INTRODUCTION

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

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 LD$_{50}$) and (b) test for determining the neutralization potency (efficacy) of induced antivenom (determination of median effective antivenom dose or ED$_{50}$). Large number of mice are injected with venom/antivenom mixtures and the number of surviving mice is statistically analyzed to give an LD$_{50}$ 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 ECVAM (European 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. (Garo viper) was collected by rolling snakes onto a lethal dose of venom (100% LD$_{50}$) in a controlled environment at room temperature (22–28°C) at the Institute of Immunology, University of Zagreb, Croatia. Plasma, collected from individuals that were bitten by Vipera ammodytes ammodytes L., was obtained from the Institute of Immunology, University of Zagreb, Croatia. Mice, both sex and age, were kept in the Institute of Immunology.

Protein concentration determination

The protein concentrations in hyperimmune horse plasma and in samples from all purification steps were estimated spectrophotometrically using the method, based on an equation: (A280 nm / A234.5 nm) × 0.2137 × mg/mL. (4)

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 3000SW (7.8 x 300 mm) column. Antigen was injected as a 1 mg/mL solution at room temperature and the absorbance was monitored at 280 nm. The running buffer was 0.1 M phosphate buffer pH 7.0. Standard proteins used for molecular weight determination were cytochrome c (Mr = 12,375), ovalbumin (Mr = 44,000) and ribonuclease A (Mr = 13,700).

ELISA

ELISA for detection of venom - specific antibodies in samples from hyperimmune horse plasma processing was performed by coating microtiter plate with 100 µL/well of venom solution (1 µg/mL) in 50 mM carbonate buffer, pH 9.6, and left overnight at room temperature (RT). After blocking with 3% (w/v) BSA in PBS at RT for 2 h, the plates or samples from each purification step were incubated in two-fold serial dilutions and left overnight at RT. The antibodies isolated from hyperimmune horses by protein A affinity chromatography were used as standards. In the subsequent steps, incubation with HRP-anti-horse IgG (Sigma, 1:2000 diluted) at 37°C for 2 h occurred, followed by the addition of OPD (0.6 mg/mL solution) in citrate-phosphate buffer, pH 5.0. After 20 min of incubation in the dark, the enzymatic reaction was stopped with 1 M H$_2$SO$_4$, and the absorbance at 405 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 preincubated with equal volumes of the venom solution containing two median lethal doses (LD$_{50}$) of the amount of dry venom (in µg) causing the death in half of the mice population used. The immunoreagents were removed by centripetal filtration and clear supernatant (i.e. administered to groups of four mice. Deaths were recorded 48 hours later. For each sample the median effective dose ED$_{50}$, the amount of undiluted venom capable of neutralizing the venom's lethal effect in 50 % of the animals was determined. The lethal toxicity neutralization potency (ED$_{50}$) was expressed as the number of LD$_{50}$ venom doses that can be neutralised by 1 mL of undiluted sample and calculated by the equation (µg/mL-ED$_{50}$) where µg represents the number of LD$_{50}$ inoculated per mouse. µg was used as a measure of the protective efficacy of each sample.

AIM

To monitor the efficiency 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 purification steps might have different venom neutralization potency in comparison to original population of polyclonal antibodies in the starting hyperimmune plasma. Consequently, this might lead to over or underexpression 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 our process of immunoglobulin purification from hyperimmune horse plasma consists of three steps: thermal coagulation, caprylic acid precipitation and dialfiltration, generating thermally treated plasma (TT plasma), crude IgG and pure 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 antigen), 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 input and output.

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

Moreover, neutralization potency per µg 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 IgG or to the loss of some IgG subset. In contrast, protein A affinity purification leads to the loss of neutralization power of IgGs (0.85 LD$_{50}$/µg), 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.

Table 1. Monitoring of purification process by ELISA

Table 2. Comparison of in vivo neutralisation potency and γ(IgG) determined in vitro

CONCLUSION

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

Figure 1. Purification step monitoring by SEC HPLC

horse plasma TT plasma crude IgG pure IgG thermal coagulation centrifugation, filtration caprylic acid precipitation centrifugation, filtration dialfiltration

Table 3. Comparison of in vivo neutralisation potency and γ(IgG) determined in vitro

Table 4. Neutralisation potency of different plasma samples

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IN VITRO CORRELATE OF IN VIVO ANTIVENOM NEUTRALIZATION POTENCY ASSAY FOR EFFICIENCY ASSESSMENT OF ANTIVENOM PURIFICATION STEPS

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