THE ROAD TOWARDS IN VITRO ALTERNATIVE TO IN VIVO SNAKE VENOM TOXICITY AND ANTIVENOM POTENCY ASSAYS

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INTRODUCTION
Continuous quality control of venoms and antivenoms is necessary for successful production of viper antivenom. Control is based on two in vitro tests: (a) the venom lethal toxicity assay (determination of median lethal dose of venom or LD50) and (b) test for determining antivenom potency (effectiveness) of produced antivenoms (determination of median effective antivenom dose or ED50). 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 (2).

Ammodutoxins (Atx) and haemorrhagins (H) are the main constituents of Vipera ammodytes ammodytes (Vaa) venom whose involvement in the generation of human pathology has been considered to be highly probable. Since development of in vitro tests requires identification of the components that contribute to the venom's overall toxicity or specificity of antibodies that effectively neutralize them, the aim of our study was to investigate participation of anti-Atx and anti-H antibodies in neutralization of the whole Vaa venom lethal toxicity in mice. Also, two 'in vitro' methods for determination of Atx content in different venom batches were developed and evaluated as possible screening tools for selection of the best antigen for immunisation and antivenom production.

MATERIAL AND METHODS
Vaa venom was extracted from snakes held at the Institute of Immunology Inc., Zagreb, Croatia (2 batches – KRRS and SLS). Atx and total haemorrhagic fraction were prepared at IoSf Stolfin Institute, Ljubljana, Slovenia.

SANDWICH ELISA
Microtiter plate was coated with rabbit anti-Atx IgG in 0.05 M carbonate buffer, pH 9.6 and left overnight at RT. After blocking with 0.5% (w/v) BSA in PBS/T (0.05% (w/v) Tween 20 in PBS buffer for 2 h at 37°C, the investigated venom batches and the pure AtxB standard used as standard were added in five serial two-fold dilutions in duplicates and incubated overnight at RT. Plate was washed; HRP-anti-guinea pig IgG was added and incubated for 2 h at 37°C. Finally, plate was washed; OD solution was added and incubated for half an hour at RT in the dark. The enzymatic reaction was stopped with 3 M H2SO4 and A405 nm was measured. Quantitative determination of Atx content was done by parallel line assay comparing each venom batch to the standard. The A405 content is expressed as percentage of A405 weight in the total venom weight.

HPLC METHOD
Samples of venom batches were analyzed as follows. Briefly, solution of venom in Tris/HC1 buffer, pH 9.0 was applied to the CIM CM disk. Bound material was eluted by stepwise NaCl gradient: 0.04 M (6th min), 0.08 M (12th min), 0.12 M (18th min), 0.2 M (24th min), and 1 M (30th min). Five elution fractions (EF1–EF5) were obtained and profiles of EF1 (containing Atx and AtxB) and EF2 (containing Atx) were compared for the analyzed venom batches.

RESULTS

Table 1. Quality attributes of KRRS and SLS samples of Vaa venom used for immunogenicity comparison. Results are given as mean ± 95% CI from indicated (in brackets) number of measurements. All tested quality attributes of the two venoms were significantly different at indicated p-values.

<table>
<thead>
<tr>
<th>Vaa venom sample</th>
<th>Lethal toxicity (LD50 dose in mg)</th>
<th>Atx content (%)</th>
<th>CM-HPLC of basic venom components</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRRS</td>
<td>45.64 ± 31.35 (5)</td>
<td>1.40 ± 0.16 (8)</td>
<td>10.1 ± 3.21 (6)</td>
</tr>
<tr>
<td>SLS</td>
<td>35.6 ± 18.5 (8)</td>
<td>1.90 ± 0.75 (6)</td>
<td>10.3 ± 6.0 (8)</td>
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<tr>
<td>p-value</td>
<td>&lt;0.1&lt;sup&gt;11&lt;/sup&gt;</td>
<td>&lt;0.3&lt;sup&gt;11&lt;/sup&gt;</td>
<td>&lt;0.2&lt;sup&gt;11&lt;/sup&gt;</td>
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</table>

The quantities of anti-Atx IgG and anti-H IgG determined by ELISA, and the protective efficacies (R) against the venom lethal toxicity, of the sera of rabbits immunized with Atx, total haemorrhagic fraction (H) or their combination. Serum of the rabbit immunized with whole venom served as positive control. Results are given as mean values ± SE from a minimum of three independent experiments.


definition of terms:

- LD50: dose in mg that causes death in 50% of the test animals.
- ED50: dose in µg that neutralizes 1 mL of undiluted serum.
- SE: standard error.
- ECVAM: European Centre for Validation of Alternative Methods.

CONCLUSION

- The results showed that functional anti-Atx antibodies were only partially involved in the neutralization of the venom toxicity.
- Functional anti-H antibodies did not provide protection at all.
- Atx content determination in different venom batches could serve as a good screening method in the selection of the best antigen for immunisation (4).
- Developed methods for anti-Atx or anti-H determination, cannot be a substitute for in vivo antivenom potency determination. Deeper insight into snake venom composition at the molecular level and the participation of the particular components in the venom toxicity has yet to be done before solving this task (5).

REFERENCES


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