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Original article

Use of immunoturbidimetry to detect venom–antivenom binding using snake venoms☆,☆☆

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ABSTRACT

Introduction: Immunoturbidimetry studies the phenomenon of immunoprecipitation of antigens and antibodies in solution, where there is the formation of large, polymeric insoluble immunocomplexes that increase the turbidity of the solution. We used immunoturbidimetry to investigate the interaction between commercial snake antivenoms and snake venoms, as well as cross-reactivity between different snake venoms. **Methods:** Serial dilutions of commercial snake antivenoms (100 μl) in water were placed in the wells of a microtitre plate and 100 μl of a venom solution (50 μg/ml in water) was added. Absorbance readings were taken at 340 nm every minute on a BioTek ELx808 plate reader at 37 °C. Limits imposed were a 30 minute cut-off and 0.004 as the lowest significant maximum increase. Reactions with rabbit antibodies were carried out similarly, except that antibody dilutions were in PBS. **Results:** Mixing venom and antivenom/antibodies resulted in an immediate increase in turbidity, which either reached a maximum or continued to increase until a 30 minute cut-off. There was a peak in absorbance readings for most Australian snake venoms mixed with the corresponding commercial antivenom, except for *Pseudonaja textilis* venom and brown snake antivenom. There was cross-reactivity between *Naja naja* venom from Sri Lanka and tiger snake antivenom indicated by turbidity when they were mixed. Mixing rabbit anti-snake antibodies with snake venoms resulted in increasing turbidity, but there was not a peak suggesting the antibodies were not sufficiently concentrated. The absorbance reading at predetermined concentrations of rabbit antibodies mixed with different venoms was able to quantify the cross-reactivity between venoms. Indian antivenoms from two manufacturers were tested against four Sri Lankan snake venoms (*Daboia russelli*, *N. naja*, *Echis carinatus* and *Bungarus caeruleus*) and showed limited formation of immunocomplexes with antivenom from one manufacturer. **Discussion:** The turbidity test provides an easy and rapid way to compare and characterise interactions between antivenoms and snake venoms.

1. Introduction

Snake envenoming is now recognised as a global health issue and antivenom is the major treatment (Kasturiratne et al., 2008). However, there are shortages of antivenom in many parts of the world and there is a need for high quality and effective antivenoms to be developed (Lalloo et al., 2002). An essential part of antivenom development is determining the ability of antivenom to bind to toxins in snake venoms and also the cross-reactivity between particular antivenoms and other snake venoms (Isbister, O'Leary, et al., 2010; Kornhauser et al., 2013). This allows effective polyvalent antivenoms to be developed to cover all snakes in a particular geographical region.

The use of immunoprecipitation to determine the extent of reactivity between venoms or venom components (toxins) and antivenoms is well-known (van der Weyden et al., 2000; Williams et al., 1994). The traditional radial immunodiffusion (RID) process, which is carried out in an agar gel, takes many hours to days to complete. Such long experimental times limit the number of studies that can be done. Immunoturbidimetry studies the same phenomenon, but in solution rather than on a gel. In solution the formation of large, polymeric insoluble immunocomplexes causes an increase in turbidity. The Ramon flocculation assay is based on this principle and is used as one of the quality check procedures in the manufacture of diphtheria and tetanus vaccines (Lyng & Bentzon, 1987; Preneta-Blanc et al., 2008). For this assay, varying ratios of standardised solutions of toxoid and antitoxin are mixed, and the resulting turbidity of the solution, referred to as flocculation, is observed by the naked eye.

The occurrence of flocculation at particular ratios of snake venoms and antivenoms has been long recognised, but there are limited studies on this phenomenon (Schottler, 1952). Solano et al. used a turbidimeter

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to monitor the interaction between *Bothrops asper* venom and antivenom to determine how long it took for this interaction to be complete (Solano et al., 2010). They noted that turbidity continued to increase over the 30 minute incubation period, but that neutralisation was not dependent on this time.

We found that sufficient turbidity develops to be detectable in the wells of a microtitre plate in a basic filter-based absorbance plate reader. We have used this method of detecting immunoprecipitation in solution to explore the interaction between venoms and commercial antivenoms, or in-house antibodies.

2. Methods and materials

Australian snake venoms including common tiger snake (*Notechis scutatus*), black tiger snake (*N. ater*), rough-scaled snake (*Tropidechis carinatus*), mulga snake (*Pseudechis australis*), red-bellied black snake (*P. porphyriacus*), common death adder (*Ancanthophis antarcticus*), coastal taipan (*Oxyuranus scutellatus*), Stephen's banded snake (*Hoplocephalus stephensii*), and the Eastern brown snake (*Pseudonaja textilis*) venoms were obtained from Venom Supplies, South Australia. Venoms from *Daboia russelli* (Russell's viper) and *Naja naja* (Indian cobra) venom were obtained from Colombo University, Sri Lanka. *Echis carinatus* (saw-scaled viper) and *Bungarus caeruleus* (Indian krait) venoms were purchased from Sigma. *Echis ocellatus* (carpet viper) venom was donated by Robert Harrison (Liverpool School of Tropical Medicine). Stock solutions of venoms of 1 or 2 mg/ml in 50% glycerol are stored at −20 °C. Russell's viper venom factor X activating factor (RVVX) was obtained from Haematologic Technologies Inc., VT USA.

Rabbit anti-snake antibodies were obtained from the Western Australian Institute of Medical Research as approximately 1 mg/ml in phosphate buffered saline (PBS). IgY from hens immunised with *P. textilis* venom was a gift from Frank Madaras (Venom Science Pty Ltd, South Australia), and is 3 mg/ml in PBS. The CSL antivenoms used were: Brown snake (BSAV; batch #0559-10801), Tiger snake (TSAV; batch #10702), Black snake (BlSAV; batch #0543-07301), Taipan (TAV; batch #0548-05601), and Death adder (DAAV; batch #0557-07701). Solutions of 20 U/ml in water were prepared. The Indian polyvalent antivenoms used were: VINS (batches #1054-2000, #01AS11114-2012, #01024/10-11-2010) and BHARAT (batch #A511006-2011). Indian polyvalent antivenoms are raised against four snake venoms — *D. russelli*, *N. naja*, *E. carinatus* and *B. caeruleus*. All commercial antivenoms are IgG of equine origin. Solutions of 40 mg/ml in water were prepared.

Serial dilutions of snake antivenom (100 µl) in water were placed in the wells of a microtitre plate. The plate was placed in a BioTek ELx808 plate reader at 37 °C, and 100 µl of a venom solution (50 µg/ml in water) was added. The final venom concentration was 25 µg/ml. Readings were taken at 340 nm every minute, including a 3 second shake step. Preliminary experiments found that after mixing venom and antivenom there was an immediate increase in the reading which either reached a plateau or continued to increase. Limits imposed were a 30 minute cut-off for the experiment and a value of 0.004 as the lowest significant maximum increase.

Reactions with rabbit antibodies were carried out similarly, except that the antibody dilutions were in PBS instead of water.

3. Results

3.1. Snake venoms and Australian antivenoms

Experiments were undertaken in water because turbidity did not develop in PBS for with CSL antivenoms. Fig. 1 shows the increase and peak in turbidity for the major Australian snake venoms mixed with the corresponding antivenom for increasing concentrations of antivenom. The antivenom concentration for the maximum turbidity was estimated by interpolation (Table 1) and varied for each venom. *P. textilis* venom and BSAV only had a small increase and no distinct peak. The combination of *P. textilis* venom and BSAV was repeated for a range of venom concentrations, but this did not result in the appearance of a well-defined maximum either.

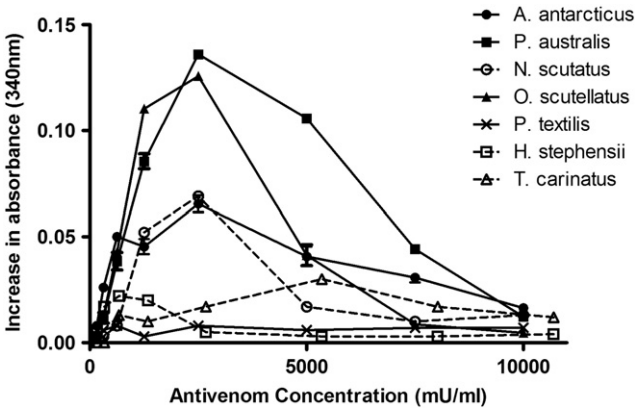

Fig. 1. Increase in absorbance resulting 30 min after the addition of seven snake venoms to their corresponding CSL Ltd. antivenoms. Tiger snake antivenom was used for *T. carinatus* and *H. stephensii*. Experiments were repeated in triplicate for *A. antarcticus*, *P. australis* and *O. scutellatus*. All venoms were at a concentration of 25 µg/ml.

The cross reactivity of *N. naja* venom (Cobra from Sri Lanka) with TSAV was tested by comparing the combinations of *N. scutatus* venom/TSAV and *N. naja* venom/TSAV for increasing concentrations of TSAV (Fig. 2). There was a peak at a TSAV concentration of approximately 2500 mU/ml which was significantly lower than the peak for *N. scutatus* venom.

3.2. Snake venoms and rabbit monovalent antibodies

Fig. 3A shows increasing turbidity with increasing concentrations of antibodies against six Australian snake venoms and rabbit anti-*N. ater* antibodies and that a peak does not occur. It appears that the preparations of these rabbit antibodies are not sufficiently concentrated to reach a peak of maximum turbidity with venom. Fig. 3B depicts absorbance reading versus time for venom with increasing concentrations of antibody, showing the rapid formation of the immunocomplex.

Cross-reactivity between different venoms and rabbit monovalent antibodies was compared at a pre-determined concentration of antibodies. Table 2 provides the absorbance found for a range of snake venoms using the antibodies. Fig. 3C shows the cross-reactivity between four Asian snake venoms (*D. russelli*, *E. carinatus*, *B. caeruleus* and *N. naja*) and antibodies to *D. russelli* venom. Fig. 3D shows

Table 1
Formation of insoluble immunocomplexes between snake venoms and antivenoms. The venom concentration was 25 µg/ml.

Venom	Antivenom	Antivenom concentration (mU/ml) for maximum absorbance
N. scutatus	TSAV	2500
N. ater	TSAV	3750
T. carinatus	TSAV	5350
H. stephensii	TSAV	670
P. porphyriacus	TSAV	No peak
P. porphyriacus	BlSAV	No peak
P. australis	BlSAV	2500
O. scutellatus	TAV	2500
P. textilis	BSAV	No peak
A. antarcticus	DAAV	2500 and 625
N. naja	TSAV	2500

TSAV — tiger snake antivenom; BlSAV — black snake antivenom; TAV — taipan antivenom; BSAV — brown snake antivenom; DAAV — death adder antivenom.