INTRODUCTION

Downstream processing of antivenom from hyperimmune plasma consists of series of purification steps. The whole process requires constant evaluation of antivenom yield in each purification step, especially during developmental phase. Predirectional evaluation of the efficacy of antivenoms in animals is required by international regulatory authorities, especially for products that will be used in humans.

Determination of antivenom efficacy is based on two in vivo tests: (a) the venom lethal toxicity assay (determination of median lethal dose of venom or LD50) and (b) test for determining the neutralization potency (effectiveness) of produced antivenom (determination of mean venom dose or LD50). Large number of mice are injected with venom/antivenom mixtures and the number of surviving mice is statistically analyzed to give an LD50 value reflecting the efficacy of that antivenom.

Both these tests, which cause suffering, pain and death of the experimental animals and also require a large number of animals, were identified by Ehresmann (Ehresmann Centre for Validation of Alternative Methods) as assays that are necessary to be replaced with alternative methods. Therefore, each laboratory that evaluates antivenom efficacy in vivo should aim to develop an in vitro alternative in accordance with 3R principles.

MATERIALS AND METHODS

Crude venom of Vipera ammodytes ammodytes L. (Vasoviven) was collected by bleeding snakes kept at the Institute of Immunology Inc. Croatia, air dried at ambient temperature and stored in the dark at 4 °C until use. Vaso-specific hyperimmune horse plasma was obtained from the Institute of Immunology Inc., Croatia. Mice, both sex 38-45 g, used for in vivo assays were bred at the Institute of Immunology. Procedures, handling and animal work were in accordance with the Croatian Law on Animal Welfare (2000) which complies with EC Directive 2010/63/UE.

Protein concentration estimation

The protein concentration in hyperimmune horse plasma and in samples from all purification steps were estimated spectrophotometrically using Bradford’s method, using bovine serum albumin (equation: A280 nm = 0.1711 μg mL−1). Appropriate dilution of each sample was independently prepared three times to obtain the mean value of the measured concentrations for further calculation of yield and purity.

SEC HPLC – size exclusion chromatography

The molecular weight of the plasma proteins was determined by HPLC size exclusion chromatography using TSK-Gel SW3000PW (7.8 x 300 mm) column. Amino acids were visualized as their rate of elution at a wavelength of 280 nm. The absorbance was monitored at 280 nm. The running buffer was 0.1 M phosphate buffer, pH 7.6. Standard proteins used for molecular weight determination were lysozyme (Mr 14300), albumin (Mr 66000), and ribonuclease A (Mr 13700).

ELISA

ELISA for detection of venom - specific antibodies in samples from hyperimmune horse plasma processing was performed by coating microtiter plate with 100 μg/mL of venom coating solution (1 μg/mL) in 50 mM carbonate buffer, pH 9.6, and left overnight at room temperature (RT). After blocking with 5 % (w/v) BSA in PBS/0.1% Tween (TT) buffer at 37 °C for 2 h, the plates or samples from each purification step were added in two-fold serial dilution and left overnight at RT. The antibodies isolated from hyperimmune plasma by protein A affinity chromatography were used as a standard. In the subsequent steps, incubation with HRP-anti-horse IgG (1:5000 dilutan) at 37 °C for 2 h occurred, followed by the addition of OPD (0.5 mg/mL solution) in citrate-phosphate buffer, pH 5.0. After 30 minutes of incubation in the dark, the enzymatic reaction was stopped with 1 M H2SO4, and the absorbance at 492 nm was measured.

The potential of plasma and pure IgG samples to neutralise the venom’s lethal toxicity was determined by lethal toxicity neutralization assay in mice, as follows. Two-fold serial dilutions of samples were prepared with equal amounts of the venom solution containing two median lethal doses (LD50), the amount of dry venom (in g) causing the death in half of the mice population used. The immunoreceptors were removed by centrifugation and clear supernatant (i.e. administered to groups of four mice). Deaths were recorded 48 hours later. For each sample the median effective dose (ED50), the amount of undiluted serum capable of neutralizing the venom’s lethal effect in 50 % of the animals was determined. The lethal toxicity neutralization potency (ED50) was expressed as the number of LD50 venom doses that can be neutralized by 1 mL of undiluted sample and calculated by the equation: 100 × ED50/LD50, where ED50 represents the number of LD50s neutralized per mouse. It was used as a measure of the protective efficacy of each sample.

AIM

To monitor the efficacy of each purification step during development of an antivenom production process, we have developed ELISA for quantification of venom-specific antibodies, in which antibodies isolated from hyperimmune horse plasma by protein A affinity chromatography have been used as a standard. Knowing that protein A does not bind all horse IgG classes with an equal affinity, affinity purified antibodies might have different venom neutralization potency in comparison to original population of polyclonal antibodies in the starting hyperimmune plasma. Consequently, this might lead to imprecise determination of venom-specific antibodies in samples from different process steps. From that reason, we have investigated the in vivo neutralisation potency of hyperimmune plasma, its affinity purified horse IgG and horse IgG purified by a novel purification procedure and compared them to venom-specific IgG quantities determined in vitro by ELISA.

RESULTS

In vitro purification of immunoglobulin purification from hyperimmune horse plasma consists of three steps: thermal coagulation, caprylic acid precipitation and diafiltration, generating thermally treated plasma (TT plasma), crude IgG and purified IgG, respectively.

To properly evaluate purity and yield of IgGs obtained in each purification step, each sample was analysed for IgG content (by ELISA quantification of venom-specific antibody), for total protein content, and for the purity (by SEC, Figure 1).

IgG quantity determined by ELISA in crude IgG and in pure IgG samples was higher than their total protein concentration (Table 1) indicating that ELISA gave inaccurate results. From that reason, ELISA results were corrected by correction factor. It was calculated from IgG in pure IgG sample determined by ELISA and IgG quantity calculated by multiplying IgG content determined by SEC with total protein concentration of the same sample. Corrected ELISA results were in accordance to the purity of each sample observed by SEC. Corrected ELISA results indicated that purification process has improved the IgG purity.

In vivo evaluation of neutralization potency (R) of starting plasma and final pure IgG proved that process has near 100% yield, indicating that developed ELISA can serve as reliable in vitro alternative.

Moreover, neutralization potency per mg of IgG calculated for both samples was 1.39 for plasma and 1.38 for pure IgG indicating that our process does not lead either to the change in the ratio of venom-specific and nonspecific IgGs or to the loss of some IgG subtypes. In contrast, protein A affinity purification leads to the loss of neutralization power of IgGs (0.85 IgG), most probably due to the loss of some IgG isotypes that bind weakly to the protein A. The use of affinity purified IgG, having different neutralisation potency, from samples in which we quantify IgG (plasma, TT plasma, crude IgG, pure IgG) is the most probable cause for the inaccurate ELISA results. However, these can be easily corrected as we clearly demonstrated here.

CONCLUSION

We have successfully developed a precise and accurate in vitro method suitable for monitoring the efficiency of antivenom purification process that adequately replaces the in vivo neutralization assay in mice.