IXINITY® [coagulation factor IX (recombinant)] (IB1001) is a human recombinant factor IX (rFIX) developed for the management of haemorrhagic episodes in individuals with haemophilia B. IB1001 is manufactured in Chinese hamster ovary (CHO) cells using a state-of-the-art process that incorporates three validated viral reduction steps and selectively purifies active forms of FIX. The purification process results in IB1001 drug product that is consistently greater than 96% purity with a low level of CHO cell protein (CHOP) as impurities. Host cell proteins (HCP) in general carry the risk of functioning as an adjuvant and thus triggering an immunologic reaction to the active ingredient [1,2]. During clinical development, immunogenicity to residual CHOP was noted in 30% of patients without any clinical significance. The anti-CHOP response was confirmed by the western blot analysis with positive patient sera [3]. This finding was not unexpected as similar reactivity to HCP was also observed in patients after treatment with other recombinant factor products such as FVIII [4]. As prolonged exposure with rFIX is required for the treatment of haemophilia B, removal of any potential antigens or material that could act as an adjuvant in IB1001 was of paramount importance [5]. Although there were no apparent anti-CHOP antibody-associated adverse events identified in IB1001 trial subjects, an additional hydrophobic interaction chromatography (HIC) step designed to further remove CHOP residue was implemented and validated for the manufacture of IB1001.

An ELISA developed using a process-specific polyclonal antibody (sheep derived) and CHOP reference standard was used to monitor CHOP levels in the drug substance. The IB1001 produced using the newly modified process (MP) with the HIC step exhibited significant reduction (~2000 fold less) in the residual CHOP levels (< 26 ng CHOP per mg of IB1001) compared to the levels (38 500 ng CHOP per mg of IB1001) in the IB1001 produced using the former process (FP). The addition of HIC step did not alter IB1001 physiochemical characterization, or its pharmacokinetic profile in animals [3]. The specific aim of this study was to compare the immunogenic potential of residual CHOP in IB1001 product produced with and without the HIC step, in rabbits. A total of 48 rabbits (24 males and 24 females) were randomly assigned into two groups (FP and MP). IB1001 was administrated intravenously at 0.5 mg kg<sup>-1</sup> twice weekly for a total of 64 days. Serum samples for anti-CHOP reactivity testing were collected prior to dosing on days 1 (baseline), 29 and 57. To measure the anti-
CHOP response, a rabbit anti-CHOP ELISA was developed and validated using process-specific CHOP antigen produced from a null vector run. Microtiter plates coated with CHOP antigen were used to capture anti-CHOP antibodies from rabbit serum. Horseradish peroxidase-conjugated goat anti-rabbit antibody was used as the secondary antibody for detection. A screening assay was conducted for all samples at a dilution of 1:40 and the sample responses were divided by the negative control response to generate a normalized response ratio. All samples with a response ratio that was greater than or equal to the validated cut-off point of 1.107 (based on the distribution of response ratios from normal rabbit serum samples) was considered positive for antibodies against CHOP. All samples that initially screened positive were retested at a higher dilution (1:160) to confirm seroconversion. A subset of confirmed positive samples was selected to assess the titer by serially diluting samples to determine the dilutions required for the response ratios to equal the cut-off point.

Anti-CHOP antibodies were not detected in any animals prior to IB1001 exposure (baseline). The level of anti-CHOP reactivity in animals treated with FP increased from 0.184 ± 0.038 (mean OD ± SEM) on day 1 to 1.168 ± 0.238 on day 29 and continued to increase to 1.98 ± 0.405 on day 57 (Fig. 1). At day 29, 79% of the rabbits (19/24) treated with IB1001 from FP were positive for anti-CHOP reactivity and whereas none (0/24) from the group treated with MP product (Fig. 1). By day 57, this incidence increased to 96% (23/24) and 4% (1/24) for groups treated with FP and MP product respectively. The difference in the incidence between the two treatment groups (FP vs. MP) was statistically significant (Fisher’s exact test). The median time to seroconversion for the FP group was 29 days. The median time to seroconversion was not calculable for the MP product treatment group due to only one positive animal for anti-CHOP antibody. The median time to seroconversion was statistically significant (P < 0.0001, log-rank test) between two treatment groups.

Due to overwhelming seroconversion rate and limited availability of the process-specific CHOP antigen for the assay, only a subset of seroconverted samples (eight from FP and one from MP group) were selected for titration to confirm and assess the magnitude of anti-CHOP reactivity. The average titer for the eight positive samples in the FP group increased significantly from 3136.9 ± 1039 (mean ± SEM) at day 29 to 15616.0 ± 4248 at day 57 indicating the increased anti-CHOP reactivity with repeat dosing (Fig. 2). The single positive sample from the MP treatment group had a titer of 117 at day 57. The minimal response from a single animal in the MP group is consistent with the effective removal of immunogenic CHOP residuals with the addition of HIC step.

Coagulation analysis of whole blood samples was conducted using a Stago Coagulation Analyzer (Diagnostica Stago, Parsippany, NJ, USA). The mean partial thromboplastin time (PTT) (mean ± SD, 7.6 ± 0.20 for FP vs. 7.6 ± 0.24 for MP) and activated partial thromboplastin time (aPTT) (Mean ± SD, 16.0 ± 1.96 for FP vs. 15.9 ± 1.56 for MP) were similar between the groups suggesting that the coagulation function between groups was not affected. Furthermore, no excessive bleeding, haemorrhage, or other abnormal adverse event was observed with either MP or FP dosing indicating that any antidrug
antibodies possibly developed during the course of the study did not neutralize the endogenous FIX activity.

Immunogenicity of biopharmaceuticals is of concern for both clinical efficacy and safety. The source of the immunogen could be the active ingredient itself, co-purified HCP or other unexpected contaminants. The immune response to the active ingredients can have severe consequences including diminished efficacy of therapeutic protein and neutralization of endogenous counterparts [6]. Therefore, it is always prudent to avoid an immune response to these HCPs by removing most of the residual proteins throughout the downstream purification process. CHO cells are used in the expression of many protein therapeutics and approximately 70% of recently approved therapeutic proteins are produced using this system [7]. In general, there is a >70% protein homology between CHO and human proteins [8] and this level of homology may be significant enough to stimulate autoreactive T cells leading to autoimmunity in patients. Additionally, the potential adjuvant effect of impurities contributes to the increasing scrutiny of CHOP impurities in products by regulatory authorities. Although a small number of patients developed antibody responses to CHOP without any associated adverse events, there was no immunogenicity to IB1001 identified in patients during clinical studies.

The intent of the current investigation was to determine whether there is elimination of the immunogenic components of the HCP by the addition of HIC during manufacturing. The study was designed to ensure that the comparison between MP and FP was valid by using a dose and frequency of administration that was similar to clinical scenario and similar exposure. Thus, the relative immunogenicity of residual CHOP was compared without bias. Most human therapeutic proteins elicit immune response in animal models and can have serious clinical implication due to neutralization of endogenous counterparts. The FIX amino acid sequence for rabbit and human has a ~82% identity including the two active regions within FIX; FXIa cleavages sites RA (145–146) and RV (180–181), and the catalytic triad of serine protease (nucleophilic Ser at 365, acidic His at 221 and basic Asp at 269) [9,10]. With high homology between two proteins, it is hypothesized that the majority of the human rFIX MHC class II peptides including the regions with active site most likely are presented as ‘self-antigen’ in rabbits. The normal coagulation (aPTT and PTT) parameters and absence of pathology findings related to excessive bleeding or haemorrhage imply that there was no neutralization of endogenous counterparts despite the close homology between the rabbit and human FIX.

This is the first report of immunogenicity of impurities retained from the CHO expression system in rabbits. In conclusion, this study provides the critical data to confirm the effective removal of immunogenic CHOP components in IB1001 produced by the MP. This reduction of CHOP in IB1001 translated to significant decrease in anti-CHOP reactivity in rabbits compared to IB1001 manufactured without the HIC step. Immunogenicity to residual CHOP will be monitored in future clinical studies to confirm these findings.

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Author contributions

PC, AE, LS, DT and SK involved in study concept and design. AE, JH and JM involved in the study execution. PC, SK and AE, contributed to the data interpretation and manuscript preparation. All authors reviewed the manuscript and approved the final version.

Disclosures

L.S., D.T., S.K. are employees of Emergent BioSolutions. P.C. and A.E. were employees of Emergent BioSolutions at the time of the Study. J.H. and J.M are employees of MPI and HTI respectively.

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