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Assessing the preclinical efficacy of antivenoms: From the lethality neutralization assay to antivenomics

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ABSTRACT

The assessment of the capacity of antivenoms to neutralize the lethal activity of snake venoms is the gold standard in the preclinical analysis of antivenom efficacy, and is routinely performed by manufacturers and quality control laboratories. However, the complexity of snake venom composition and toxicological profile demands that, for many venoms, such as those of viperid snakes and some elapids, the neutralization of lethality be complemented with the analysis of the neutralization of other relevant toxic activities, such as hemorrhagic, myotoxic, necrotizing, procoagulant and defibrinogenating effects. This expanded protocol for preclinical testing of antivenoms should be used when a new antivenom is developed or when an existing antivenom is introduced in a new geographical setting for the neutralization of either homologous or heterologous venoms. In recent years, the assessment of the immunological reactivity of antivenoms has been enriched by the use of proteomic tools, with a methodology named 'antivenomics'. This allows the identification of venom components to which antivenoms have, or lack, antibodies, and thus complements the data gathered in neutralization tests, paving the way for a knowledge-based improvement of antivenom design and efficacy. International projects involving participants of manufacturing, quality control and academic research groups should be promoted in order to gain a deeper understanding on the preclinical neutralizing spectrum of antivenoms.

1. Assessing the efficacy of antivenoms at the preclinical level

Snakebite envenoming constitutes a highly relevant public health problem on a global basis (WHO, 2007; Williams et al., 2010; Warrell, 2010; Gutiérrez, 2012). The parenteral administration of animal-derived antivenoms constitutes the mainstay in the therapy of these envenomings (WHO, 2007, 2010; Warrell, 2010; Gutiérrez et al., 2011a). Antivenoms are raised in horses, and in few cases in other animals such as sheep or donkeys, which are immunized with the venom of either a single snake species (to generate monospecific antivenoms) or several species (for polyspecific antivenoms) (Theakston et al., 2003; Gutiérrez et al., 2011b). Once the immunized animals have developed a satisfactory antibody response, they are bled, and the plasma or serum is then fractionated to obtain either whole IgG molecules or fragments $F(ab')_2$ or Fab (Theakston et al., 2003; Gutiérrez and León, 2009; WHO, 2010). After formulation and sterile filtration, antivenoms

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are dispensed in vials; they are manufactured in either liquid or freeze-dried presentations, which have different stability and expiry times (WHO, 2010; Gutiérrez et al., 2011b).

Antivenoms should be safe and effective for use in humans and animals. Antivenom effectiveness must be demonstrated at both preclinical and clinical levels (WHO, 2010). The assessment of the preclinical efficacy of antivenoms has been based on the so called antivenom potency assay, which evaluates the ability to neutralize the lethal activity of a venom in an animal model, usually mice (Theakston et al., 2003; WHO, 2010). In the cases of new antivenoms, or antivenoms being introduced to a new geographical setting for the neutralization of either homologous or heterologous venoms, the preclinical assessment of efficacy and safety should be followed by proper clinical trials (WHO, 2010). Failure to comply with a satisfactory neutralizing efficacy at the preclinical level precludes the initiation of clinical trials. When antivenoms have been already registered in national regulatory authorities, after passing the requirements established in each country, every new batch should pass the standard quality control tests before distribution.

Despite the fact that the lethality potency assay is well established as the gold standard in assessing the preclinical efficacy of antivenoms, the complexity of snake venoms and of the pathophysiology of snakebite envenoming demands a more in-depth scrutiny of antivenom efficacy, in order to have a broader perspective of its neutralizing spectrum. The present work analyzes the lethality neutralization assay, highlighting areas that require further investigation and standardization. In addition, other tests for assessing antivenom preclinical efficacy are discussed, including antivenomics, which now allows for a more detailed characterization of the immunoreactivity of antivenoms.

2. Neutralization of lethality: the gold standard for antivenom efficacy estimation at the preclinical level

Since the early times of antivenom development, the assessment of preclinical efficacy has been based on the ability of antivenoms to neutralize the lethal effect of venoms, using animal models (Calmette, 1896; Brazil, 1918). Mice have become the most commonly used experimental model for neutralization assays (Christensen, 1955; Bolaños, 1977; WHO, 1981, 2010; Theakston et al., 2003), although other animals are used by some laboratories (WHO, 2010). Current protocols involve, initially, the determination of the Median Lethal Dose (LD_{50}), using either the intravenous (i.v.) or the intraperitoneal (i.p.) routes of injection (WHO, 2010). In this test, groups of mice of defined weight and strain are injected with various doses of venom and deaths occurring within 24 h (when using the i.v. route) or 48 h (when using the i.p. route) are recorded. LD_{50}, i.e. the dose of venom that induces death in 50% of injected animals, is estimated by either probits (Finney, 1971), Spearman-Karber (WHO, 1981) or alternative procedures such as non-parametric tests (WHO, 2010). For estimating the neutralizing efficacy of antivenoms, generally a fixed dose of venom ('challenge dose') is incubated with various dilutions of antivenom, usually for 30 min at 37 °C, followed by the injection of the mixture, either by i.v. or i.p. routes (WHO, 2010). Deaths occurring within the time spans described above are recorded and the neutralizing efficacy is determined by the same methods used for assessing the LD_{50}. Neutralizing ability is expressed as Median Effective Dose (ED_{50}), i.e. the volume of antivenom, or the antivenom/venom ratio, which protects 50% of the injected mice. On the basis of the value of ED_{50}, some laboratories then express the preclinical efficacy of antivenoms in terms of 'potency' (Araujo et al., 2008). Owing to the large variability in composition of snake venoms, international reference antivenoms are not useful. However, the use of national standard antivenoms as references has been recommended and may contribute to the standardization of antivenom manufacture and quality control (WHO, 1981; Fukuda et al., 2006).

Despite the widespread use of this basic methodology, there are variations between laboratories and Pharmacopeias in the performance of the antivenom potency assay. For instance, the 'challenge dose' of venom varies from 3 to 6 LD_{50}s (WHO, 2010). In some cases, the challenge dose is defined in terms of Minimum Lethal Dose (MLD, minimum dose of venom that induces death in all animals injected) instead of LD_{50} (Villalta et al., 2012). Such variations have evident implications in the estimation of ED_{50}, since the higher the challenge dose of venom, the lower the estimated neutralizing efficacy of the antivenom (see for example Bogarín et al., 2000). Most laboratories use the i.v. route (WHO, 2010), although several manufacturers in Latin America use the i.p. route (Araújo et al., 2008; Segura et al., 2010). Since the predominant pathophysiological effects of venoms may vary depending on the injection route, this has implications in the results, with different ED_{50}s for the same antivenom when tested by different routes (Kocholaty et al., 1968; Arce et al., 2003; Solano et al., 2010). Likewise, when an immunologically-dominant toxin is neutralized by antibodies, other toxins, for which the antivenom has a lower titer, might induce lethality by a mechanism different from that of the dominant toxin (Christensen, 1966). Furthermore, the way in which ED_{50} is expressed also varies between laboratories (μL antivenom per challenge dose of venom, mg venom/mL antivenom, μL antivenom/mg venom, mg antivenom/mg venom, or number of LD_{50}s neutralized per mL antivenom) (WHO, 2010). Some manufacturers and regulatory bodies utilize other ways to express neutralizing capacity of antivenoms, such as neutralizing Units in Taiwan (Villalta et al., 2012), Australia (Fry et al., 2001), and Japan (Fukuda et al., 2006). Therefore, manufacturers should provide details of the conditions in which the ED_{50}s of their products are determined. It is noteworthy that, in spite of the relevance of the lethality assay and its widespread use, relatively few studies have been published on its design, analytical properties, and factors determining the outcome of the assay (Christensen, 1966; Chaniot and Netter, 1971; Krifi et al., 1998; Solano et al., 2010).

A key aspect in the interpretation of the lethality neutralization assays is the understanding of the mechanism of death in experimental animals, i.e. which pathophysiological mechanisms predominate. In the case of

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