Identification of Activated FXI as the Major Biochemical Root Cause in IVIG Batches Associated with Thromboembolic Events. Analytical and Experimental Approaches Resulting in Corrective and Preventive Measures Implemented into the Octagam® Manufacturing Process

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Abstract

During the year 2010 a surprising increase of thromboembolic events (TEEs) was observed after administration of distinct Octagam® 5% (human normal immunoglobulin 50 mg/ml) batches to patients. Immediately, Octapharma initiated a massive voluntary recall of Octagam batches from EU and US markets in August 2010. In Europe, the marketing authorization was suspended in September 2010. In order to identify the biochemical root cause(s) of the TEEs in concerned Octagam batches, suitable assays had to be evaluated. In particular, a Thrombin Generation Assay (TGA) turned out to be the most sensitive and predictive test-system to assess an increased procoagulant potential. A number of experiments and analyses, including thrombelastometry, an activated Factor XI (FXIa) assay as well as all coagulation system and contact phase related assays identified FXIa as the major procoagulant activity. Other procoagulant compounds were excluded. Only moderate Kallikrein activities were found in distinct Octagam batches, which had a negligible contribution to thrombin generation. The Wessler stasis model in rabbits confirmed the biochemical results and finally provided the in vivo proof of the successful removal of FXI(a) by implementation of corrective and preventive measures into the manufacturing process. FXI minimization is now controlled by in-process sample testing. In addition, TGA was validated and implemented for routine Octagam batch release. Consequently, based on the elucidated biochemical and technical root causes and corresponding implemented measures, the CHMP recommended the lifting of the suspension of the marketing authorization in Europe in April 2011, with confirmation following by the EMA in May 2011. A corresponding file is currently under review by the FDA.

Introduction

Octagam®5% has been in use for more than 17 years with an excellent efficacy and tolerability and an overall adverse event rate of less than 0.35% in 10 years (1,2). However, during the year 2010 a surprising increase in thromboembolic events (TEEs) was observed after administration of distinct Octagam®5% batches to patients (3). The predisposition of patients with underlying diseases and thrombotic risk factors had to be taken into consideration, which meant that only a limited number of patients developed TEEs per Octagam batch. A massive voluntary recall of Octagam batches was initiated by Octapharma in August 2010. At the same time the biochemical root cause analysis of these batches had started (4). The lifting of the suspension of the MA in Europe from September 2010 was recommended by the CHMP in April 2011 (5). A corresponding file is currently under review by the FDA.

This decision was taken after Octapharma had identified the biochemical and technical root causes. Corrective and preventive measures were implemented into the manufacturing process including sensitive assays for in-process and final product testing, the latter as an additional validated routine Quality Control release test. In order to facilitate the efficient, robust and consistent minimization of the procoagulant potential in the final product, test-systems had to be explored, which were suitable and sufficiently sensitive to support the elucidation and correction of the technical root during the manufacturing process. Global coagulation assays such as PT or APTT did not show a sufficiently sensitive performance for our purposes. Therefore, additional assays such as the Thrombin Generation
Assay (TGA), thrombelastometry and, at a later stage, on targeted approaches such as quantification of distinct compounds of the coagulation system, the contact phase and beyond were performed. The analytical approaches and experiments confirming the major biochemical root cause and excluding additional factors are described in this report. These accompanied the implementation of corrective and preventive measures into the manufacturing process, finally succeeding in the production of Octagam batches with minimized procoagulant potential, which was confirmed in an in vivo thrombogenicity model. Other Octagam quality parameters are not negatively affected by the implemented measures. Recent publications discussed the presence of FXIa as the potential root cause of the observed TEEs, while Kallikrein (like) activities in particular were found in batches of several intravenous immunoglobulin (IVIG) concentrates as a major proteolytical impurity (6,7).

Results

Experimental and analytical results
Our experimental and analytical approaches proved the presence of FXIa as the biochemical root cause in the TEE associated Octagam batches. This was confirmed not only by established assays but also by tailored highly specific settings. Analyses using different chromogenic or fluorogenic substrates, combined with plasmatic, corn, bean and synthetic inhibitors suggested FXIa as the procoagulant principle (not shown), and essentially revealed results as reported by another working group recently (7).

Due to (more or less) limited specificities of these tools, immune-depletion experiments with immobilized antibodies to FXI and assays such as combined immune-adsorption and subsequent activity measurements added evidence to this conclusion. Immune-adsorption of FXI and FXIa

Repeated contact of the procoagulant activity containing Octagam samples with a FXI-antibody, showed a significant reduction of FXIa activities per cycle, finally resulting in complete (below detection limit) removal of FXIa activity. This was confirmed by parallel reduction of the peak thrombin concentration (PTC) in TGA. A representative experiment is shown in Illustration1.

Adsortion of the procoagulant principle and identification of FXIa

Several commercially available adsorbents such as ion-exchange and affinity chromatography resins were investigated to remove the procoagulant principle of distinct Octagam samples. Therefore, the starting material, the column flow-through fractions and the resin eluates were investigated in TGA. If no procoagulant activity was detected in the flow-through fractions, resin eluates were further investigated. SDS-PAGE and Western blotting results using a labeled anti-FXI antibody preparation and substrate exposure were used to visualize the FXI protein bands. Illustration2 shows three prominent bands. Because the PAGE was run under reducing conditions both, the FXI zymogen (upper band) and the activated FXI heavy- and light-chains are visible. All three bands were investigated by MALDI-TOF analyses and confirmed the mentioned FXI(a) identity.

Exclusion of other procoagulant factors
Analyses using a number of chromogenic and fluorogenic substrates, combined with available plasmatic, corn, bean as well as synthetic inhibitors confirmed FXIa as the major biochemical cause for the increased procoagulant in vitro potential. Most importantly, this activity could be inhibited by incubation with a surplus of a monoclonal antibody preparation against FXI, although not specific for FXIa. Furthermore, using deficient plasmas in TGA and NATEM settings exclusively revealed activity when using a FXI deficient plasma, but not in plasmas deficient in FII, FVII, FIX or FX (not shown). As a conclusion, contributing factors were most likely were proteases of the contact phase system, namely (Pre-)Kallikrein and/or FXII(a). Spiking of FIXa, FXa and FXIa clearly revealed a reaction in the tests as expected (Illustration3). FXIIa (PKa) had a minor impact on PTC, dependent on the FXIIa preparation used (not shown). Spiked rKallikrein activities caused a concentration dependent increase of PTC, but this was minor compared to the influence of FXIa. Kallikrein contents below one µg per ml were measured in concerned Octagam batches. It is interesting to note that rKallikrein (i.e. free from plasmatic cross-contaminations) revealed this effect in FXI-deficient plasma, which deserves further investigation.

The current Kallikrein assay has a limit of quantification of 0.05 µg activity equivalents per ml. After implementation of the measures into the Octagam manufacturing process, Kallikrein activities were below 0.05 µg/ml in recent validation batches. However, in some batches of other IVIG brands, Kallikrein activities were detectable, with one in particular showing significant amounts (IVIG #11, Illustration4).

Thrombin Generation Assay
The TGA employed was based on the commercially available Technothrombin® (Technoclone GmbH,
Austria) kit using the RC High setting. In order to avoid a bias in the setting, we started with standard human plasma (SHP). Four parts of SHP were mixed with one part (pH adjusted) IVIG samples, which provided an optimal baseline to signal ratio (not shown). After identification of FXIa as the major root cause, FXI-deficient plasma was used, which provided a wider measurement range due to a lower baseline PTC at the comparable maximum PTC obtainable. Certain other activated factors clearly have an influence on the TGA signals as expected, thus this setting was introduced only after the identification of FXIa as the only biochemical root cause for procoagulant activity. It has to be considered that potential adverse events, in particular TEEs, with other IVIGs may deserve other assay designs than the setting described, if other root causes must be identified.

The TGA setting employed by us reveals a high sensitivity for FXIa, which reaches far below one mU FXIa/ml, as demonstrated in Illustration5. In the range from zero to one mU FXIa, the PTC increases from baseline (below 50 nM PTC) to approximately 350 nM PTC. The steepness of the PTC curve in the sub-microunit FXIa range provides a very sensitive test-system. The rationale for a cut-off level of 350 nM PTC is discussed below, considering PTCs measured in TEE-associated and non-associated Octagam batches and their correlation with Wessler test results.

**Additional test results**

Further information was obtained by NATEM® (TEM Innovations GmbH, Munich, Germany) measurements in SHP and FXI-deficient plasma, which characterizes clot formation and propagation properties. A very good correlation between increasing FXIa levels and the shortening of clotting times was observed as presented in Illustration6. Additional studies excluded different cytokine and growth factor signal patterns compared to non-associated Octagam batches using the EliSpot® system (AID Diagnostika GmbH, Strassberg, Germany) as potential contributing root causes. This also holds true for microparticles/microvesicles (with tissue factor) and certainly as well for traces of other (activated) coagulation factors in the final product as well (not shown).

**Wessler stasis model**

This state-of-the-art in vivo model was performed to detect traces of thrombogenic compounds. It is essentially independent of the inducing principle and has been used for the investigations of a number of available products, in particular coagulation factor concentrates, but is also recommended to be performed for platelet and platelet substitute products (8). Standardization and stringent control of all performance criteria renders the model an adequate, sensitive and highly reproducible model (9). After application of Octagam batches (one gram IgG/kg bw each) and subsequent ten minutes stasis of the jugular vein, excision and ex vivo scoring (0 to 4, in steps of 0.5) was performed. Activated prothrombin complex concentrate (FEIBA®, Baxter Healthcare GmbH, Vienna, Austria) was used as a positive control, whereas a comparable volume of physiological sodium chloride solution served as negative control.

The results of Octagam batch testing (TEE associated and non-associated batches) revealed a threshold concentration for visible thrombi between two and seven mU FXIa per ml of the 5% IgG solution (40-140 mU FXIa per gram IgG), administered at a dose of one gram IgG per kilogram body weight. The interrelation with FXIa, TGA and TEE associated batches is shown in Illustration5, and is further discussed below.

**Discussion**

Intravenous immunoglobulin concentrates (IVIG) have been used for decades for the prophylaxis and treatment of different indications (10,11). A frequency of TEEs in less than one per 10,000 infusions/patients treated is considered an anticipated rate of occurrence and can be traced back to underlying diseases or thrombotic risk factors. General IVIG features were reported potentially contributing to such impact. However, there was no externally introduced contamination described causing TEEs. Among those characteristics are the increase of plasma viscosity due to transient state of hyperproteinemia with subsequent pseudo-hyponatremia, arterial vasospasm, high IVIG administration doses at high infusion rates and (activated) coagulation factors (12-18). Surprisingly, in 2010, an increased number of TEEs were reported, including venous and arterial thrombotic events, which were associated with the administration of distinct Octagam 5% batches to patients.

FXIa was identified as the major biochemical root cause for the increased procoagulant potential and excluded numerous other factors. This underlines the principle importance of FXIa as an inducing factor of thromboembolic events. The clear-cut correlation of enhanced FXIa levels with an overall elevated procoagulant potential can obviously lead to thrombosis, probably dependent on the individual risk factors, which deserve further investigation. As early as 2000, Wolberg and colleagues suggested FXIa in
Correspondingly, the FXIa threshold concentration to induce thrombi in the Wessler test lies between two and seven milliunits FXIa per ml. In conclusion, the Wessler stasis model was confirmed to be an adequate and sensitive in vivo model to detect or to exclude an enhanced thrombogenic potential, independent of the inducing compound. These results contributed to the definition of a preliminary TGA based acceptance limit for Octagam batches of 500 nM PTC, which was finally lowered to 350 nM PTC in order to increase the safety margin with respect to a minimized procoagulant potential. This limit corresponds to a FXIa content of less than one milliunit per milliliter in a 5% IgG solution. In addition, a TGA lag time of equal or longer than 11 minutes as well as a time-to-peak period of equal or larger than 19 minutes must be met for release.

TGA had turned out to be a suitable and sensitive assay to detect the procoagulant principle. The assay was further developed and characterized in communication with US and EU regulatory authorities. The numeric PTC and other parameters deserve consideration, because they are characterized by the TGA setting employed. Technothrombin® based determinations of the PTC of plasma from healthy donors revealed 597 nM (SD: 226 nM) (29). Other TGAs reported normal plasma values of 497 nM (SD: 37), 458 nM (SD: 60) and even 200-800 nM, respectively (30-32). Our recent comparison of Octaplas® with fresh frozen plasma (FFP; n=18 batches or donations each) showed PTCs of 361 nM (SD: 54) and 331 nM (SD: 74), respectively. Octaplas® (and Uniplas®) represent a solvent-detergent treated plasma products derived from a pool of on average 1,000 plasma donations of healthy donors, thus providing a representative average. PTCs ranged from 262 nM to 472 nM in Octaplas/Uniplas batches and from 195 nM to 437 nM in FFP, respectively (33). Accordingly, a cut-off value of 350 nM PTC is in agreement with the thrombin generation potential of normal plasma. A recent comparison of PTCs from healthy donors (n=20) with patients suffering from symptomatic thrombophilia (n=32) revealed mean PTCs of 475 nM and 811 nM, respectively (34). While absolute PTCs reported deserve further evaluation, the differences between a normal PTC and an inherent prothrombotic potential are striking. Certainly the individual risk factors of patients must be considered, which may result in TEEs even after application of a less pronounced procoagulant challenge.

It has to be considered that a comparison with other TGA or TGT (thrombin generation test) systems always requires the detailed description of the assay.
performance and reagents used, such as the tissue factor and lipid concentrations. Assay impact factors such as the use of (deficient) plasma with or without corn trypsin inhibitor are discussed, as are different substrates, signal quenching effects and comparability of absolute values (6, 35). Therefore, each assay used has to be validated individually, as we performed it for the Technothrombin® based assay. It is important to note, that during a recent workshop on the ‘Risk Mitigation Strategies to Address Potential Procoagulant Activity in Immune Globulin Products’, data from several groups including regulatory authorities were presented, which appeared to have comparable abilities to identify IVIGs with increased procoagulant potential when compared to controls, despite varying different absolute PTC values or lag times (36). Therefore, a collaborative study has been initiated to evaluate the inter-laboratory performance of TGAs and their characteristics in procoagulant activity evaluation in IVIG (37). The study includes NAPTT testing, which is discussed as a potential alternative to TGA (6).

In concerned Octagam batches, FXIa was unambiguously the major root cause for the enhanced procoagulant potential. The potential presence of other or additional properties was investigated in studies analysing a contribution of microvesicles (TF), microparticles or cytokine and growth factors. These factors could be excluded, and will be reported on in more detail in separate communications. Kallikrein was identified as a detectable protease in distinct Octagam batches, but was found to be below one microgram per milliliter (corresponding activity). In this concentration range, Kallikrein has a negligible effect in the TGA. Importantly, all assay characteristics regarding a procoagulant potential in concerned Octagam batches were mimicked by FXIa spiking (Illustration5), adding further evidence as the major biochemical cause and excluding a significant contribution by other compounds.

However, high Kallikrein activities (more than one microgram per milliliter, i.e. more than 20 microgram activity equivalents per gram IgG) can contribute to the procoagulant potential as demonstrated by Kallikrein spiking experiments (Illustration4). Therefore, the TGA does not differentiate in this setting between the inducing agent, but in principle may also be suitable to detect high Kallikrein activities. This deserves consideration with respect to potential adverse events other than TEEs and requires individual validation for other IVIGs. Kallikrein or Kallikrein-like activities in the microgram range were found in several IVIGs as reported recently (7). Furthermore, a recent report regarding a subcutaneous immunoglobulin concentrate assumed a correlation between a high procoagulant potential and an increased TEE risk (38). While the mechanism of TEE induction by s.c. application remains elusive, it demonstrates that processes of other products also deserve in-depth evaluation. The EDQM European Pharmacopoeia Commission communicated that an amendment to the Ph.Eur. IVIG was proposed, which includes the identification of potential procoagulants during processing and effective reduction steps (39). This was performed extensively for Octagam and was a prerequisite for lifting of the suspension of the marketing authorization.

The individual patient predisposition to compensate or to react to a certain procoagulant challenge is hard to evaluate. As a consequence, the FXIa levels have to be minimized in the applied product. The clear identification of the biochemical root cause of the enhanced procoagulant activity in distinct Octagam batches and the correlation with in vitro and in vivo test results as well as with TEEs facilitated a targeted and efficient evaluation of the technical root cause. Therefore, understanding of the Octagam manufacturing process steps with respect to FXIa generation and removal capacities was supported by the knowledge and detection abilities of the designed or adjusted assays. Identified technical reasons for the unexpected presence of FXIa in distinct Octagam batches were discussed recently (40). Consequently, this led to corrective actions and, most importantly, to the implementation of a routine and validated FXI adsorption step early in the process using a commercially available adsorbent, which is widely used for fractionation and purification of plasma proteins. Efficacy of FXI removal is monitored by a FXI ELISA after the adsorption step, while the minimized procoagulant activity is confirmed by TGA of the final product with a validated release assay, as described above. Consequently, the European Commission has lifted the suspension of the Octagam marketing authorization in Europe on May 2011 (41). The file is currently under review by the FDA.

Abbreviations

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<tr>
<td>CHMP</td>
<td>Committee for Medicinal Products for Human Use</td>
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<td>APTT</td>
<td>Activated Partial Thromboplastin Time</td>
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<tr>
<td>EDQM</td>
<td>European Directorate for the Quality of Medicines &amp; Healthcare</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked ImmunoSorbent Assay</td>
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<td>EMA</td>
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References


Illustrations

Illustration 1

Immune-adsorption of FXI/FXIa by immobilized antibodies to FXI. An immobilized polyclonal FXI antibody was used to adsorb FXI/FXIa from distinct Octagam batches; flow-through fractions were collected. FXIa was determined in the starting material (Cycle 0) and after repeated cycles using a fluorogenic substrate. A representative experiment is shown.
Illustration 2

SDS-PAGE and Western blotting with subsequent immune-detection with labeled antibodies to FXI. This result shows that only a distinct portion of the FXI zymogen was activated. Notably, the non-activated FXI antigen (in the absence of FXIa) was not active in a clotting assay. This is in agreement with results from final container storage studies, in which only decreasing FXIa activities were observed, but not an increase (not shown).
Illustration 3

Impact of activated coagulation factors FIIa, FXa, FXIa and Kallikrein on PTC of TGA. The impact of commercially available proteases on PTC of FXI-deficient plasma was investigated. PTCs of representative experiments are shown.
Illustration 4

Kallikrein activities in Octagam batches and different batches of 11IVIG brands (blinded). Kallikrein contents were quantified in concerned Octagam batches (Octagam mid 2010) and compared with some recently produced batches, which demonstrate a significant reduction of Kallikrein below the LOQ. In addition, batches of other IVIG products were analysed. In particular one other IVIG brand contained elevated Kallikrein activity (IVIG 11).
Illustration 5

Impact of FXIa on PTC, correlation with TEEs and sensitivity of the Wessler test. Box A covers the FXIa/PTC range found in TEE associated batches. Box B summarizes the range and sensitivity of the Wessler stasis test. Box C covers a safe range in terms of FXIa and PTC levels. Box D describes the release limits for Octagam batches, providing a large safety margin.
Illustration 6

Correlation of FXIa activities with the clotting time (CT) measured in the FXI-deficient plasma NATEM setting using the ROTEG reg; system (n=100 data points).
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