Quantitative Determination of Relevant Amounts of Blood Coagulation Factor XI Activity in a Specific Brand of Intravenous Immunoglobulin

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The authors are employees of Grifols.
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**Abstract**

Background: Residual content of activated procoagulant components, particularly FXIa, has been suggested to play a relevant role in the increased thrombogenic potential of intravenous immunoglobulin (IVIG) from a specific manufacturer.

Methods: Flebogamma, Flebogamma DIF and other marketed IVIG preparations (products A 5%, A 10%, B1 5%; B2 10%, C 10%, D 5% and E 10%) were tested for the presence of procoagulant activities: coagulation (pro)enzymes (one stage clotting assays for FXII, FXI, FIX, FX, FVII and FII using the corresponding prothrombin time (DG-PT) and APTT (DG-APTT) reagents); FXI antigen level (FXI:Ag) (ELISA); thrombin generation (TGT using the Technothrombin TGA) and non-activated partial thromboplastin time (NaPTT).

Results: Clotting activity was detected in lots of product A 5% corresponding to the year 2010 (0.08-0.10 IU/ml FXI:C), while in all other products and coagulation factors clotting activity was below the detection limit of the assays (between DIF, Flebogamma and product A 5% from a lot corresponding to the year 2007 showed clotting times similar to those of the assay controls (233-272 s).

Conclusion: Lots from the year 2010 of 5% and 10% products from manufacturer A showed significant procoagulant potential. FXIa seemed to be present in significant amounts in lots corresponding to 2010 of product A 5%.

**Introduction**

Intravenous immunoglobulin (IVIG) products are prepared from pools of plasma obtained from a thousand or more healthy donors. IVIG represents a source of natural immunoglobulin G (IgG), mainly used to treat Primary Immunodeficiency (PID) and a variety of autoimmune, infectious and chronic diseases [1]. A major goal of plasma derivative manufacturers is to strengthen the production performance and the quality testing methods to guarantee that products are both effective and safe. The manufacturing process is key to ensuring that therapeutic IVIG is free of unwanted impurities including activated coagulation factors [2]. Changes in the production process may have the potential to induce deleterious effects in the quality or purity of the product.

Recently, an increased incidence of thromboembolic adverse events (TAEs) associated with the use of an IVIG brand from a specific manufacturer has raised serious safety concerns [3]. The TAE outbreak has tentatively been ascribed to increased residual content of activated procoagulant components; including coagulation factor XI (FXIa) and possibly other impurities in that product, apparently due to a modification in the production process. The incident has lead to the suspension of the corresponding marketing authorization by the European Commission [3]. In fact, TAEs rarely occur with the use of IVIG, with the exception of cases of known association with underlying diseases and cardiovascular risk factors [4-6].

Under physiological conditions, FXI is activated to FXIa by thrombin bypassing the contact activation pathway, although under non-physiological conditions, FXI can be activated by activated FXII. FXIa activates FIX, leading to thrombin generation [7]. High plasma concentrations of FXI are known to be associated with thrombotic risk and may be explained by an increase in endogenous thrombin potential [8]. Moreover, it has been observed that infusion of FXIa induces activation of the intrinsic coagulation in vivo [9]. For this reason, contaminant traces of FXIa have been suggested to play a relevant role in the thrombogenic potential of IVIG [10, 11].

To develop safe IVIG preparations, plasma derivative manufacturers apply different strategies. For instance, Flebogamma® and Flebogamma® DIF are the only products among the main IVIG therapies currently marketed in Europe and/or USA which include pasteurization [12, 13] in their production process; an important step with demonstrated capacity to inactivate clotting enzymes during production [2].

There are a number of in vitro tests that have long been used to assess the procoagulant activity of blood derivatives, particularly those with a significant risk of
thrombogenicity [14-17] that might be applicable to the characterization of IVIG, in order to further enhance the knowledge on possible residual impurities with procoagulant potential. Recently, modifications to the established thrombin generation test (TGT) test have been developed to detect minor quantities of potential activators such as FXIa or others in IVIG preparations [2, 18]. In this study, a thorough characterization of the presence of procoagulant activities in different commercial IVIG preparations, including Flebogamma and Flebogamma DIF, was performed employing well established analytical methods.

Material and Methods

Study products: Flebogamma 5%, and Flebogamma 5% DIF or Flebogamma 10% DIF (Instituto Grifols S.A., Barcelona, Spain) and other marketed IVIG products from 5 different manufacturers identified as A (liquid, 5% and 10%), B (B1: lyophilized, 5%; B2: liquid, 10%), C (liquid, 10%), D (liquid, 5%) and E (liquid, 10%) were tested for the presence of procoagulant activities according to the methodologies hereafter described. All lots were within their charted shelf-life, except product A5% for which a lot corresponding to the year 2007 was also tested (about one year after expiry date). The remaining lots of product A were released to the market in 2010.

Determination of coagulation (pro)enzymes (one stage clotting assays): FXII, FXI, FIX, FX, FVII and FII were determined by one stage clotting assays [19] performed in a Q Hemostasis Analyzer (Diagnostic Grifols, Barcelona, Spain) using the corresponding prothrombin time (DG-PT) and APTT (DG-APTT) reagents as well as the factor deficient plasmas from the same manufacturer. For each factor, a working reference curve of at least four dilutions was prepared with a calibrator plasma (DG-Ref, Diagnostic Grifols, Barcelona, Spain) and Owren buffer (DG-Owren). Fifty µl of each plasma dilution were mixed with 50µl of deficient plasma and APTT reagent (addition of 50µl of DG-APTT after 3 minutes incubation at 37ºC) or PTs (addition of 100µl of DG-PT after 2 minutes incubation at 37ºC). Samples (IVIG products) were tested at three independent dilutions prepared with DG-Owren. The reference and sample results were inspected for parallelism and linearity. A minimum of at least one, sample test result should fall within the readable range of the working reference curve to accept a quantifiable result calculated as the average of the three dilutions assayed. Results were expressed in IU/ml.

The FXI activities of product A 5% (lots from 2010) were tested at 1/5, 1/10 and 1/20 dilutions, and in some cases also at the 2/5 dilution. The products with clotting activities below the detection limit of the assays were tested at 1/5 dilution or lower. Determination of FXI antigen (ELISA): FXI antigen level (FXI:Ag) was assayed by enzyme-linked immunosorbent assay (ELISA Total Human Coagulation Factor XI Assay, Dunn Laborteknich, Asbach, Germany). The antigen assay consists of a sandwich type ELISA in which the samples (IVIG concentrates) and a properly diluted control (lyophilised plasma. DG-REF, Diagnostic Grifols, S.A. Barcelona, Spain) were tested according to the manufacturer's instructions using an affinity purified capture antibody coated on the microtitre plate and a horseradish peroxidase anti-human FXI polyclonal antibody. A standard calibration curve is prepared along with the samples and control using dilutions of human Factor XI provided in the same kit. The results were expressed in Plasma Equivalent Units (PEU/ml of product). The equivalence between PEU and µg was established as the average of 3 different lots of human normal plasma in 9 different assays and represents 3g/ml (according to the original standard provided by the ELISA kit manufacturer).

Determination of peak of thrombin by thrombin generation (TGT): Thrombin generation was determined in Flebogamma® 5%, Flebogamma® 5% DIF, product A 5% (2007), product A 5% and product A 10% using the Technothrombin® TGA (Technoclone, Vienna, Austria), with a fluorescence reader and specially adapted software according to the manufacturer’s instructions. The reaction mix contains the test sample and the plasma vehicle in a ratio of 1 to 4. Briefly, thrombin generation is initiated in a mixture of platelet poor plasma (PPP; DG-REF, Diagnostic Grifols, Barcelona, Spain) and sample (32 µl of PPP and 8 µl of sample) by the addition of 10 µl of a reagent containing a low concentration of phospholipid micelles with 0.5 pM recombinant human tissue factor in Tris Hepes buffer and 50 µl of TGA fluorogenic substrate (0.5 mM Z-G-G-R-AMC, 15 mM CaCl2). For the assay control (vehicle) the test sample is replaced with 8 µl of 0.15 M NaCl. The fluorimetric measurements at 360 nm and 460 nm (excitation/emission) are conducted at 37ºC. From the changes in fluorescence over time, the peak concentration of thrombin (nM) in the sample was calculated using a thrombin calibration curve. The value of the peak of thrombin of the vehicle in the same microplate was subtracted from the result of each test sample.

Determination of clotting factors activation (NaPTT): The non-activated partial thromboplastin time (NaPTT)
was performed as described in the European Pharmacopoeia (Ph. Eur.) monograph [15] on samples of Flebogamma® 5% DIF, Flebogamma® 10% DIF, product A 5% (2007), product A 5% and product A 10% tested after dilutions 1/5 and 1/10 in the assay buffer. Briefly, 0.1 ml of PPP (DG-REF, Diagnostic Grifols, Barcelona, Spain) and 0.1 ml of a suitable dilution of a phospholipid preparation (Diagnostica Stago, Ansiers sur Seine, France), acting as a platelet substitute, were preincubated for 1 min at 37ºC. Then, 0.1 ml of the tested sample (neat or diluted with 0.06 M tris(hydroxymethyl)aminomethane, 0.09 M NaCl buffer pH 7.5) or buffer (control) was added. Immediately after the addition of 0.1 ml of 3.7 g/l calcium chloride (0.025 M), the clot formation time was monitored. Results are expressed either as seconds or as ratio between the products and the normal plasma control included in the same run of the test according to the European Pharmacopoeia monograph (which must yield clotting times between 200 and 350 seconds).

Results

Coagulation pro(enzymes) content: As shown in illustration 1, clotting activities were below the detection limit of the assays for all products and coagulation factors (detection limits: FXI:Ag determinations: FXI:Ag levels (n=1 to 4 batches per product) ranged from non-detectable (TGT determinations: With the TGT test, the peak of thrombin generation for Flebogamma 5%, Flebogamma 5% DIF, Flebogamma 10% DIF and product A 5% lot from 2007 was comparable to the vehicle. By contrast, peak of thrombin generation increased in lots from 2010 of product A 5% (168±7 nM) and product A 10% (172±24 nM). These results are included in illustration 3.

NaPTT determinations: Using NaPTT, Flebogamma 5%, Flebogamma 5% DIF, Flebogamma 10% DIF and product A 5% lot from 2007 showed clotting times similar to those of the assay controls, with values ranging 233-272 s for 1/5 dilution samples, and a ratio sample to reference control very close to 1. By contrast, product A 5% and product A 10% showed shortened clotting times, with means ranging 173-217 s, also for 1/5 dilution samples, meaning a ratio sample to control with means ranging 0.62-0.78. The results are summarized in illustration 3. Samples tested after 1/10 dilution did not significantly differ from those buffer-diluted 1/5 (data not shown).

Discussion

A recent TAEs outbreak has been associated with the use an IVIG preparation from a specific manufacturer [3]. Our findings suggest that the presence of procoagulant activities, particularly FXIa may be related to these undesired events, although the role of additional impurities cannot be ruled out with these assays [2]. The industrial human plasma-derived fractions obtained by cold ethanol fractionation and used to obtain immunoglobulin contain relevant markers of blood coagulation enzymes activation [2]. These enzymes may have contributed in the past to the well established lack of tolerability for intravenous administration of the immune serum globulin (ISG) intramuscular preparations [11].

Several strategies have been employed to derive IVIGs from the above mentioned fractions. The first attempts were done using enzyme digestion, acid pH treatment and/or anion exchange chromatography [20]. However, anion exchange chromatography (with positively charged resins at the usually employed chromatographic conditions with pH of 6.0 to 7.0) has also shown limited or no capacity to remove positively charged impurities (which are present in the industrial fractions used as starting materials for IVIG production), like FXI or more relevant, FXIa, thrombin [2] and possibly others such as FVII, FXII, kallikrein or tissue factor, with predicted pls (based on primary sequence) of 6.92, 8.04, 8.60 and 6.64 according to the ExPASY proteomics server. In our previous paper (2) we found relevant indications of activation of FVII and important amounts of kallikrein in the industrially extracted fraction employed for IVIG production.

In our previous paper, we also showed that pasteurisation, as applied in the context of Flebogamma® and Flebogamma® DIF production processes, has the capacity to inactivate FXIa and other markers of blood clotting enzymes, while acid pH only showed capacity to inactivate PKA [2]. In this paper we studied analytical tools that could be useful to differentiate products with a higher risk of thromboembolic complications when applied to the final product containers.

From our results, lots from 2010 of 5% and 10% products from manufacturer A showed potential prothrombotic capacity, expressed as significant thrombin generation capacity (TGT assay) and slightly shortened clotting times (NaPTT). As observed in a previous report, the lot from 2007 of product A 5% showed less obvious prothrombotic profile with respect
This difference in behavior may be due to lower residual procoagulant activity as a consequence of testing after expiry date, and/or it could be ascribed to a true difference in product quality/purity, probably caused by a change in the starting fraction for IVIG production that took place between both dates, from FrI+II+III to FrI+II+III [2], overcoming the purification capacity of the production process.

Factor XI and its activated form FXIa are among the IVIG impurities with most relevant risk of thrombogenic potential [8-11]. In our results, FXI was the only clotting factor that showed detectable activity by one stage assays, more precisely, 0.08-0.10 IU/ml, again in product A 5%, lots from 2010. This means that a 70 kg adult being infused this product at a rate of 2 g IVIG/kg b.w. would be administered 140 g of protein, that is 2800 ml of product A 5% containing around 250 IU of FXI, apparently at least partially activated. As was reported using a chimpanzee model [9], an iv bolus of 100 µg FXIa induced coagulation activation, which sustained for 240 min for some evaluation parameters. Highly purified commercial FXIa reagents show a specific activity, in one-stage clotting assays of around 450 plasma equivalent Units (PEU)/mg. Therefore, 100 µg of FXIa are equivalent to approximately 45 PEU of FXI:C activity. This means that the hypothetical infused dose of 2 g of product A 5% (lots from 2010)/kg would include an amount of FXIa (assuming that all FXI is activated) 7-8 fold higher than the proven coagulation-activator in chimpanzees (100 mg of FXIa). To the best of our knowledge, the batches of product A from 2010 analyzed in this study were not among those published as being involved in thromboembolic adverse events and, therefore, might represent a “best case” estimation of the true amounts of FXI(a) received by the patients with these events, which in most cases appeared without underlying previous risk conditions [18].

It is interesting to point out that, apart from FXI in product A 5%, no other clotting factor showed detectable activity using one-stage assays in any tested product. This strongly suggests that those factors are efficiently removed during the IVIG manufacturing processes down to below the detection threshold of the technique.

FXI protein (FXI:Ag) was detected in most products, although only in product A 5%, lots from 2010 was it detected in significant amounts, as high as tenfold PEU/ml with respect to other products and similar to the values of FXI:C observed in the functional clotting assay. Nevertheless, the presence of FXI:Ag is not a direct indicator of procoagulant activity because the protein can be inactivated during the production process, e.g., during pasteurisation or otherwise. According to these results, a regular NaPTT assay as described in the European Pharmacopeia (15) or with some improvements as described by Grundmann and coworkers [18, 21] might suffice to discriminate the product involved in the thromboembolic events outbreak, although additional research and standardization is required. The wide array of additional laboratory tests, either with a pharmacopoeia or an investigational basis, should be sufficient to guarantee the safety of newly developed products as well as existing products when manufacturing changes are being explored.

To summarize, lots from 2010 of 5% and 10% products from manufacturer A showed significant procoagulant potential, with marked thrombin generation capacity and slightly shortened NaPTT times. Moreover, FXIa seemed to be a major procoagulant present in significant amounts of lots manufactured in 2010 of product A 5%. Flebogamma® DIF, Flebogamma® and other products showed results comparable to the corresponding assay controls.

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Illustrations

Illustration 1

Clotting activities determined on the studied products by one-stage clotting assay. * denotes measurement below assay detection limit; NT: not tested.

<table>
<thead>
<tr>
<th>Product</th>
<th>FXI</th>
<th>FII</th>
<th>FVII</th>
<th>FIX</th>
<th>FX</th>
<th>FXII</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 5% (lot of 2007)</td>
<td>1 *</td>
<td>1 *</td>
<td>1 *</td>
<td>1 *</td>
<td>1 *</td>
<td>1 *</td>
</tr>
<tr>
<td>A 5%</td>
<td>3</td>
<td>0.08-0.10</td>
<td>3 *</td>
<td>3 *</td>
<td>3 *</td>
<td>3 *</td>
</tr>
<tr>
<td>A 10%</td>
<td>3 *</td>
<td>1 *</td>
<td>1 *</td>
<td>1 *</td>
<td>1 *</td>
<td>1 *</td>
</tr>
<tr>
<td>B1</td>
<td>1 *</td>
<td>NT</td>
<td>1 *</td>
<td>NT</td>
<td>NT</td>
<td>1 *</td>
</tr>
<tr>
<td>Flebogamma® 5%</td>
<td>2 *</td>
<td>2 *</td>
<td>2 *</td>
<td>2 *</td>
<td>2 *</td>
<td>2 *</td>
</tr>
<tr>
<td>Flebogamma® 5% DIF</td>
<td>5 *</td>
<td>2 *</td>
<td>2 *</td>
<td>2 *</td>
<td>2 *</td>
<td>2 *</td>
</tr>
<tr>
<td>Flebogamma® 10% DIF</td>
<td>4 *</td>
<td>1 *</td>
<td>1 *</td>
<td>1 *</td>
<td>1 *</td>
<td>1 *</td>
</tr>
</tbody>
</table>
Illustration 2

FXI antigen level (FXI:Ag) assessed by ELISA in IVIG products. The results are expressed in Plasma Equivalent Units/ml (PEU/ml, 3 micro g/ml ~ 1PEU/ml).

<table>
<thead>
<tr>
<th>Product</th>
<th>FXI:Ag</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N</td>
</tr>
<tr>
<td>A 5% (lot f 2007)</td>
<td>1</td>
<td>0.005</td>
</tr>
<tr>
<td>A 5%</td>
<td>3</td>
<td>0.089 - 0.116</td>
</tr>
<tr>
<td>A 10%</td>
<td>4</td>
<td>0.004 - 0.009</td>
</tr>
<tr>
<td>B1</td>
<td>2</td>
<td>0.003 - 0.006</td>
</tr>
<tr>
<td>B2</td>
<td>2</td>
<td>0.005 - 0.010</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>E</td>
<td>2</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Flebogamma® 5%</td>
<td>2</td>
<td>&lt;0.003 - 0.011</td>
</tr>
<tr>
<td>Flebogamma® 5% DIF</td>
<td>4</td>
<td>&lt;0.003 - 0.008</td>
</tr>
<tr>
<td>Flebogamma® 10% DIF</td>
<td>4</td>
<td>&lt;0.003 - 0.025</td>
</tr>
</tbody>
</table>
Illustration 3

Non-activated partial thromboplastin time (NaPTT, dilution 1/5) and thrombin generation test (TGT) performed on IVIG products. The value of peak concentration of thrombin in the vehicle is subtracted from the result of each test sample. Negative results indicate results below the value obtained for the vehicle. NT: not tested.

<table>
<thead>
<tr>
<th>Product</th>
<th>NaPTT</th>
<th>TGT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Range (s)</td>
</tr>
<tr>
<td>A 5% (lot of 2007)</td>
<td>1</td>
<td>272</td>
</tr>
<tr>
<td>A 5%</td>
<td>3</td>
<td>173-217</td>
</tr>
<tr>
<td>A 10%</td>
<td>1</td>
<td>202</td>
</tr>
<tr>
<td>Flebogamma® 5%</td>
<td>2</td>
<td>314-315</td>
</tr>
<tr>
<td>Flebogamma® 5% DIF</td>
<td>3</td>
<td>233-269</td>
</tr>
<tr>
<td>Flebogamma® 10% DIF</td>
<td>3</td>
<td>242-272</td>
</tr>
</tbody>
</table>
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