INTRODUCTION & AIM

Besides therapeutic actions, the whole IgG antivenoms obtained from the hyperimmune animal plasma still commonly cause clinical side effects attributable to contaminating proteins and/or aggregates. There has been a hypothesis in the literature that some purification procedures (pseudopurification of IgGs, low pH-mediated elution of IgGs in chromatography, etc.) induce transient conformational changes of IgG molecules, making their structure less stable and more prone to aggregation, in contrast to others that leave them in solution throughout (caprylic acid precipitation of non-IgG plasma proteins). Our goal was to verify this hypothesis by comparing IgGs isolated by five different commonly employed protocols from the same plasma in terms of stability, purity and immunoglobulin subclass composition. The fractionation methods were: ammonium sulphate precipitation (ASP), anion- (AEC) and cation-exchange chromatography (CEC), affinity chromatography (AC) and caprylic acid precipitation (CAP).

EXPERIMENTAL

The IgG antibodies venom-specific IgG subclass distribution within IgG purified fractions was determined by the respective in-house ELISA for IgG, IgGb and IgG(T) subclass detection as reported. The concentration of tested samples gave curves described either by 2nd step polynomial or natural logarithm that were used for determination of IgG concentration giving absorbance at 492 nm of 1.0 voxel in each ELISA.

The protein concentration was measured spectrophotometrically by using the equation (ng/mL) = [A280 nm + 0.0025 A230 nm] / 1000 dilution factor, where Ehrlich's factor 'f' = 0.0025 was used. The purity was examined by SDS-PAGE on 4-12% gel with MOPS as running buffer under non-reducing conditions and CB2 R250 staining.

The impact of different refinement protocols on the IgG thermal stability was determined by thermal shift assay (TSA) as described. The IgG samples (1 mg/mL 0.2 phosphate buffer, pH 6.0) with or without 2 M sorbitol as stabilising agent, were analysed, and samples were stored at -37 °C.

Hyperimmune horse plasma refinement protocols

The starting material for each purification procedure was hyperimmune horse plasma (HHP) thermally treated at 1 h for 56 °C.

Caprylic acid precipitation (CAP) was performed by adding caprylic acid (CA) to the HHP so that final concentration of 3% (v/v) in 2-fold diluted reaction mixture was achieved. Following vigorous stirring (750 rpm for 1 h at 23 °C) and centrifugation (2800 g, 45 min), IgG enriched supernatant (CAP) was obtained.

Anion-exchange chromatography (AEC) was performed in batch mode. HHP, 5-fold diluted with 25 mM Tris/HCl binding buffer, pH 8.0, containing 35 mM NaCl was incubated with Toyopearl SuperQ-650S stationary phase for 1 h at 23 °C. Following centrifugation, IgG enriched supernatant (AEC) was collected. The remaining impurities were removed by 0.5% CA (v/v) precipitation and the pure IgG fraction (AEC2) was obtained.

Cation-exchange chromatography (CEC) was performed by loading HHP, 10-fold diluted with 20 mM MES binding buffer, pH 6.0 ([14 mM run] to CIMultus S03-1 column at a flow rate of 1 mL/min. The bound antibodies were eluted with binding buffer containing 1 M NaCl and dialysated into 50 mM MES buffer, pH 5.5 using 100 kDa MWCO membrane. After precipitation of non-IgG proteins from eluted fraction (CEC1) with 1% CA (v/v), pure IgG sample (CEC2) was obtained.

Protein A based affinity chromatography (AC) was performed by applying 2-fold diluted HHP (7 mL/ per run) on MabSelect columns (V = 2 ± 1 mL) with 20 mM Tris/HCl binding buffer, pH 7.4, at a flow rate of 1 mL/min. The bound antibodies were eluted with 20 mM citric acid and the pure IgG sample was obtained (AC).

Ammonium sulphate precipitation (ASP) protocol was followed as described. The precipitate was dissolved in 50 mM MES buffer, pH 5.5. The obtained IgG-enriched preparation (ASP1) was dialysated in the same buffer using 100 kDa MWCO membrane and further purified with 2% CA (v/v) precipitation (ASP2).

Each IgG-enriched fraction was dialysated into 200 mM phosphate buffer, pH 6.0 using 100 kDa MWCO membrane.

RESULTS

The highest purity was achieved by AC and CAP (Fig. 1), while the highest aggregates content was observed in samples from AC, CEC1 and ASP1 (Table 1).

Additional purification step by CAP performed on IgG fractions of lower purity (ASP1, AEC1 and CEC1) reduced aggregates content in preparations obtained by ASP and CEC (Table 1).

In samples from all studied protocols, melting temperature (Tm) of IgGs was around 70 °C, except for the one obtained by (Fig. 2), which was slightly decreased. That might be the consequence of its different subclass composition (Fig. 3) as well as the influence of conditions during the process of purification. Addition of sorbitol improved IgG thermal stability in all samples (Fig. 2).

Aggregates content (Table 2) and Tm of analysed preparations (Fig. 2) remained without significant change during one-month stability study.

AC had the greatest impact on the subclass composition since it caused substantial loss of IgG(T) (by factor 5) and significant enrichment of IgGa subclass (by factor 332) (Fig. 3).

CEC procedure showed the same phenomenon albeit to a lesser extent (Fig. 3).

Other purification protocols did not have substantial impact on subclass composition, even though some loss of IgGa has been observed in samples gained by ASP, AEC and CAP. This loss might not be highly relevant for IgG function, since IgGa is one of the minor IgG subclasses in horse plasma (Fig. 3).

CONCLUSION

The comparison of the whole IgG snake antivenom refinement methods regarding stability, purity and immunoglobulin subclass composition of the final product proved that employed conditions have noticeable impact on every feature we have studied which might affect both safety and effectiveness. Still, conformational changes during purification procedures might not be the trigger for increased aggregation.

REFERENCES