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Paraspecificity of *Vipera a. ammodytes*-specific antivenom towards *Montivipera raddei* and *Macrovipera lebetina obtusa* venoms

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ABSTRACT

Antivenom raised against the venom of nose-horned viper, *Vipera ammodytes* (*V. a.*) *ammodytes* (European viper venom antiserum, Zagreb antivenom), contains neutralising equine $F(ab')_2$ fragments that are clinically successful against homologous venom, but also against the venoms of several others medically important European snakes due to its paraspecific action. In this work we demonstrated that Zagreb antivenom is preclinically effective in neutralising lethal toxicity and hemorrhagicity of venoms of Armenian mountain snakes – *Montivipera raddei* and *Macrovipera lebetina obtusa* as well. In order to better understand the biochemical basis of the observed paraspecificity, the ability of anti-*V. a. ammodytes* serum to recognise and neutralise proteinases of the two venoms was also investigated. Anti-*V. a. ammodytes* serum showed surprisingly low capacity to inhibit metalloproteinases of both venoms included in the study, probably due to weak immunorecognition of their P-I representatives. Also, it completely failed to abolish enzymatic action of serine proteinases from *Macrovipera lebetina obtusa* venom. Relevance of such finding is yet to be established.

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1. Introduction

Snake bite envenoming is a common and frequently devastating environmental and occupational disease, especially in rural areas of tropical developing countries, which is increasingly being recognised as a highly relevant public health issue on a global basis also (Chippaux, 1998a, 1998b; Theakston et al., 2003). Its specific treatment is critically dependent on the availability of safe and effective animal-derived antivenoms (Calvete et al., 2009). Production of antivenoms claims the adequate selection of snake specimens as candidates for collection of immunising venom mixtures which is generally based on recognising the ones that are responsible for the largest burden of envenoming in a particular geographic region, their range of distribution, and particularly, immunological relationships between their venoms and the venoms of other taxonomically related species (Gutiérrez et al., 2009). Namely, despite being subjected to accelerated molecular evolution, venoms of snakes belonging to the same or even different genera often tend to share antigenic determinants creditable for their immunoreactive cross-neutralisation, also known as paraspecificity (Calvete, 2010). Paraspecificity refers to the capacity of antivenom to neutralise the venom of the species not included in the immunisation mixture at therapeutically useful doses, *i.e.* not excessively beyond those necessary for specific neutralisation (Archundia et al., 2011). Animal experimentation, notably lethality neutralisation assay in mice as the golden

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standard for ensuring the antivenom potency, has usually been performed for paraspecific immunoreactivity determination (WHO, 2010). Additionally, more reliable assessment of the preclinical efficacy can be achieved by enrichment of such traditional analysis with other approaches measuring the ability of antivenom to abrogate the spectrum of toxic and enzymatic activities of probed venom, as well as by application of "antivenomics", a proteomics-based protocol for assessing the immunological profiles of antivenoms (Gutiérrez et al., 2009). Although extrapolation of the results obtained in laboratory to real clinical setting has to be undertaken with caution because of physiological limitations of *in vivo* and *in vitro* tests (Theakston et al., 2003; WHO, 2010), determination of paraspecific immunoreactivity may have important implications for antivenom design and use, especially in medical situations with urgent need for extension of neutralisation coverage to species whose venom is not included in the immunisation mixture (Calvete et al., 2009; Calvete, 2010). Additionally, knowledge concerning paraspecificity also may be of great significance for accessibility and supply improvements in regions experiencing inadequate coverage or complete lack of antivenoms produced against medically important venoms, an issue often associated with lack of epidemiological data, disorganisation of health services and high manufacturing costs in developing countries (Chippaux, 1998a).

Antivenom produced by hyperimmunisation of horses with the venom of nose-horned viper, *Vipera ammodytes* (*V. a.*) *ammodytes* (European viper venom antiserum, in the literature also known as Zagreb antivenom, Institute of Immunology Inc., Croatia), contains neutralising antibodies that are clinically successful against homologous venom, as well as against the venoms of several others medically important European snakes, as demonstrated by its continuous, over 30 years long use for the treatment of envenomings induced by *Vipera aspis* (Italy), *Vipera berus* (UK, Sweden), *Macrovipera lebetina* and *Montivipera xanthina* (Greece, Turkey). To possibly broaden its clinical coverage, we decided to screen paraspecificity exhibited by Zagreb antivenom towards the venoms of geographically more distant specimens by investigating the extent of their cross-neutralisation and chose *Montivipera* (*Mo.*) *raddei* and *Macrovipera lebetina* (*Ma. l.) obtusa*, both from the Armenian region, as a suitable candidates. These snakes are important cause of snake bite in western Asia each year, causing mild to severe local effects, such as tissue blistering, oedema, hemorrhage and necrosis, as well as hypotension shock and coagulopathy, especially clotting disorders and hypofibrinogenemia (Göçmen et al., 2006; Sanz et al., 2008; Ayvazyan and Ghazaryan, 2012). Although the venoms of *Mo. raddei* and *Ma. l. obtusa* have been thoroughly characterised at proteomic level (Sanz et al., 2008), their toxinological profile is less well studied and the information about the correlation with the clinical picture of envenoming still lacks. Despite high medical importance of those two Armenian mountain vipers it is noteworthy to mention that at the moment specific therapy against *Mo. raddei* envenoming, representing rare but potentially serious hazard, is not available. On the other hand, Zagreb antivenom shows sufficient level of protection against the venom of *Ma. lebetina*, as proved by its clinical effectiveness in treatment of snake bites occurring in Turkey. However, concerning the well documented phenomenon of geographical intraspecies variability of snake venoms in general, it would be of great importance to confirm therapeutic potential of Zagreb antivenom against bites of *Ma. lebetina* specimens from neighbouring regions as well.

To summarise, our objectives were i) to gain the information about the relevant biological and toxinological activities of *Mo. raddei* and *Ma. l. obtusa* venoms of Armenian origin; ii) to determine the degree of logical cross-reactivity between *V. a. ammodytes* and *Mo. raddei* venoms that has never been studied before and iii) to revise the preclinical efficacy of Zagreb antivenom against the venom of *Ma. l. obtusa*.

2. Materials and methods

2.1. Reagents and chemicals

Azocasein, Tween 20, ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (Pefabloc) and N_α-benzoyl-L-phenylalanine-L-valine-L-arginine-*p*-nitroanilide hydrochloride, iodoacetamide and DL-dithiothreitol (DTT) were from Sigma–Aldrich, USA. Horseradish peroxidase-conjugated goat anti-rabbit IgG (HRP-anti-rabbit IgG) was from Bio-Rad Laboratories, USA. Chemicals for buffers and solutions were from Kemika, Croatia.

2.2. Snake venoms and antivenoms

Crude venom of *V. a. ammodytes* was collected by milking snakes kept at the Institute of Immunology, air dried at ambient temperature and stored in the dark at 4 °C until use. Crude *Ma. l. obtusa* and *Mo. raddei* venoms were supplied by Orbeli Institute of Physiology, National Academy of Sciences (Yerevan, Armenia) and stored at 4 °C until use. Commercial European viper venom antiserum (Zagreb antivenom) was from Institute of Immunology Inc., Croatia. Experimental rabbit serum against *V. a. ammodytes* venom (anti-venom serum) was produced according to production immunisation scheme on a small scale.

2.3. Animals for in vivo assays

All animal work was in accordance to the Croatian Law on Animal Welfare (2013) which strictly complies with EU Directive 2010/63/EU. Mice (18–20 g) used for the assays of lethal toxicity and neutralisation of lethal toxicity were of NIH Ola/Hsd strain, bred at the Institute of Immunology. Adult rats for the assays of hemorrhagic activity and neutralisation of hemorrhagic activity were of Lewis strain, also bred at the Institute of Immunology.

2.4. Assay of lethal toxicity

The lethal toxicity, expressed as the median lethal dose (LD_{50}), was determined according to method of Theakston

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