95% confidence intervals for pairwise comparisons) were used to examine the nature and magnitude of differences between IVIG products for these ratios. P-values below .05 were considered statistically significant. Statistical analysis used The SAS System for Windows version 9.3, and graphs were obtained from Minitab.

3. Results

3.1. Specific antibody binding of IVIG products to non-pTau peptide

Anti-nonpTau antibody ratios (means and medians) for the IVIG products are shown in Fig. 1. The mean values for these ratios were: Gammagard, 1.341; Gamunex-C, 1.295; Gammaked, 1.264; Flebogamma, 1.152; Privigen, 1.225; and Octagam, 1.353. There were statistically significant differences among these mean ratios ($F = 25.40$, $p < .0001$). Estimated differences between mean anti-nonpTau antibody ratios, Tukey–Kramer adjusted p-values, and 95% Tukey–Kramer confidence intervals for pairwise comparisons between the mean ratios are shown in Table 1. Octagam had a higher mean ratio than Gamunex-C, Gammaked, Privigen and Flebogamma, Gamunex-C had higher mean ratio than Privigen, and Flebogamma had a lower mean ratio than Gamunex-C, Gammaked, Octagam, and Privigen. The differences between the mean ratio of Gammagard and the mean ratios of the other IVIG products were not statistically significant.

3.2. Specific antibody binding of IVIG products to pTau peptide

Anti-pTau antibody ratios for the IVIG products are shown in Fig. 2. The variability for these ratios was greater than for the anti-nonpTau antibody ratios. In one experiment no specific binding of Flebogamma to the non-pTau peptide was detected (the anti-nonpTau antibody ratio in that experiment was 0.979); assuming that specific binding of an IVIG product to pTau should not be present in the absence of its specific binding to non-pTau, no anti-pTau antibody ratio was calculated for Flebogamma for that experiment. There were statistically significant differences among the

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