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Mini-review

Phage display as a novel promising antivenom therapy: A review

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ABSTRACT

In this work, we present recent advances in the use of phage display technology for the preparation of antivenoms for animal toxin neutralization. Even though classical antivenoms have been used since the early 20th century, envenomation remains a global public health problem. Recently, the phage display technique has been used in an attempt to circumvent some of the difficulties associated with traditional preparations of antivenom. Here, we review studies that developed antibody fragments with potential inhibitory effects against animal toxins and discuss the most current technical issues and perspectives regarding phage display technology in this field.

1. Envenomations by animal toxins

Animal bites are considered a major public health problem worldwide (WHO, 2013). According to the World Health Organization, more than five million people are bitten each year by snakes alone. Approximately 2.4 million of these people are poisoned, and 94,000 to 125,000 die as a result (WHO, 2013). The numbers involving scorpion stings are similarly high. Approximately 2.3 billion people live in risk areas such as Africa, the Middle East, Southern India, Mexico, the Eastern Andes, and Southern Latin America, where more than 1.2 million stings are reported per year, causing at least 3250 deaths (Rodriguez de la Vega et al., 2010). Regarding spider bites, the 30th Annual Report of the National Poison Data System (NPDS, 2012) documented approximately 9343 cases in the United States alone. That same year, 5444 cases of accidental bee, wasp, and hornet stings were registered (Mowry et al., 2013). Among many other venomous animals, the caterpillar *Lonomia obliqua* is considered the one most dangerous in southeast of Brazil. The number of accidents involving this insect is growing as a consequence of extensive deforestation and uncontrolled agricultural expansion. However, the current incidence of accidents related to this insect is still highly underestimated (Pinto et al., 2010).

A considerable number of accidental bites are related to animal defense, one of the primary functions of venoms (Brodie, 2009). These complex mixtures are generally composed of a rich set of enzymes, bioactive peptides, amino acids and inorganic salts, and their composition may vary among genera, species, subspecies, geographic region, individuals, season, eating habits, habitat, age, gender, regeneration time and even the method of venom extraction (Chippaux et al., 1991; Rodriguez de la Vega et al., 2010). Snake, scorpion, and spider venoms are composed of neurotoxic components that affect muscles and nerves and are used to immobilize prey (Brodie, 2009). Moreover, phospholipases as well as some proteinases, which are found in snake and spider venoms, help digest the prey after an attack (Kordis and Gubensek, 2000) and may cause hemolysis in the envenomed victims (Soto et al., 1988). Bees also produce neurotoxins mainly as a form of defense (Brodie, 2009) to delay or limit an attack of a possible predator, especially when the colony is disturbed (Vetter et al., 1999).

The use of classic antivenoms obtained from the processing of hyperimmunized horse serum has served as the main resource in treatments of accidental envenomation for more than a century. The use of antivenoms has led to a significant reduction in morbidity and mortality in a number of registered accidents involving complex animal venoms (Gutierrez et al., 2007).

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2. Classical antivenoms and the advent of phage display technology

At the end of the 19th century, Behring and Kitasato initiated in Berlin what would later be considered the starting point of the so-called serotherapy. They reported that the serum derived from immunized animals with diphtheria or tetanus toxins presented anti-toxin properties against these same toxins (von Behring and Kitasato, 1991). Using the anti-toxic properties of serum from rabbits and guinea pigs immunized with snake venom, Phisalix and Bertrane, obtained results similar to those previously reported. (Phisalix, 1894). Similar findings were simultaneously reported by Calmette (Calmette, 1894). In the following year, the first antivenom produced by Calmette in hyperimmunized horses was used by Haffkine in India and by Lépinay in Vietnam. In 1897, Calmette published the first successful experiment using antivenom serotherapy (Calmette, 1897).

Following Calmette's contribution, many scientists around the world effectively contributed to the development of anti-venom therapies. Researchers such as Vital Brazil, who together with Calmette is considered as one of the pioneers in serotherapy, used hyperimmunized horse serum in the preparation of anti-venom (Chippaux and Goyffon, 1998). The active molecules responsible for the therapeutic action of the serum were further identified as immunoglobulins, which started to be purified and used instead of the whole serum, as described in a historical review by Browning (1955). It was subsequently discovered that pepsin could cleave an immunoglobulin molecule, thus producing $F(ab)'_2$ fragments (Pope, 1939a,b). This approach aimed to minimize the side effects caused by using whole immunoglobulin molecules. In response to concerns regarding animal care for hyperimmunized donors, the technique of plasmapheresis aseptically returns used erythrocytes to the donor, which prevents the development of anemia in cases that entail multiple blood draws (WHO, 2010).

Even when the animal toxin activity is reduced, the use of heterologous immunoglobulins may produce adverse effects (Hoogenboom, 2005). In the guidelines published by the World Health Organization for the production, control and regulation of snake antivenom immunoglobulins the adverse effects caused by antivenoms are classified as early and late reactions (WHO, 2010). Early reactions may occur within the first 24 h of antivenom administration and can be classified based on their mechanisms, such as pyrogenic reactions induced by endotoxins, IgE mediated and non-IgE mediated reactions. The last two manifestations are characterized by fever, pruritus, urticaria, tachycardia, hypotension, smooth muscle spasms, gastrointestinal symptoms, bronchospasm, respiratory collapse, angioedema, shock, and even death. The late effects may occur between 5 and 24 days after antivenom administration and are induced by immune complexes formed by patient's immunoglobulins against heterologous antibodies or venom proteins. These reactions are known as serum sickness, which is a type III hypersensitivity reaction, and are characterized by fever, myalgia, arthralgia, arthritis, urticaria, lymphadenopathy and gastrointestinal disorders. Complications rarely observed include laryngeal edema, glomerulonephritis, and vasculitis (Leon et al., 2013). Several efforts have been made to mitigate these effects. Chimeric antibodies, in which the constant portion (Fc) of the immunoglobulin is of human origin, significantly decreased the immunogenicity of the compound (Morrison et al., 1984). Later on, researchers developed a technique in which peptides and/or immunoglobulin domains are presented on the surface of filamentous phages. This technique has been named Phage Display. The peptides and antibody fragments in Phage Display might be selected according to their specificity and binding affinities (Fig. 1) (McCafferty et al., 1990; Smith, 1985).

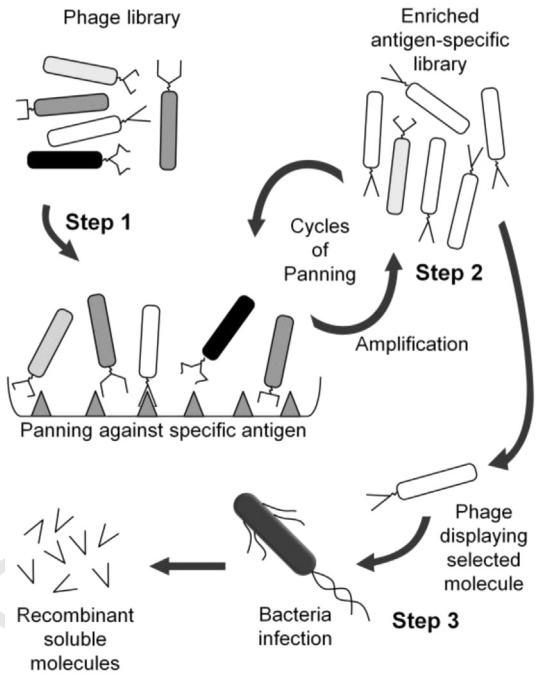

Fig. 1. Schematic representation of a round of panning in a Phage Display selection. Phages displaying a pool of different molecules are selected by binding to specific antigens (Step 1). Non-binding phages are washed away. The enrichment of specific-antigen clones is possible by repeated exposures to the antigen, and by increasing the stringency of the following washes (Step 2). Phages expressing selected molecules are used to infect bacteria in order to express their soluble molecules (Step 3).

The application of this technique for the production of antivenoms would no longer require the use of immunized animals or the constant need for antigens. However, facing the complexity of animal's venoms, monoclonal antibodies specific to different active substances would be required in order to inhibit as much toxicity as possible. Conversely, many components of the venom are not toxic, and phage display allows the production of antibodies exclusively against those that are active, different from the traditional animal immunization with the whole venom.

It should be noted that animal venoms also have toxins presenting particular similarities that are sometimes conserved among different species (Menez, 1998). This feature allows the development of a single antibody molecule that is capable of inhibiting a number of different toxins, as demonstrated by Pucca et al. (2013) and Riano-Umbarila et al. (2011). Thereby, as part of the process of antibody development, proteomic approaches should identify the most relevant toxins in each medically relevant venom (Peiren et al., 2005), and thus refining the selection of more useful molecules.

On the next topic, we will discuss some studies that already generated promising results in the development of functional antibodies or their fragments.

3. Phage display as a new antivenom therapy

PubMed and Google Scholar searches combining the keywords “phage display” and “venom” resulted in 39 research articles published up until September of 2014. Importantly, 60% of these studies were published in the last six years, confirming Phage Display as a recent and growing research area.

Gazarian et al. (2000), Hernandez et al. (2002) and de Moura et al. (2011) selected toxin mimotopes from peptide libraries to

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