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Pros and cons of different therapeutic antibody formats for recombinant antivenom development

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ABSTRACT

Antibody technologies are being increasingly applied in the field of toxinology. Fuelled by the many advances in immunology, synthetic biology, and antibody research, different approaches and antibody formats are being investigated for the ability to neutralize animal toxins. These different molecular formats each have their own therapeutic characteristics. In this review, we provide an overview of the advances made in the development of toxin-targeting antibodies, and discuss the benefits and drawbacks of different antibody formats in relation to their ability to neutralize toxins, pharmacokinetic features, propensity to cause adverse reactions, formulation, and expression for research and development (R&D) purposes and large-scale manufacturing. A research trend seems to be emerging towards the use of human antibody formats as well as camelid heavy-domain antibody fragments due to their compatibility with the human immune system, beneficial therapeutic properties, and the ability to manufacture these molecules cost-effectively.

1. Introduction

The world fauna presents a vast variety of venomous animals including snakes, scorpions, spiders, bees, wasps, caterpillars, sea anemones, jellyfishes, lizards, fishes, and cone snails as examples. Many of these animals can cause severe envenomings by their sting or bite, inflicting pain, tissue damage, and systemic pathologies, and may in some cases cause fatalities. The true number of these accidents is unknown, as even the World Health Organization (WHO) does not report epidemiological data for envenomings by all classes of venomous animals. However, it has been estimated that snakes alone cause 1.8 to 2.7 million envenomings each year, resulting in 81,000 to 138,000 deaths (Gutiérrez et al., 2017a), while scorpion stings result in 1.2 million envenomings per year, leading

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to around 3000 deaths (Chippaux and Goyffon, 2008). In particular, snakebite envenoming is classified by the WHO as a Neglected Tropical Disease (NTD), a group of diseases that prevail in tropical and subtropical parts of the world and mainly affect populations living in poverty with very limited access to healthcare.

The specific medical treatment for envenomings caused by animals is the use of antivenoms. Heterologous antivenom serotherapy is a century-old treatment described simultaneously by Césaire Auguste Phisalix, Gabriel Bertrand, and Albert Calmette in France in 1894 (Calmette, 1894; Phisalix and Bertrand, 1894). Later (1901), in Brazil, Vital Brazil Mineiro da Campanha demonstrated that antivenom specificity is essential for treating envenomings from particular species (Hawgood, 1992). Since that time, the use of antivenoms has saved countless lives. Nowadays, different heterologous antivenoms are manufactured in many countries with the aim of neutralizing venoms from diverse venomous animal species (Laustsen et al., 2016a). Supplies of these life-saving medicines are, however, still critically scarce in many regions (Brown and Landon, 2010), and efforts are being carried out to improve their availability and accessibility (Gutiérrez, 2012).

Although heterologous antivenoms are, to this date, the only effective treatment for snakebite envenomings, these therapeutic agents present some documented undesirable problems (Fig. 1): (i) Antivenoms can cause anaphylactic reactions, which can be either IgE-mediated or, more commonly, non-IgE-mediated (due to complement activation); both types are known as early adverse reactions (up to 24 h) (de Silva et al., 2011; Isbister et al., 2008a,b; León et al., 2013). (ii) Antivenoms are composed of whole immunoglobulins (IgGs) or antigen-binding fragments ($F(ab')_2$s or Fabs) raised against whole venom(s) via immunization of a host animal (Laustsen et al., 2016a, 2016c; Rodríguez-Rodríguez et al., 2016). However, the majority of these antibodies are not directed towards medically relevant venom toxins (Laustsen et al., 2015), but are instead directed against antigens that the immunized animal has encountered during its life (environmental antigens, microorganisms, and parasites). As a consequence, most antivenoms carry a large portion of immunoglobulins that are not directed against venom components (about 70%) (Laustsen et al., 2016a; Segura et al., 2013). (iii) The large amount of antivenom antibodies combined with the elicited human anti-horse antibodies (IgGs and IgMs) may result in the generation of immune complexes (ICs) that have a long elimination half-life. This can trigger IC deposition in target tissues (such as blood vessels, glomeruli, and joints), mediating inflammation and promoting serum sickness – a late adverse reaction associated with type III hypersensitivity (1–2 weeks after antivenom therapy) (Cunningham et al., 1987; Descotes and Choquet-Kastylevsky, 2001).

Taken together with the high cost of antivenom production, which is dependent on both animal immune systems and procurement of venoms, a need for innovation within envenoming therapies exists. Several approaches, including the use of immunization with DNA, synthetic epitope strings, or recombinant toxins, have been pursued (Alvarenga et al., 2002; Araujo et al., 2003; Harrison, 2004; Laustsen et al., 2016a, 2016c). However, despite a promising potential for eliminating the need for keeping venomous animals in captivity and "milking" them to obtain their venoms, these novel immunization techniques all retain the drawbacks of creating heterologous antivenoms with compromised compatibility with the human immune system. A more recent avenue that has been taken is the development of recombinant antibodies and antibody fragments of camelid and/or human origin (Harrison et al., 2011; Laustsen et al., 2016a, 2016c; Pucca et al., 2012; Pucca et al., 2011a,b; Richard et al., 2013). These molecules have very low immunogenicity and are easy to engineer using standard approaches that are well-investigated in other fields. This allows for the design of more optimized envenoming therapies with better safety profiles and potentially higher efficacy, as such recombinant antibodies would be completely compatible with the human immune system. Furthermore, only therapeutically active antibodies targeting medically relevant toxins would be included in a novel recombinant antivenom (Laustsen et al., 2015). Additionally, in the future it is projected that the production of recombinant antivenoms based on mixtures of such antibodies may be cost-effective compared to traditional antivenom manufacturing methods (Laustsen et al., 2017, 2016b). However, although several antibody formats have been investigated for use in recombinant antivenoms (Fig. 2), a clear indication of which format represents the optimal molecular scaffold to be used does not exist. In this review, we therefore aim at presenting all available data on different antibody formats that have been investigated for neutralization of animal toxins, and discuss their pros and cons in relation to toxin targeting in clinical scenarios.

2. Pharmacodynamics: ability to neutralize venom toxins

Pharmacodynamics (PD) plays a key role in the successful outcome of antivenom immunotherapy. Within the field of antivenom, PD refers to the ability of therapeutic molecules to neutralize *in vivo* specific venom toxins present in a given venom, which is one of the key determinants of antivenom efficacy. Independent of their antibody format, antivenoms derive their PD efficacy from high affinity interactions between each antibody-toxin pair, although antibody stability is also considered important for neutralization capacity (Juárez-González et al., 2005). In the simple situation involving only a single antibody and a single toxin, affinity is often reported using the dissociation constant, K_d. However, several factors complicate such measurements when comparing classical polyclonal antivenoms: (i) several different antivenom antibodies (with different specificities) may recognize the same or various epitopes in a single toxin; (ii) each individual antivenom antibody may recognize similar (homologous) toxins with different affinities; (iii) the concentration of each antibody that recognizes a given toxin is unknown. For these reasons, it is only feasible to measure the avidity (a measure of the strength between a venom and multiple antibodies), also interpreted as functional affinity (Casewell et al., 2010; Vauquelin and Charlton, 2013). To our knowledge, no studies have systematically investigated the effect on avidity after enzymatic treatment of polyclonal IgGs to Fabs (or $F(ab')_2$s). However, one may expect a higher avidity of an IgG or $F(ab')_2$-based antivenom than a Fab-based antivenom due to the bivalent nature of the IgG and $F(ab')_2$ formats. The two independent binding sites on these antibody formats provide a larger probability that a toxin will become rebound to the antibody if the toxin is released due to molecular proximity effects. Additionally, cross-linking to other toxin-antibody complexes can take place, making it less likely that a toxin may escape during transient dissociation of the complex (Rudnick and Adams, 2009). This cross-linking effect may potentially lead to high therapeutic relevance of weaker interactions. Nevertheless, at least one Fab-based antivenom has proven to be at least as effective in the clinical setting than an IgG-based antivenom (Dart and McNally, 2001).

Monoclonal IgGs and single-chain variable fragments (scFvs) are the primary recombinant antibody formats that have been investigated for neutralization of animal toxins. *In vivo* lethality studies assessing the neutralization capacity of several monoclonal IgGs have shown positive results for snake toxins (Charpentier et al., 1990; Frauches et al., 2013; Stiles et al., 1994), spider toxins (Alvarenga et al., 2003; Boulain et al., 1982), and scorpion toxins

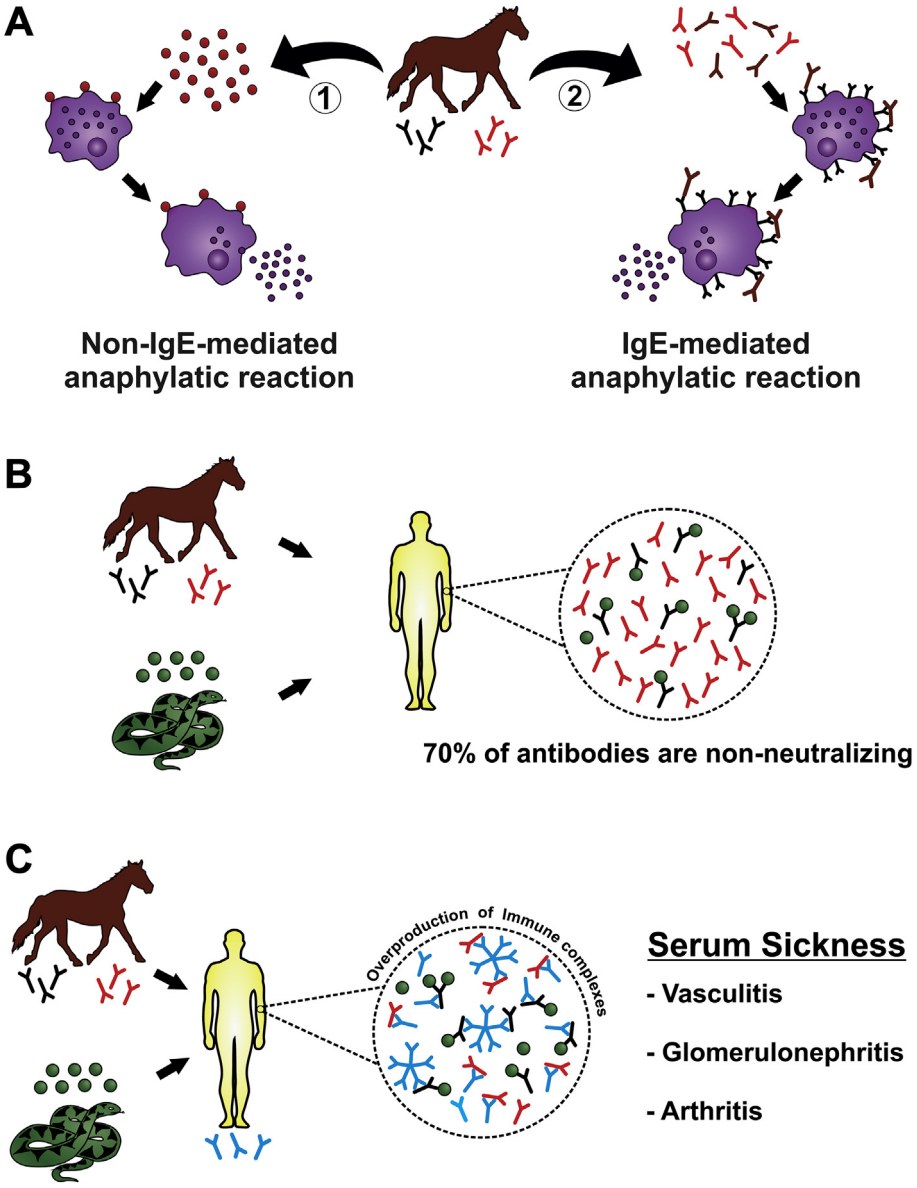

Fig. 1. Disadvantages of current animal plasma-derived antivenoms. Early adverse reactions occur within 24 h after administration of antivenoms. **(A1)** Patients may develop early adverse reactions (within 24 h) resulting from *de novo* complement activation (non-IgE reactions) or, (**A2**) in cases of previous exposure to animal antibodies, due to IgE-mediated anaphylactic reactions. **(B)** Around 70% or more of the antivenom antibodies are not directed towards medically relevant venom toxins. Therefore, envenomed victims will receive a larger than necessary dose of equine antibodies, which have no therapeutic value, but which may cause adverse reactions. **(C)** The large amount of antivenom antibodies combined with elicited human anti-horse antibodies (IgGs and IgMs) may result in overproduction of immune complexes. These may be deposited in blood vessels, glomeruli, and joints, mediating inflammation and promoting serum sickness 1–2 weeks after administration of antivenom therapy. **Black antibodies:** equine antibodies specific to target toxins. **Red antibodies:** equine antibodies against non-venom antigens. **Blue antibodies:** human antibodies against equine antibodies. **Green circles:** Snake toxins. For the sake of simplicity, examples illustrating the disadvantages of heterologous antibody therapy refer to equine antivenoms, but the same principles apply to antivenoms derived from other animal species. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Bahraoui et al., 1988; Clot-Faybesse et al., 1999; Zamudio et al., 1992) as summarized in Tables 1–3, respectively. Studies testing neutralization through biochemical assays have additionally found promising results against both snake toxins (Boulain et al., 1982; Charpentier et al., 1990; Trémeau et al., 1986) and scorpion toxins (Alvarenga et al., 2005) for the IgG format. In addition, many studies focusing on snake venom toxins have discovered IgGs with neutralizing abilities against multiple specific toxins responsible for myonecrosis (Frauches et al., 2013; Li et al., 1993; Lomonte et al., 1992; Lomonte and Kahan, 1988), hemorrhage (Fernandes et al., 2010; Frauches et al., 2013; Iddon et al., 1988; Morine et al., 2008; Perez et al., 1984; Schneider et al., 2014; Tanjoni et al., 2003b), and proteolytic effects (Morine et al., 2008; Schneider et al., 2014), as shown in Table 1.

Another of the most commonly investigated antibody formats is the Fab format. Two different studies produced Fabs against snake toxins. In one study, a Fab targeting cardiotoxin from *Naja nigricollis* venom was developed (Guillon et al., 1986), and in another study a Fab was developed against β1-bungarotoxin from *Bungarus multicinctus* venom (Yang and Chan, 1999). Both Fabs were shown to neutralize *in vitro* and *in vivo* effects of the toxins, respectively. Four studies have developed monoclonal Fabs against spider and scorpion toxins. Of these, three exhibited neutralizing effects *in vivo* against spider toxins (Bugli et al., 2008) and

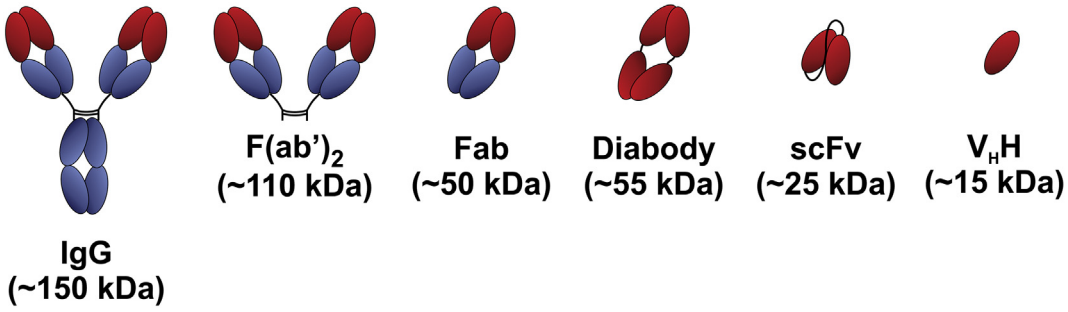

Fig. 2. Schematic overview of the different antibody formats used in existing plasma-derived antivenoms and experimental recombinant antivenoms. IgG: whole IgG antibody. **F(ab')$_2$**: pepsin-digested IgG antigen-specific region. **Fab**: papain-digested antigen-specific region. **Diabody**: non-covalent dimers of scFv fragments. **scFv**: single-chain variable fragments. **V$_H$H**: single-domain antigen-specific fragments.

Table 1
Reported monoclonal antibodies and antibody fragments against snake venom toxins.

Antibody format	Snake	Toxin	Origin	K_d (nM)	Therapeutic effects				Ref.
					Myotoxic effects	Haemotoxic effects	Biochemical assay	Lethality	
IgG1	*Echis carinatus*	Whole venom	Murine			*In vivo*			(Iddon et al., 1988)
IgG2a, IgG2b	*Naja naja oxiana*	Neurotoxin I	Murine					*In vivo*	(Stiles et al., 1994)
IgM, IgG1, IgG2b	*Bothrops asper*	BaP1	Murine	In the nM range		*In vivo*			(Fernandes et al., 2010)
IgG	*Naja naja siamensis*	α-cobratoxin	Murine	Estimated at 2.1–3.7			*In vitro*		(Charpentier et al., 1990)
IgG1, IgM	*Bothrops atrox*	Atroxlysin-I	Murine	8.52–15.10		*In vivo*			(Schneider et al., 2014)
IgG	*Bothrops atrox*		Murine		*In vivo*	*In vivo*		*In vivo*	(Frauches et al., 2013)
IgG2a	*Naja nigricollis, Laticauda semifasciata, Laticauda colubrina, Naja mossambica* and *Naja naja atra*	Toxin α, Erabutoxin b and c, Toxin d, Toxin I and III and Cobrotoxin	Murine	2–1.500			*In vitro*		(Trémeau et al., 1986)
IgG1	*Bothrops jararaca*	Jarahagin	Murine			*In vivo*			(Tanjoni et al., 2003b)
IgG1	*Agkistrodon contortrix lacticinctus*	Myotoxin	Murine		*In vivo*				(Li et al., 1993)
IgG	*Crotalus atrox*		Murine			*In vivo*			(Perez et al., 1984)
IgG2a	*Naja nigricollis*	Toxin α	Murine	0.35			*In vitro*	*In vivo*	(Boulain et al., 1982)
IgG1, IgM	*Bothrops asper*	Myotoxin	Murine		*In vivo*				(Lomonte and Kahan, 1988)
IgG1	*Probothrops flavoviridis*	HR1a	Human			*In vivo*			(Morine et al., 2008)
scFv	*Crotalus durissus terrificus*	Crotoxin	Murine	0.2–7.4				*In vivo*	(Meng et al., 1995)
scFv	*Crotalus durissus terrificus*	Subunit B Crotoxin (II-PLA$_2$)	Human	10,000					(Lafaye et al., 1997)
scFv	*Naja kaouthia*	Long alfa neurotoxin	Human					*In vivo*	(Kulkeaw et al., 2009)
scFv	*Bothrops asper*	BaP1	Not specified			*In vivo*		*In vivo*	(Castro et al., 2014)
scFv	*Vipera ammodytes meridionalis*	Vipoxin (PLA$_2$)	Human			*In vitro*			(Stoyanova et al., 2012)
scFv	*Bothrops jararacussu*	All the PLA$_2$ isoforms of the venom	Human		*In vivo*			*In vivo*	(Roncolato et al., 2013)
scFv	*Bothrops jararacussu*	BthTXI and BthTX-II PLA$_2$	Human		*In vivo*				(Tamarozzi et al., 2006)
scFv	*Crotalus durissus terrificus*	Subunit B Crotoxin (II-PLA$_2$)	Human		*In vivo*			*In vivo*	(Oliveira et al., 2009)
scFv	*Crotalus durissus terrificus*	Crotoxin	Human					*In vivo*	(Cardoso et al., 2000)
V$_H$H	*Naja kaouthia*	α-cobratoxin	Camelid	2.0–3.0					(Stewart et al., 2007)
V$_H$H/IgG	*Naja kaouthia*	α-cobratoxin	Camelid	0.4–25				*In vivo*	(Richard et al., 2013)
VH/V$_H$H	*Naja kaouthia*	PLA$_2$	Camelid				*In vitro*		(Chavanayarn et al., 2012)

scorpion toxins (Licea et al., 1996; Selisko et al., 2004), whereas the last study did not obtain neutralizing Fab antibodies (Aubrey et al., 2004). As previously mentioned, the scFv antibody format has also been widely studied. scFvs showing neutralization of lethality *in vivo* have been reported for both snake toxins (Cardoso et al., 2000; Castro et al., 2014; Kulkeaw et al., 2009; Lee et al., 2015; Meng et al., 1995; Oliveira et al., 2009; Roncolato et al., 2013) and scorpion toxins (Amaro et al., 2011; Devaux et al., 2001a; Hmila et al., 2012; Mousli et al., 1999; Riaño-Umbarila et al., 2016, 2013, 2011, 2005; Rodríguez-Rodríguez et al., 2016). To obtain more biochemical details regarding scorpion toxin neutralizing capacity, electrophysiological studies involving the two-electrode voltage clamp technique using *Xenopus laevis* frog oocytes showed that activation of sodium channels by *Tityus*

Table 2
Reported monoclonal antibodies and antibody fragments against spider venom toxins.

Antibody format	Spider	Toxin	Origin	Therapeutic effects	Reference
				Lethality	
IgG	*Loxosceles intermedia*	unknown	Murine		(Alvarenga et al., 2003)
Fab	*Latrodectus tredecimguttatus*	α-latrotoxin	Murine	*In vivo*	(Bugli et al., 2008)
IgG	*Loxosceles intermedia*	SmaseD	Murine	*In vivo*	(Dias-Lopes et al., 2014)

Table 3
Reported monoclonal antibodies and antibody fragments against scorpion venom toxins.

Antibody format	Scorpion	Toxin	Origin	K_d (nM)	Therapeutic effects			Reference
					Biochemical assay	Lethality	Not neutralizing	
IgG	*Androctonus australis*	AahII	Murine	0.8		*In vivo*		(Bahraoui et al., 1988)
IgG1, IgG2a, IgG2b	*Androctonus australis hector*	AahI	Murine			*In vivo*		(Clot-Faybesse et al., 1999)
IgG	*Buthus martensi*	BmK AS-1	Murine	0.0278-0.152				(Jia et al., 2000)
IgG2a, IgG1	*Centruroides noxius*	Cn2	Murine			*In vivo*		(Zamudio et al., 1992)
IgG1	*Tityus serrulatus*		Murine		*In vitro*			(Alvarenga et al., 2005)
Fab	*Androctonus australis*	AahI	Murine	0.082			x	(Aubrey et al., 2004)
Fab	*Centruroides noxius*	Cn2	Murine			*In vivo*		(Selisko et al., 2004)
Fab	*Centruroides noxius*	Cn2	Murine			*In vivo*		(Licea et al., 1996)
scFv	*Androctonus australis*	AahII	Murine			*In vivo*		(Mousli et al., 1999)
scFv	*Androctonus australis*	AahI	Murine			*In vivo*		(Devaux et al., 2001b)
scFv	*Centruroides noxius*	Cn2	Human	1.01		*In vivo*		(Riaño-Umbarila et al., 2005)
scFv	*Centruroides noxius*	Cn2	Murine	0.075			x	(Juárez-González et al., 2005)
scFv	*Centruroides noxius* and *Centruroides suffusus suffusus*	Cn2 and Css2	Human	0.05–16.60		*In vivo*		(Riaño-Umbarila et al., 2011)
scFv	*Centruroides suffuses, Centruroides limpidus, Centruroides noxius and Centruroides tecomanus*	Css2, Css4, Cll1, Cll2, Cn2, Ct1a	Human	1–290		*In vivo*		(Rodríguez-Rodríguez et al., 2016)
scFv	*Centruroides noxius* and *Centruroides limpidus limpidus*	Cll1 and Cn2	Human	8.1–25.1		*In vivo*		(Riaño-Umbarila et al., 2013)
scFv	*Centruroides noxius.*	Cn2	Human	5.4–93.7		*In vivo*		(Riaño-Umbarila et al., 2016)
scFv	*Tityus serrulatus*	Ts1	Human			*In vivo*		(Amaro et al., 2011)
scFv	*Tityus serrulatus*	Whole venom	Human					(Pucca et al., 2012)
scFv	*Tityus serrulatus*	Ts1 and Ts2	Human		*In vitro*			(Pucca et al., 2014)
V_HH	*Androctonus australis*	AahI	Camelid	1.21–55.8		*In vivo*		(Hmila et al., 2008)
V_HH	*Androctonus australis*	AahII	Camelid	0.12–76.00		*In vivo*		(Abderrazek et al., 2009)
V_HH	*Androctonus australis*	AahI and AahII	Camelid			*In vivo*		(Hmila et al., 2010)
V_HH	*Androctonus australis*	AahI and AahII	Camelid			*In vivo*		(Hmila et al., 2012)
Diabody mixture	*Androctonus australis*	AahI and AahII	Murine			*In vivo*		(di Tommaso et al., 2012)
Diabody	*Centruroides noxius*	Cn2	Human	0.0369–0.095		*In vivo*		(Rodríguez-Rodríguez et al., 2012)

serrulatus venom toxins Ts1, Ts2, and Ts5 could be neutralized by human scFvs (Pucca et al., 2014). Also, scFvs capable of neutralizing myonecrosis have been reported for snake venom toxins (Oliveira et al., 2009; Roncolato et al., 2013; Tamarozzi et al., 2006). Other scFvs have been discovered, which can neutralize melittin and phospholipase A_2 (PLA_2) from Africanized bees *in vitro* and prolong survival *in vivo* (see Table 4). However, scFvs that lack neutralizing abilities have also been reported (Juárez-González et al., 2005). In addition to assessing their neutralization potential, a few studies of scFv antibodies developed against snake venom toxins also include a structural and sequencing analysis to determine the regions involved in toxin binding (Kulkeaw et al., 2009; Lafaye et al., 1997; Meng et al., 1995).

Several studies have involved two other small antibody formats, variable fragments of heavy chain antibodies (V_HHs) and dimers of scFvs (diabodies), used against snake and scorpion toxins. Of these, one V_HH has shown neutralization of lethality against snake toxins (Richard et al., 2013), whereas both V_HHs (Abderrazek et al., 2009; Hmila et al., 2012, 2008, 2008) and diabodies (di Tommaso et al., 2012; Rodríguez-Rodríguez et al., 2012) have shown neutralization of lethality against scorpion toxins. For IgGs (Bahraoui et al., 1988; Boulain et al., 1982; Charpentier et al., 1990; Fernandes et al., 2010; Iddon et al., 1988; Jia et al., 2000; Schneider et al., 2014; Trémeau et al., 1986), Fabs (Aubrey et al., 2004), scFvs (Juárez-González et al., 2005; Lafaye et al., 1997; Lee et al., 2015; Meng et al., 1995; Riaño-Umbarila et al., 2016, 2013, 2011, 2005; Rodríguez-Rodríguez et al., 2016), and V_HHs (Abderrazek et al., 2009; Hmila et al., 2008; Richard et al., 2013; Stewart et al., 2007) some studies have determined the K_d between the antibodies and their respective toxins. The K_ds range from 10 μM as the highest reported for an scFv against crotoxin from the venom of the South American rattlesnake (Lafaye et al., 1997) to the lowest K_d of 28 pM for an IgG developed against BmK AS-1 from the Chinese scorpion *Buthus martensii* Karsch (Jia et al., 2000). The reported K_ds seem to corroborate the notion that high affinity frequently correlates with better neutralization ability, where antibodies with neutralizing abilities have K_ds in the lower nanomolar range, as shown in Tables 1 and 3.

All reported monoclonal antibody formats that have been developed against snake, scorpion, spider, and bee venom toxins seem to neutralize toxins equally well (see Tables 1–4). No conclusion can thus be drawn on which format binds and neutralizes animal toxins best. However, one major challenge when comparing different antibody formats is that studies have

Table 4
Reported monoclonal antibodies and antibody fragments against bee venom toxins.

Antibody format	Bee	Toxin	Origin	Therapeutic effects			Reference
				Myotoxic effects	Hemotoxic effects	Lethality	
scFv	*Apis mellifera*	Mellitin and PLA_2	Murine		*In vivo*	*In vivo*	(Santos et al., 2013)
scFv	*Apis mellifera*	Mellitin and PLA_2	Human	*In vivo*			(Funayama et al., 2012; Pessenda et al., 2016)

employed very different approaches for assessing toxin neutralization. For better comparison of neutralization potentials of different antibodies, it would be beneficial if a common approach could be employed, such as that recommended by the WHO for assessing the preclinical efficacy of antivenoms. Following this approach, *in vivo* neutralization is assessed by pre-incubation of toxin and antibody prior to injection into rodents, as this has been shown to yield the best reproducibility of results and allow for better comparability between antivenoms (Gutiérrez et al., 2017b). This protocol does, however, not mimic a real life envenoming and subsequent treatment scenario, and antibodies showing neutralization potential when pre-incubated with the toxin prior to injection may not show efficacy if administered after venom injection (Charpentier et al., 1990). It would therefore be more relevant to evaluate antivenom neutralizing capacity in experiments involving independent administration of venoms and antibodies, i.e. 'rescue experiments'. Overall and unsurprisingly, no final conclusion can be drawn based purely on pharmacodynamics regarding which antibody format represents the most optimal format for toxin neutralization. To allow for better comparison between different antibody formats it would be beneficial to test a single monoclonal antibody and its derived formats against the same toxin target. In this context, no prior studies have been performed within the field of toxinology.

2.1. Modes of neutralization

Understanding the modes of neutralization of antibodies may guide the design of novel antivenom components. Nonetheless, only limited efforts have been invested in this area, and it is therefore not possible to determine any general trend in how different antibody formats neutralize various animal toxins. However, studies of single antibodies targeting mainly snake venom toxins have proposed five different mechanisms to explain the mode of neutralization. Firstly, direct inhibition where antibodies interfere with the site of interaction between the toxin and its target by competitive inhibition (Fig. 3A). This mechanism has been demonstrated for an anti-long chain neurotoxin monoclonal antibody (Charpentier et al., 1990) and has been suggested as a general mode of neutralization of small neurotoxins by polyvalent antivenoms (Engmark et al., 2017a, 2016). Secondly, for enzymatic toxins, direct inhibition may be equivalent to blocking the catalytic site (Fig. 3B). Similar to direct inhibition, binding of a relative large antibody (fragment) to a region near the site of interaction may result in a steric hindrance effect (Fig. 3C). However, to the best of our knowledge no record of such situation is available, although it is structurally feasible. A third mechanism is allosteric inhibition (Fig. 4), where binding of the antibody induces a conformational change making a toxic site inaccessible or locking the toxin in a much less toxic, or even inactive, conformation. As an example, a polyvalent Crotalinae antivenom has been reported to recognize linear peptides mimicking a known allosteric site from snake venom serine proteases (Engmark et al., 2017b). Fourthly, antibodies can prevent the dissociation of toxin complexes responsible for forming the active toxins (Lafaye et al., 1997) (Fig. 5). Fifthly, even if an antibody does not block the active site of the toxin nor an allosteric site, the formation of toxin-antibody complexes may preclude the toxin from interacting with its target, and may facilitate its elimination by the mononuclear phagocytic system (Gutiérrez and León, 2009).

On the more general level of venom toxicity, neutralization of single toxins by antibodies may reduce the clinical manifestations dramatically. This may be explained by high individual toxicity and/or high concentration of a single toxin in a venom (Laustsen et al., 2015), and when this toxin is neutralized, only weakly toxic or non-toxic components remain. However, abrogation of venom toxicity by a single antibody can also be caused by an interruption of synergistic effects between toxins, if a key toxin (or key component) is neutralized (Fig. 6). Toxin synergism is a well-known feature of certain snake venoms (Laustsen, 2016). Each venom toxin may exhibit low toxicity on its own, but when the individual toxins are combined in a whole venom, they amplify the effect of each other resulting in actions such as destabilization of phosphorylative oxidation and increased tissue necrosis (Gasanov et al., 2014). Consequently, understanding the toxicity and interplay between individual toxins, as well as possible mechanisms of neutralization, is key to rational design of future recombinant antivenoms. Therefore, despite the great biochemical complexity of snake venoms (Calvete, 2017) and other animal venoms, it is likely that, in some cases, the neutralization of a few key toxins by antibodies may result in a drastic reduction in overall venom-induced toxicity.

3. Pharmacokinetics: distribution and elimination of antibodies and antibody fragments

The efficacy of treatment for a therapeutic antibody is strongly influenced by the speed and concentration at which it reaches the site of action, as well as its residence time in the body and consequent elimination. Upon injection, the pharmacological effect of the antibody will vary according to its absorption, distribution, metabolism, and excretion (ADME), pharmacokinetic (PK) processes that depend largely on the structural and biophysical properties of the molecule (Deng et al., 2012; Liu, 2017; Mould and Green, 2010). The combination of these processes provides an antibody with a PK profile, generally described by parameters such as volume of distribution (V_d), bioavailability (F), clearance (CL), maximum concentration in plasma (C_{max}), and elimination half-life ($t_{1/2}$), among others, that are calculated after measuring the concentration in plasma of the antibody over a period of time after its administration (Fan and de Lannoy, 2014).

Generally, for antibodies and their fragments, there is a strong relationship between the molecular mass of the molecule and its distribution and elimination characteristics. The PK profiles of recombinant monoclonal IgG antibodies used for therapeutic purposes (isotypes IgG1, IgG2, and IgG4) are characterized by limited tissue distribution and long elimination half-lives (Fig. 7A–B), displaying either linear or non-linear (dose-dependent) profiles (Kamath, 2016; Keizer et al., 2010; Tabrizi et al., 2006). Distribution of IgGs, which involves extravasation to the interstitial space and elimination from tissue, occurs mainly by convection, as diffusion

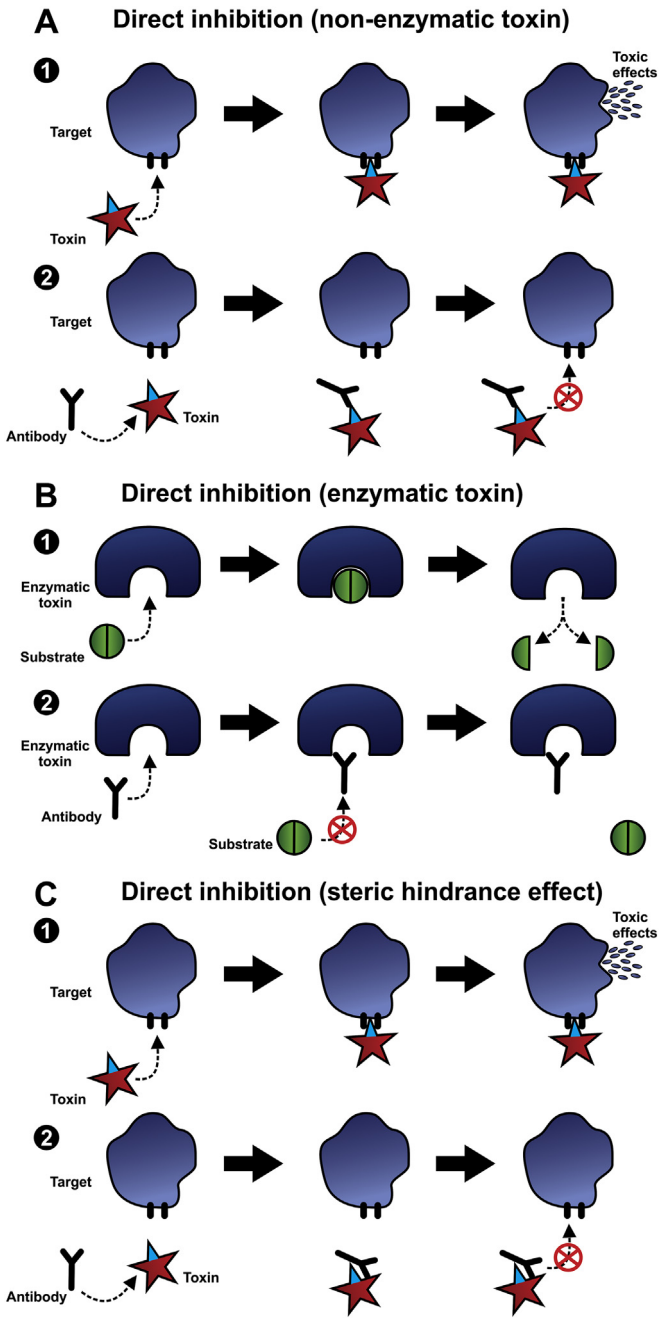

Fig. 3. Modes of neutralization: Direct inhibition of non-enzymatic toxins. (A1) A non-enzymatic toxin binds to its target (toxin binding region in blue), resulting in a toxic effect. **(A2)** The antibody interferes with the functional site of the non-enzymatic toxin, thereby preventing the toxin binding to the target. **Direct inhibition of enzymatic toxins. (B1)** An enzymatic toxin binds to the substrate resulting in enzymatic degradation of the substrate. **(B2)** The antibody blocks (or distorts) the catalytic site of the enzymatic toxin, thereby preventing substrate degradation. **Inhibition by steric hindrance**. **(C1)** A toxin binds to its target (toxin binding region in blue), resulting in a toxic effect. **(C2)** The antibody binds to a region near the site of interaction, thereby preventing the toxin from binding to the target. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

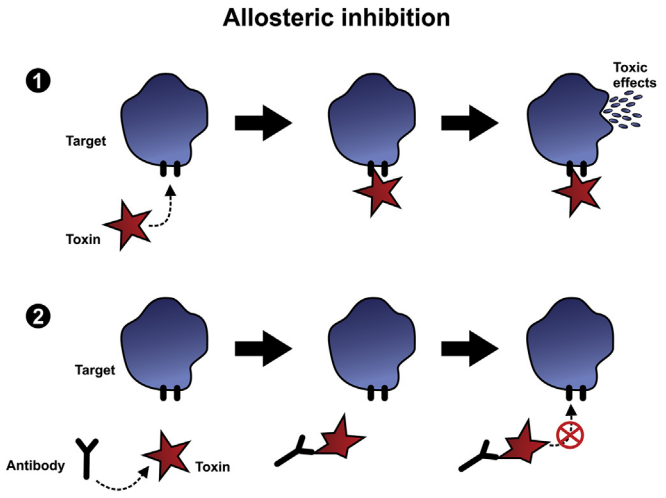

Fig. 4. Modes of neutralization: Allosteric inhibition. (1) A toxin binds to its target, resulting in a toxic effect. **(2)** The antibody binds to a distal site of the toxin, which induces conformation changes, resulting in a less or non-functional toxin (allosteric inhibition).

across endothelial cells is very slow due to the large size and hydrophilicity of the molecule (Lobo et al., 2004). Their large size also impedes IgGs from being enzymatically metabolized by cytochrome P450 (Mould and Green, 2010), and cleared by the kidneys (glomerular filtration cut-off ~50 kDa) (Wang et al., 2008). Instead, the main route for their elimination is via intracellular catabolism in the lysosomes, upon fluid-phase endocytosis (pinocytosis) or receptor-mediated endocytosis, including binding to Fcγ receptors (FcγR) expressed by phagocytic cells (Keizer et al., 2010; Tabrizi et al., 2006; Wang et al., 2008). However, a major fraction of the internalized IgGs is rescued from rapid degradation through binding to the neonatal Fc receptor (FcRn) of cells in the mononuclear phagocytic system (Fig. 7C), which transports the IgGs back to the cell surface and facilitates their release into the extracellular fluid (Brambell et al., 1964; Junghans, 1997); this is a saturable, pH-dependent, recycling mechanism that confers a long half-life (21–28 days) to human IgGs (Keizer et al., 2010; Raghavan et al., 1995; Tabrizi et al., 2006; Wang et al., 2008). Of note, the affinity of IgGs for FcRn is species dependent (Ober et al., 2001). Human IgGs have a higher affinity for human FcRn than chimeric IgGs and murine IgGs, which explains the shorter elimination half-lives of the latter in humans (8–10 days and 1–3 days, respectively) (Deng et al., 2012; Tabrizi et al., 2006).

In contrast to whole IgGs, the smaller sizes of antibody fragments, such as Fabs, scFvs (monomers and dimers), V_HHs, and minibodies, account for a larger volume of distribution and faster rate of tissue penetration (Harmsen and De Haard, 2007; Keizer et al., 2010; Wu et al., 1999, 1996). Due to the lack of an Fc region on these antibody fragments, they are unable to bind to the FcRn. Also associated with their small size, the main route for their clearance is via glomerular filtration by the kidneys (Lobo et al., 2004; Tabrizi et al., 2006). Owing to these features, these antibody fragments possess considerably shorter half-lives (0.5–30 h) (Tabrizi et al., 2006). $F(ab')_2$ fragments, also devoid of Fc region, possess a shorter half-life than IgGs, since recycling by the FcRn rescue mechanism is not possible (Tabrizi et al., 2006). However, their distribution profile resembles that of IgGs, and similarly, elimination occurs mainly by non-renal mechanisms, as their size exceeds the cut-off for renal filtration (Seifert and Boyer, 2001; Tabrizi et al., 2006).

In addition to the structural and biophysical properties of the antibody molecule, PK of IgGs and their fragments can be influenced by specific patient conditions, such as age, gender, health status (renal and hepatic function), or concomitant administration of other drugs (Deng et al., 2012; Tabrizi et al., 2006). The interaction between the antibody and the antigen may also affect PK (Bauer et al., 1999; Meijer et al., 2002), as well as immune responses

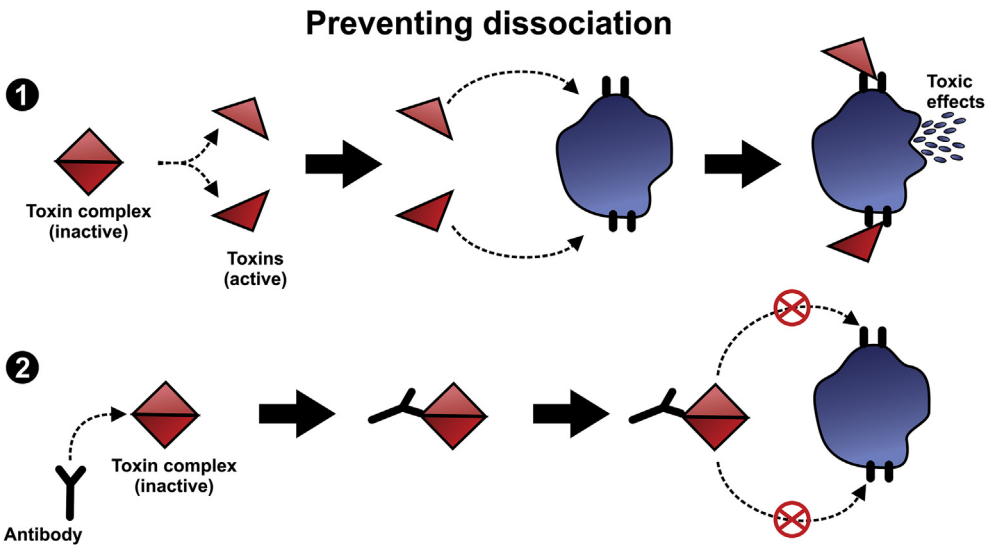

Fig. 5. Modes of neutralization: Preventing dissociation. (1) A toxin complex is dissociated and the active toxins bind to their targets, resulting in a toxic effect. **(2)** Antibody binding inhibits the dissociation of the toxin complex, thereby preventing the formation of active toxins.

against the antibody itself that may shorten its half-life (de Vries et al., 2007; Xu et al., 2008). Recently, several strategies to prolong the half-lives of antibodies and their fragments have been explored, such as mutations in the Fc region to increase affinity towards FcRn (Dall'Acqua et al., 2006; Finch et al., 2011; Monnet et al., 2014), *N*-glycosylation (Stork et al., 2008), polysialylation (Chen et al., 2012; Constantinou et al., 2008), PEGylation (Chapman et al., 1999), modification of the isoelectric point (Boswell et al., 2010; Kobayashi et al., 1999), and fusion or binding to proteins having an extended half-life (e.g. albumin, immunoglobulin) (Andersen et al., 2011; Evans et al., 2010; Hutt et al., 2012; Müller et al., 2007; Sleep et al., 2013; Smith et al., 2001; Unverdorben et al., 2012).

In agreement with the PK parameters displayed by whole recombinant IgGs and their fragments used as therapeutics, kinetic studies of plasma-derived antivenoms have shown the same strong relationship between the molecular mass of the molecules and their PK profiles (Gutiérrez et al., 2003). Antivenoms based on IgGs have low volumes of distribution, long elimination half-lives, and a high number of cycles through the interstitial spaces (Ho et al., 1990; Ismail et al., 1998; Ismail and Abd-Elsalam, 1996). Conversely, antivenoms based on Fab fragments, much smaller than IgGs, have larger volumes of distribution, diffuse faster into extravascular compartments, and have shorter elimination half-lives (Ariaratnam et al., 2001, 1999; Brvar et al., 2017; Meyer et al., 1997; Rivière et al., 1997; Vázquez et al., 2010a,b; Vázquez et al., 2005). A negative consequence of the short elimination half-life of Fab fragments is the higher incidence of recurrent peaks in plasma venom levels, and therefore envenomation symptoms, compared to IgG and $F(ab')_2$ antivenoms. This is most probably due to rapid clearance of Fab fragments from circulation that impedes the neutralization of venom toxins released from the bite site in later stages of the envenoming (Boyer et al., 2013; Gutiérrez et al., 2003; Seifert and Boyer, 2001). In accordance with their intermediate molecular mass, the PK profile of $F(ab')_2$-based antivenoms constitutes a middle point between that of IgGs and Fab fragments (Boyer et al., 2013; Gutiérrez et al., 2003; Ho et al., 1990; Isbister et al., 2015; Kurtović et al., 2016; Maung-Maung-Thwin et al., 1988; Pépin-Covatta et al., 1996; Sevcik et al., 2004). However, in general, due to the heterologous nature of antivenoms derived from horse or sheep plasma, these antibodies are eliminated faster than expected for a homologous human antibody (Scherrmann, 1994). The molecular mechanisms behind this observation are not fully understood, but could be the result of impeded binding to FcRn and/or development of anti-antibodies by the patient's immune system (Tabrizi et al., 2006; Wang et al., 2008).

While information on animal plasma-derived antivenom PK is somewhat available, only two studies have reported the PK profiles of recombinant antibody fragments targeting animal toxins. Aubrey et al. investigated the *in vivo* kinetics of a homodimeric diabody (50 kDa) derived from the anti-AahI murine 9C2 antibody after intraperitoneal injection into mice (Aubrey et al., 2003). The diabody displayed rapid diffusion, being detected in plasma only 5 min after its administration. Consequently, the maximum concentration (C_{max}) was reached shortly after (30–60 min post-injection). High concentrations (>50% of C_{max}) were detected for at least 6 h, and complete clearance of the diabody took approximately 24–32 h (Aubrey et al., 2003). In the other study, Hmila et al. compared the distribution and kinetics of two nanobodies (NbAahIF12 and NbAahII10, 14 kDa each) and a bispecific nanobody construct (NbF12-10, 29 kDa) to those of a $F(ab')_2$-based (110 kDa) scorpion antivenom after intravenous administration in mice and rats (Hmila et al., 2012). *In vivo* monitoring of radiolabeled nanobodies and $F(ab')_2$ fragments revealed that the nanobody-based molecules were cleared from blood faster than the $F(ab')_2$ antivenom, most likely due to the lower molecular mass of nanobodies. Additionally, a major difference was observed in the organ accumulation of the antitoxins. Monovalent nanobodies and the bispecific construct accumulated mainly in the kidneys, whereas $F(ab')_2$ fragments were predominantly retained in the liver (Hmila et al., 2012).

PD has implications on PK profiles of antibodies, and this will further have implications on efficacy, which highlights the importance of choosing the right antibody format for rational development of novel antivenoms. Often, venoms consist of complex mixtures containing both low and high molecular mass toxins, acting locally and/or systemically (Fig. 7D). On one hand, antivenoms should ideally provide antitoxins able to rapidly reach locally acting toxins and toxins that reach systemic extravascular targets very fast, such as low molecular mass neurotoxins. On the other hand, antivenoms should also provide antitoxins with extended half-lives that remain in circulation for prolonged periods of time (many hours to days). This will allow the antitoxins with

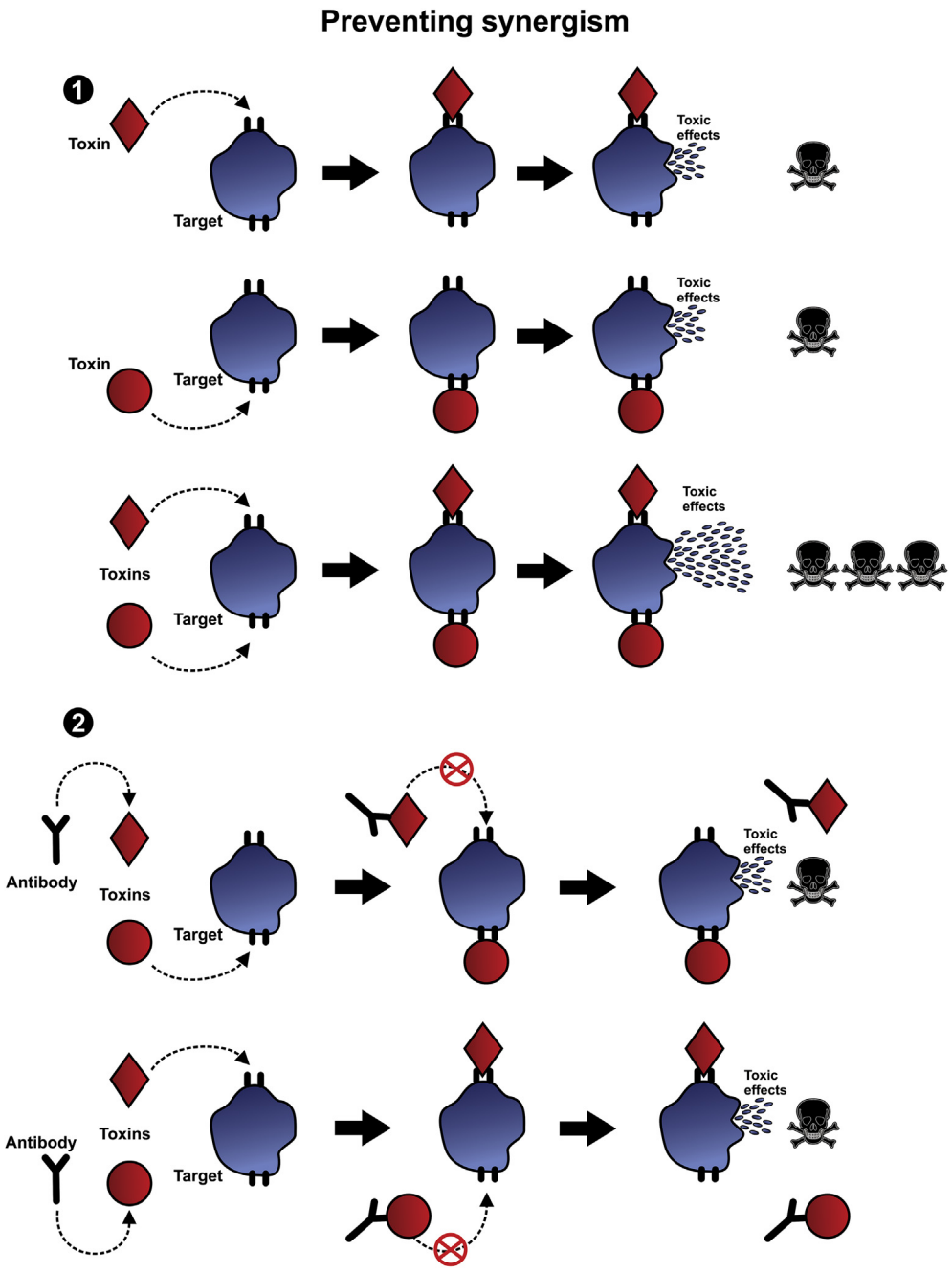

Fig. 6. Modes of neutralization: Preventing synergistic effects. (1) Synergism between toxin A (diamond) and B (circle) results in synergistically enhanced toxicity. **(2)** Antibody binding to one of the toxins results in milder toxic (or no) effects due to disruption of synergism.

long half-lives to intercept and neutralize systemically acting toxins in the circulatory system before these toxins reach their target site (Gutiérrez et al., 2003). Thus, an antivenom comprised of a mixture of different antibody formats could be necessary to target all medically relevant toxins present in complex venoms (Gutiérrez et al., 2003). Regarding the route of administration, notable differences have been found when comparing intravenous administration with intramuscular administration. Intravenous injections directly deliver the antibodies to the bloodstream, avoiding the absorption step and providing complete bioavailability (Liu, 2017). Hence, it is considered the preferred route of administration for antivenoms in a hospital setting. In contrast, intramuscularly injected antivenoms have shown poor efficacy due to slow absorption and reduced bioavailability of the antibodies or their fragments (Isbister et al., 2008a,b; Pépin-Covatta et al., 1996, 1995; Vázquez et al., 2010a,b). Nevertheless, it could still be considered an option, as antivenoms are occasionally required to be administered in the field (Warrell, 1995). Although the PK of a specific antibody format may be predicted based on the general distribution and elimination characteristics typical for its molecular mass, more PK studies are required to increase the current knowledge and guide the development of recombinant antivenoms based on in-depth understanding of the PK-PD relationship of each antibody format on an individual case basis. Additionally, favorable PK-PD for a given antibody format may very well depend on the toxicokinetics of the target toxin(s).

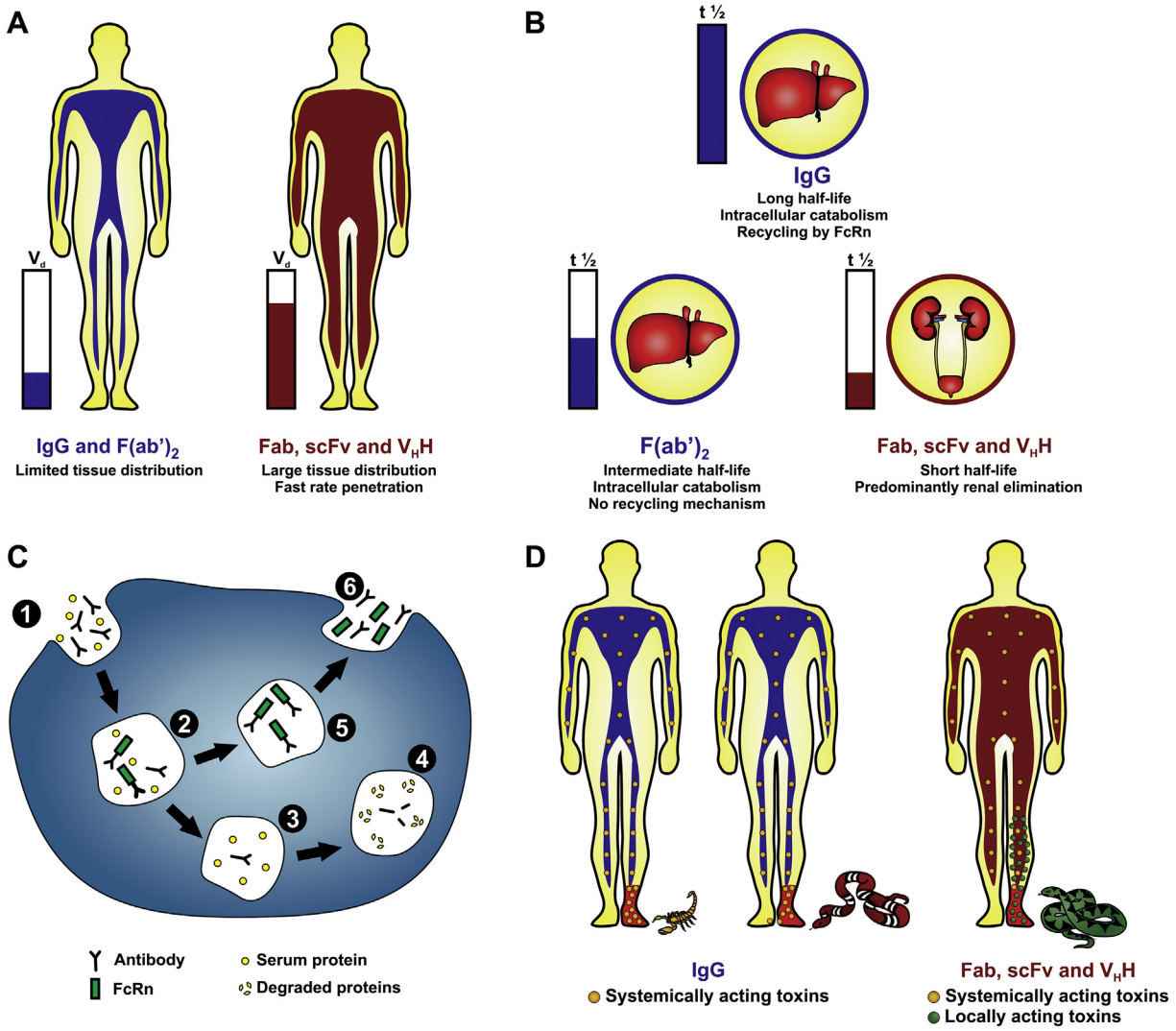

Fig. 7. Antivenom pharmacokinetics. **(A)** Distribution profiles for different antibody formats, showing their volume of distribution (V_d). **(B)** Elimination mechanism for different antibody formats, indicating their elimination half-life ($t_{1/2}$). **(C)** IgG recycling by FcRn receptor. 1. IgGs and plasma proteins are internalized in vesicles by endocytosis. 2. IgGs bind to FcRn receptors in the acidic endososome. 3. Non-FcRn bound proteins. 4. Proteins are degraded in the lysosome. 5. IgG-FcRn complexes are transported to the cell surface. 6. IgGs are dissociated from the FcRn receptors at physiological pH. **(D)** The influence of the antibody format on pharmacokinetics in relation to toxicokinetics. The distribution of the larger IgG antibody format is largely restricted to the intravascular compartment, where it is effective in neutralizing systemically acting toxins over a period of many days due to its long elimination half-life. Smaller antibody fragments may both neutralize toxins in circulation, toxins present in or around the bite wound, and toxins that have reached systemic targets in tissues, i.e. neuromuscular junctions, due to their larger volumes of distribution, which allow these smaller fragments to more effectively penetrate tissue compartments. However, antibody fragments have a shorter elimination half-life. Systemically acting toxins are represented by scorpion stings and elapid snakebites, whereas viper snakebites represent locally and systemically acting toxins, although all three types of bite/sting contain both locally and systemically acting toxins in their venom.

4. Propensity for adverse reactions of different antibody formats

Adverse reactions to animal plasma-derived antivenoms are relatively common, with 6–59% of patients experiencing early-onset reactions, depending on the particular antivenom being used. In rare cases, administration of animal plasma-derived antivenoms may result in severe life-threatening anaphylaxis (Schaeffer et al., 2012; Stone et al., 2013). Further, 5–23% of treated patients experience delayed-onset serum sickness (typically observed 1–2 weeks after exposure), with symptoms such as high fever, rash, urticaria, and arthralgia (LoVecchio et al., 2003). The propensity of an animal-derived antivenom to generate early and late adverse reactions depends on the microbiological and physicochemical quality of the product, its format (i.e. Fab, $F(ab')_2$, or IgG), and the total amount of protein injected in a treatment (León et al., 2013). A relatively low rate of early adverse reactions (5–7%) has been reported for a highly purified Fab antivenom in use in the USA, which includes an affinity chromatography purification step in its manufacture (Cannon et al., 2008; Farrar et al., 2012). In comparison, $F(ab')_2$ and IgG antivenoms of good physicochemical quality induce early adverse reactions in 13–26% of treated patients (see review by (León et al., 2013)). In these cases, the majority of such reactions are mild, including mostly cutaneous manifestations. In contrast, other antivenoms of poor physicochemical quality, or containing pyrogens, are known to induce a rate of adverse reactions as high as 80%, with some of these reactions being severe (León et al., 2013). Most early adverse reactions to antivenoms are *de novo* reactions, i.e. occurring in people who have not been previously sensitized with antivenoms and are, therefore, non-IgE-mediated. In fact, only a small proportion of early adverse reactions are IgE-mediated (León et al., 2013).

Administration of animal-derived antivenoms also induce late adverse reactions, a type III hypersensitivity phenomenon associated with serum sickness (León et al., 2013). This occurs approximately 1–2 weeks after antivenom infusion as a consequence of the generation of human antibodies against animal IgGs, and the consequent formation of antigen-antibody complexes, which exert effects in the microvasculature and the joints, causing arthralgia, fever, and urticaria (Gutiérrez et al., 2017a). The incidence of serum sickness after antivenom administration has not been analyzed in depth, although it seems to depend on the total load of foreign protein administered (LoVecchio et al., 2003) and on the format of the antivenom preparation. In particular, Fab antivenoms have been shown to induce a much lower incidence of serum sickness than IgG and F(ab')$_2$ antivenoms (Lavonas et al., 2013; León et al., 2013). A detailed account of the studies reporting incidences of adverse reactions to animal-derived antivenoms can be found in (see reviews by Descotes (2009) and Hansel et al. (2010)).

There are no antivenoms in clinical use that are comprised of monoclonal antibodies or of any type of recombinant product. Information on safety of other biotherapeutics based on monoclonal antibodies may instead be utilized to shine light on the potential challenges that recombinant antivenoms may face, when they become available in the future. Murine monoclonal antibodies have been shown to induce early and late adverse reactions in humans (see reviews by Descotes (2009) and Hansel et al. (2010)), owing to their heterologous nature, including anaphylactic reactions in a few cases and serum sickness. As a result, biotherapeutics based on murine antibodies are no longer put into development and enter clinical trials. The propensity to generate adverse reactions has, however, been greatly reduced by the generation of chimeric, humanized, and fully human monoclonal antibodies, although it is still possible to generate anti-idiotype antibodies with such products (Hansel et al., 2010). For example, a humanized monoclonal antibody against an integrin has been reported to induce early adverse reactions (urticaria) in 4% of patients (Ransohoff, 2007). Despite these observations, the introduction of humanized or fully human monoclonal antibodies in the development of new antivenoms is likely to greatly reduce the incidence of early and late adverse reactions, currently observed for animal plasma-derived antivenoms, owing to the greater compatibility of these products with the human immune system (Laustsen, n.d.). Likewise, the fact that antivenoms are usually used only once in a single individual further reduces the likelihood of development of adverse reactions. From a theoretical viewpoint, it is also probable that recombinant antivenom antibodies of low molecular mass formats, such as Fab, scFv, V_HH, diabodies, bivalent constructs, and other binding protein formats, will be less prone to induce adverse reactions than whole IgG preparations. However, this should be carefully balanced with other aspects such as PK profile and the possible role of the Fc part of the immunoglobulin in its biological action. Finally, optimization of antibody glycosylation to better resemble human patterns may lead to recombinant antivenom formats with even better compatibility with the human immune system. All these issues demand renewed research vis-à-vis the current upsurge in the development of recombinant antivenoms.

5. Formulation

Owing to the proteinaceous nature of antibodies, antivenoms face many of the generic issues commonly related to high-protein-concentration solutions. Antivenom antibodies are especially susceptible to degradation when exposed to heat, freezing, light, pH extremes, shear-stress, agitation, as well as to some metals and organic solvents (Lowe et al., 2011). Particularly heat stability is important for long term storage in tropical regions, where most envenomings occur (Gutiérrez et al., 2006; Warrell, 2007). Liquid antivenom should generally be stored at 2–8 °C, but this requirement is not always possible to fulfil in rural areas where the cold-chain is often interrupted or non-existent. When stored at room temperature, formation of turbidity over time is observed in liquid formulations, indicating physical instability and decrease in biological activity (Segura et al., 2009). To overcome this issue, many antivenom manufacturers lyophilize their antivenoms, although this adds to the cost of manufacture (Segura et al., 2009). As an example, two studies on EchiTAb-Plus-ICP antivenom used to treat snakebite victims in rural sub-Saharan Africa attempted to determine the optimal state for antivenom stability. These studies indicated that freeze-drying offered the best thermal stability of the antivenom compared to liquid formulation without stabilizer and liquid formulation stabilized with sorbitol (Herrera et al., 2017, 2014). Most of the current research efforts are, however, focused on finding a stable liquid formulation that can be stored at room temperature. As an example, Solano et al. (2012) described that an acetate buffered (pH 4.0) formulation stabilized antivenoms for at least six months at room temperature without the presence of a protective carbohydrate excipient (Solano et al., 2012).

Some antivenom formulation additives have been reported to have varying levels of effects depending on the combination of additive molecules used and on whether the additives are added to liquid or lyophilized formulations. In a study that compared the stabilizing effects of sorbitol, sucrose, and mannitol in lyophilized antivenom, Herrera et al. (2014) showed that antivenoms lyophilized with mannitol lost efficacy against the lethal effects of *B. asper* venom (Herrera et al., 2014). Furthermore, it was shown that a 5% (w:v) sucrose formulation exhibited the best stability, indicating that sucrose could perform better as a stabilizer than mannitol and sorbitol in lyophilized antivenoms. Of the additives used in antivenom formulation, the most commonly used are phenol, cresol, and sodium chloride (see Table 5). These additives stabilize and preserve the antivenom by preventing aggregation of IgGs and/or antibody fragments, by providing an isotonic solution, and by having antifungal and bacteriostatic effects (Rodrigues-Silva et al., 1999; Segura et al., 2009). Preventing aggregation for therapeutic antibodies is crucial, as aggregation may significantly contribute to their immunogenicity (Rosenberg, 2006; van Beers et al., 2010).

Other less conventional formulations explored at the experimental level focus on enhancing the neutralization ability through conjugation of protein nanoparticles and/or facilitating the administration through encapsulation. Renu et al. (2014) used soy protein nanoparticles conjugated to F(ab')$_2$ fragments to optimize the neutralizing effects of *Bungarus caeruleus* antivenom (Renu et al., 2014). They achieved to produce the smallest size of self-stabilized soy protein nanoparticle reported within antivenom research, which displayed improved neutralization capacity against toxins from *B. caeruleus* venom at a much lower concentration compared to the non-conjugated antivenom. The conjugated antivenom particles also showed enhanced thermal stability (Renu et al., 2014).

Certain formulations could allow for alternative routes of antivenom administration. These formulations are being explored to allow non-physicians to aid snakebite victims before the victim reaches a clinic or hospital. Currently, all antivenoms are administered by intravenous bolus injection and/or intravenous infusion (Ahmed et al., 2008). Compared to other common routes of administration (e.g. intramuscular route), intravenous injection offers the fastest route to maximum concentration of antivenom in

Table 5
Different formulations used for antivenoms.

Trade name	Format	Formulation	Additive molecule	Benefit of additive	References
Studies performed on antivenom formulation					
	Ovine Fab	Liquid	Acetate buffer	Buffer and stabilization[a]	(Al-Abdulla et al., 2003)
	Equine $F(ab')_2$	Liquid	Sorbitol, phenol	Buffer and preservation[b]	(Solano et al., 2012)
	Equine	Lyophilized	Sorbitol/Mannitol/ Sucrose	Stabilization	(Herrera et al., 2014)
	Equine $F(ab')_2$	Liquid	Alginate encapsulation	Oral delivery	(Bhattacharya et al., 2014)
	Equine IgG	Liquid	Phenol, sorbitol, sodium chloride	Preservation, protection against heat denaturation	(Segura et al., 2009)
	Equine	Liquid	Phenol/Sorbitol	Preservation, protection against heat denaturation	(Rodrigues-Silva et al., 1999)
	Equine IgG/Equine $F(ab')_2$	Liquid	Sorbitol	Protection against heat denaturation	(Rodrigues-Silva et al., 1997)
	Equine $F(ab')_2$	Liquid	Conjugated soy protein NP's	Improvement of venom neutralization efficiency	(Renu et al., 2014)
	mAb	Liquid	PBS	–	(Bugli et al., 2008)
EchiTAb + ICP	Equine IgG	Liquid/ Lyophilized	Sorbitol/sucrose	Stabilization, protection against heat denaturation	(Herrera et al., 2017)
Snake antivenoms currently on market with disclosed formulation					
ViperaTAb	Equine $F(ab')_2$	Liquid	Sodium acetate buffer	Buffer	("Product information. ViperaTAb.," n.d.)
Snake Antivenin (Polyvalent) I.P.	Equine	Liquid	Phenol	Preservation	("Product information. Snake Antivenin (Polyvalent) I.P.," n.d.)
Snake Venom Antiserum I.P.	Equine	Liquid	Cresol	Preservation	("Product information. Snake Venom Antiserum I.P.," n.d.)
Anavip	Equine $F(ab')_2$	Lyophilized	Sodium chloride, sucrose, glycine	Stabilization	("Product information. Anavip.," n.d.)
CroFab	Ovine Fab	Lyophilized	PBS	Buffer	(("Crofab Crotalidae Polyvalent Immune Fab Ovine," n.d.))
Suero Antiofidico polivalente	Equine $F(ab')_2$	Liquid	Phenol, sodium chloride	Preservation	("Suero Antiofidico polivalente. Centro de Biotechnologia Facultad de Farmacia.," n.d.)
Black Snake Antivenom	Equine $F(ab')_2$	Liquid	Phenol, sodium chloride	Preservation	("Product information. Black Snake Antivenom.," n.d.)
Brown Snake Antivenom	Equine $F(ab')_2$	Liquid	Phenol, sodium chloride	Preservation	("Product information. Brown Snake Antivenom.," n.d.)
Death Adder Antivenom	Equine $F(ab')_2$	Liquid	Phenol, sodium chloride	Preservation	("Product information. Death Adder Antivenom.," n.d.)
Polyvalent Snake Antivenom	Equine $F(ab')_2$	Liquid	Phenol, sodium chloride	Preservation	("Product information. Polyvalent Snake Antivenom.," n.d.)
Sea Snake Antivenom	Equine $F(ab')_2$	Liquid	Phenol, sodium chloride	Preservation	("Sea Snake Antivenom - Current Consumer Medicine information.," n.d.)
Taipan Antivenom	Equine $F(ab')_2$	Liquid	Phenol, sodium chloride	Preservation	("Taipan Antivenom - Curent Consumer Medicine information Nov 2017.," n.d.)
Tiger Snake Antivenom	Equine $F(ab')_2$	Liquid	Phenol, sodium chloride	Preservation	("Tiger Snake Antivenom - Current Consumer Medicine Information Oct 2016.," n.d.)
Soro Antielapídico (bivalente)	Equine $F(ab')_2$	Liquid	Phenol, sodium chloride	Preservation	(("Product information. Soro Antielapidico bivalente.," n.d.))
Soro Antibotrópico (pentavalente) e Antilaquético	Equine $F(ab')_2$	Liquid	Phenol, sodium chloride	Preservation	(("Product information. Soro Antibotropico pentavalente e antilaquetico.," n.d.))
Soro Anticrotálico	Equine $F(ab')_2$	Liquid	Phenol, sodium chloride	Preservation	("Product information. Soro Anticrotálico.," n.d.)
Soro Antibotrópico (pentavalente) e Anticrotálico	Equine $F(ab')_2$	Liquid	Phenol, sodium chloride	Preservation	(("Product information. Soro antibotropico pentavalente e anticrotalico.," n.d.))
Soro Antibotrópico (pentavalente)	Equine $F(ab')_2$	Liquid	Phenol	Preservation	("Product information. Soro Antibotropico (pentavalente).," n.d.)
Suero Antibotrópico polivalente	Equine IgG	Liquid	Phenol, thimerosal	Preservation	(Biológicos et al., n.d.)
Suero Anticrotálico monovalente	Equine IgG	Liquid	Phenol, thimerosal	Preservation	(Biológicos et al., n.d.)
Suero Antilachésico monovalente	Equine IgG	Liquid	Phenol, thimerosal	Preservation	(Biológicos et al., n.d.)
Suero Antiloxoscélico monovalente	Equine $F(ab')_2$	Liquid	Phenol, thimerosal	Preservation	(Biológicos et al., n.d.)
EchiTAbG antivenom	Ovine IgG	Liquid	PBS	Buffer	(Casewell et al., 2010)
EchiTAb-Plus-ICP	Equine IgG	Liquid	Phenol, sodium chloride	Preservation,	(Segura et al., 2010)

Table 5 (*continued*)

Trade name	Format	Formulation	Additive molecule	Benefit of additive	References
Banded Krait Antivenin	Equine IgG	Lyophilized	Glycine, phenol, sodium chloride	Preservation, stabilizer	("Product information. Banded Krait Antivenin.," n.d.)
Viper Venom Antitoxin	Equine IgG	Liquid	Phenol, sodium chloride	Preservation	("Product information. Viper Venom Antitoxin.," n.d.)
Snake Venom Antiserum	Equine $F(ab')_2$	Lyophilized	Cresol, glycine, sodium chloride	Preservation, stabilizer	("Premium Serums. Snake Venom Antiserum - Lyophilized.," n.d.)
Snake Venom Antiserum I.P	Equine $F(ab')_2$	Liquid	Cresol, glycine, sodium chloride	Preservation, stabilizer	(Premium Serums. Snake Venom Antiserum I.P., n.d.)
Snake Venom Antiserum (Central Africa)	Equine $F(ab')_2$	Lyophilized	Cresol, glycine, sodium chloride	Preservation, stabilizer	("Premium Serums. Snake Venom Antiserum (Central Africa).," n.d.)
Snake Venom Antiserum (Pan Africa)	Equine $F(ab')_2$	Lyophilized	Cresol, glycine, sodium chloride	Preservation, stabilizer	("Premium Serums. Snake Venom Antiserum (Pan Africa).," n.d.)
Snake Venom Antiserum (African − Ten)	Equine $F(ab')_2$	Lyophilized	Cresol, glycine, sodium chloride	Preservation, stabilizer	("Premium Serums. Snake Venom Antiserum (African - Ten).," n.d.)
Snake Venom Antiserum (North Africa)	Equine $F(ab')_2$	Lyophilized	Cresol, glycine, sodium chloride	Preservation, stabilizer	("Premium Serums. Snake Venom Antiserum (North Africa).," n.d.)
Scorpion antivenoms currently on market with disclosed information					
Scorpion Venom Antiserum (India)	Equine $F(ab')_2$	Lyophilized	Cresol, glycine, sodium chloride	Preservation, stabilizer	("Premium Serums. Scorpion Venom Antiserum (India).," n.d.)
Scorpion Venom Antiserum (North Africa)	Equine $F(ab')_2$	Lyophilized	Cresol, glycine, sodium chloride	Preservation, stabilizer	("Premium Serums. Scorpion Venom Antiserum (North Africa).," n.d.)
Suero antiescorpiónico	Equine $F(ab')_2$	Liquid	Phenol, sodium chloride	Preservation, stabilization	("Suero antiescorpiónico. Centro de Biotechnologia Facultad de Farmacia.," n.d.)
Alacramyn	Equine Fab	Lyophilized	Cresol	Preservation	("Instructions for use. Alacramyn®.," n.d.)
Soro antiarachnidico	Equine $F(ab')_2$	Liquid	Phenol	Preservation	("Package leaflet: Soro antiarachnidico - Butantan.," n.d.)
Soro antiescorpiônico	Equine $F(ab')_2$	Liquid	Phenol	Preservation	("Package leaflet: Soro antiescorpionico - Butantan.," n.d.)
Polyvalent Scorpion Antivenom	Equine $F(ab')_2$	Liquid	Cresol, PBS	Preservation	("Polyvalent Scorpion Antivenom | National Antivenom and Vaccine Production Center.," n.d.)
Scorpifav	Equine $F(ab')_2$	Liquid	Sodium chloride, polysorbate 80	Preservation	("MAVIN Poison Centre Munich - Scorpifav.," n.d.)
Scorpion Venom Antiserum	Equine IgG	Lyophilized	Ortho-cresol	Preservation	("Scorpion Anti Serum | VINS BioProducts Limited," n.d.)
Soro Antiescorpiônico (FUNED)	Equine $F(ab')_2$	Liquid	Phenol, sodium chloride	Preservation	("Product information. Soro Antiescorpiônico (FUNED).," n.d.)
Anti-scorpion Venom Serum	Equine IgG	Lyophilized	Phenol	Preservation	("Antitoxins & Sera: Antiscorpion venom serum," n.d.)
Spider antivenoms currently on market with disclosed information					
Funnel Web Spider Antivenom	Leporid IgG	Lyophilized	Glycine, sodium chloride, sodium phosphate − dibasic, sodium phosphate - monobasic	Preservation, stabilization	("Funnel Web Spider Antivenom - Current Cunsumer Medicine Information Jan 2017.," n.d.)
Red Back Spider Antivenom	Equine IgG	Liquid	Phenol, sodium chloride	Preservation	("Red Back Spider Antivenom - Current Consumer Medicine Information Febr, 2017.," n.d.)
Aracmyn PLUS	Equine $F(ab')_2$	Lyophilized	Cresol	Preservation	("Aracmyn Plus - Bioclon | PR Vademecum Mexico," n.d.)
Reclusmyn	Equine $F(ab')_2$	Lyophilized	Cresol	Preservation	("Our Products - Reclusmyn.," n.d.)
Soro antiarachnidico	Equine $F(ab')_2$	Liquid	Phenol	Preservation	("Package leaflet: Soro antiarachnidico - Butantan.," n.d.)
Suero antiloxoscélico monovalente	Equine IgG	Liquid	Thimerosal, phenol	Preservation	("Detalle del Producto. Suero antiloxoscélico monovalente.," n.d.)
Soro Antilatrodéctico	Equine $F(ab')_2$	Liquid	Phenol, sodium chloride	Preservation	("Instituto Vital Brazil - Soro Antilatrodéctico.," n.d.)
Antivenin (*Latrodectus mactans*)	Equine IgG	Lyophilized	Thimerosal	Preservation	("Antivenin (Latrodectus mactans).," n.d.)

[a] Stabilization implies benefits that prevent aggregation of IgGs and/or IgG fragments.
[b] Preservation implies antifungal and bacteriostatic benefits.

the circulatory system (Gutiérrez et al., 2003), although rapid infusion of foreign antivenom proteins may result in adverse reactions often experienced by patients upon antivenom administration (León et al., 2013). An approach to minimize the adverse effects of antivenom, that has only been explored once experimentally, involves oral administration of alginate encapsulated antivenom (Bhattacharya et al., 2014). However, even if antibodies can be properly formulated for oral administration, oral delivery of an emergency medicine will come at a cost to bioavailability and the delayed arrival of antibodies may not be optimal for efficient toxin neutralization. Thus, even if such formulations may one day be useful in the field, they will have to be supplemented with intravenously administered antivenom once the snakebite victim reaches a clinic or hospital.

In conclusion, it is observed that the majority of antivenoms currently on the market are formulated with one or more of the excipients phenol, cresol, sodium chloride, glycine in some products and, in the case of freeze-dried antivenoms, sucrose. Most of the available data on antivenom formulation is based on plasma-derived equine or ovine polyclonal F(ab')$_2$s, possibly due to the early stage of development for recombinant antivenoms based on monoclonal antibodies. It seems likely that antivenom research will increasingly focus on more modern approaches involving the use of recombinant human antibodies (Laustsen, n. d.; Laustsen et al., 2017). With such a shift, more research is needed in order to develop and optimize formulations of mixtures of monoclonal antibodies. These future efforts will fortunately not start from scratch. In other fields, (mixtures of) human monoclonal antibodies have been extensively used, and existing formulation solutions from these fields are likely to also be applicable for recombinant antivenoms (Heijtink et al., 1999; Robak et al., 2012).

6. Expression of different antibody formats

To enable large-scale production of novel antivenoms consisting of recombinant antibodies or antibody fragments, a suitable expression system is essential. To the best of our knowledge, so far, no monoclonal antibody nor antibody fragment targeting an animal toxin has been produced in larger scale. Several different research efforts have, however, employed different expression hosts, which will be reviewed in the following for their suitability for Research and Development (R&D) purposes and scale up.

6.1. Key differences between eukaryotes and prokaryotes in antibody expression

Antibodies and antibody fragments can be expressed in either prokaryotic or eukaryotic cells, depending on the structure of the protein product and the application of the desired antibody fragment. These cell types are inherently different and thus offer different advantages and disadvantages in relation to antibody expression (Berlec and Strukelj, 2013).

Advantages of prokaryotic expression of antibodies include low cost of media and ease of handling. For these reasons, *E. coli* has been a much-used organism for expression of several different antibody formats within antivenom research. However, the inability of prokaryotes to glycosylate antibodies limits the range of antibody formats that can be expressed with these systems. Therefore, *E. coli* has mainly been used to produce diabodies, scFvs, Fabs, and V_HHs (see Table 6). Furthermore, the tendency to form incorrectly folded proteins and insoluble aggregates in the reducing environment of the bacterial cytoplasm decreases expression yields. Other prokaryotes that are more promising than *E. coli* for production of biotherapeutics could be strains of the genus *Bacillus*, which have a long track record of successful use for expression of both heterologous and homologous proteins (Lakowitz et al., 2017). These have, however, not yet been employed within the field of antivenom.

In contrast to prokaryotic cells, mammalian cells are capable of performing more advanced post-translational modifications, such as glycosylation, and possess more complex cellular machinery for folding and secretion (Chadd and Chamow, 2001; Frenzel et al., 2013). Mammalian cells are capable of yielding more diverse antibody formats with lower immunogenicity (Chadd and Chamow, 2001; Frenzel et al., 2013) and are the primary production system for full IgG molecules (Walsh, 2014). Also, mammalian cells typically deliver close to 100% fully functional proteins, in contrast to prokaryotic expression systems, where the yield of active protein may be significantly lower than the overall protein yield. However, drawbacks for mammalian cells include high cost of media and consumables, difficulty in handling, and (arguably) slow growth rate. Productivity has, however, been increased significantly in recent decades by optimization of protein expression levels for many of the mammalian cell lines employed in industrial processes, which compensates for the slower growth of mammalian cells compared to prokaryotes. Previously, pathogenic contaminations of cell cultures also posed a threat, but modern protocols for avoiding such contaminations limit this issue (Frenzel et al., 2013).

6.2. scFvs are typically expressed in E. coli

The use of *E. coli* as an expression host appears to be the most commonly used system within antivenom research, not only for scFvs, but also for other antibody fragments (see Table 6). In 1999, Mousli et al. expressed an scFv in *E. coli*. capable of neutralizing the AahII toxin of the desert scorpion, *Androctonus australis hector* (Mousli et al., 1999). More recently, scFv expression in *E. coli* cells has been optimized, leading to improved expression yields. As an example, signal peptides that localize antibody fragments to the oxidative environment of the periplasm are often added to the expression plasmid (Amaro et al., 2011; Juárez-González et al., 2005; Juste et al., 2007; Pucca et al., 2012; Roncolato et al., 2013). The oxidative environment allows for the formation of disulphide bond, which is normally unattainable in the reducing cytoplasmic environment of *E. coli*, wherein expression tends to lead to non-functional aggregates. Research groups outside of the field of antivenom have attempted different strategies as alternatives to localizing antibodies to the periplasm to achieve a higher degree of correct folding. These strategies include: (i) denaturation and refolding of cytoplasmic, aggregated antibodies, (ii) increased expression of cytoplasmic chaperones in addition to altering the cytoplasmic environment by creating mutations in reductases, (iii) creating cysteine-free antibodies, and (iv) cytoplasmic oxidase expression (Frenzel et al., 2013; Gaciarz et al., 2016; Veggiani and De Marco, 2011). These methods have been employed with varying degrees of success. Denaturation and refolding does often not prove efficient, whereas increasing the expression of chaperones and cytoplasmic oxidases have successfully increased yields for Fab and V_HH fragments, respectively (Frenzel et al., 2013; Gaciarz et al., 2016).

Engineering of expression vectors, such as optimization of codons, promotor, Shine-Dalgarno sequence, leader sequence, and transcript stability, can further improve scFv expression in *E. coli* (Frenzel et al., 2013). Furthermore, cultivation of *E. coli* in bioreactors instead of shake flasks has in some cases significantly increased scFv yields. As an example of shake flask cultivation, Kipriyanov et al. obtained a yield of 16.5 mg/L for an scFv against the T cell surface antigen CD3 by expression in *E. coli* cultivated in

Table 6
Expression of antibody formats targeting spider, scorpion, and snake toxins.

Format	Origin	Expression system	Yield	Note	Reference
Diabody mixture	Human	*E. coli* (strain: TG1)	1.5 mg/L & 2.4 mg/L		(Rodríguez-Rodríguez et al., 2012)
	Murine	*E. coli* (strain: HB2151)			(di Tommaso et al., 2012)
			0.5 − 0-8 mg/L		(Aubrey et al., 2003)
Nb/V_HH	Camelid	*E. coli* (strain: WK6)			(Hmila et al., 2012)
	Camelid (dromedary)	*E. coli* (strain: WK6)	3.75 mg/L		(Hmila et al., 2010)
			3 mg/L		(Hmila et al., 2008)
			1:15000		(Abderrazek et al., 2009)
	Camelid (camel)	*E. coli* (strain: BL21 (DE3))		Humanized	(Chavanayarn et al., 2012)
	Camelid (llama)	*E. coli* (strain: BL21 (DE3))		Pentamerised	(Stewart et al., 2007)
		E. coli (strain: HB2151)	12-18 mg/L. Titre: 3.0×10^5	VhH2-Fc	(Richard et al., 2013)
scFv	Human	*E. coli* (strains: TG1 and HB2151 *E. coli* non-suppressor lineages [K12, ara, D(lac-pro), thi/F'proA þ Bþ, laclqZDM15])	1.24×10^{16}		(Pessenda et al., 2016)
		E. coli (strain: HB2151)	3.27×10^8 CFU/mL		(Tamarozzi et al., 2006)
			0.4–0.6 mg/L		(Roncolato et al., 2013; Tamarozzi et al., 2006)
					(Pucca et al., 2014, 2012)
			1.3×10^7 CFU/mL		(Oliveira et al., 2009)
		E. coli (strain: TG1)			(Riaño-Umbarila et al., 2016)
			1.5 mg/L		(Riaño-Umbarila et al., 2013)
			1.0–2.4 mg/L		(Riaño-Umbarila et al., 2011)
			0.7 mg/L		(Riaño-Umbarila et al., 2005)
					(Rodríguez-Rodríguez et al., 2016)
					(Cardoso et al., 2000)
			1.0 mg/L		(Amaro et al., 2011)
		E. coli (strains: BL21 (DE3) & HB2151)			(Danpaiboon et al., 2014; Kulkeaw et al., 2009)
	Murine	*E. coli* (strain: C43 (DE3))	280 μg/L		(Castro et al., 2014)
		E. coli (strain: TG1)	0.3–1.0 mg/L		(Juárez-González et al., 2005)
					(Devaux et al., 2001b)
		E. coli (strain: BL21 (DE3))	1 mg/mL after purification		(Meng et al., 1995)
		E. coli (strain: HB2151)			(Mousli et al., 1999)
		E. coli (strain: HB2151 [K12, ara, Δ(lac-pro), thi/F′ proA+ B+, laclq lacZΔM15])	0.1 mg/L	Tandem scFv	(Juste et al., 2007)
		E. coli (strain: W3110)			(Mérienne et al., 1997)
Fab	Murine	*E. coli* (strains: XL1-Blue, BL21(DE3)pLysS, and Rosetta 2(DE3)pLysS)	0.5–1.5 mg/L		(Bugli et al., 2008)
		E. coli (strain: TOPP2)	1 mg/L		(Selisko et al., 2004)
		E. coli (strain: HB2151)	0.02 mg/L	Recombinant	(Aubrey et al., 2004)
IgG	Equine, murine	Hybridoma cells (*in vivo* hybridoma cultivation/ascite)			(Alvarenga et al., 2003)
	Human	Hybridoma cells (*in vitro* hybridoma cultivation)	1:4000		(Morine et al., 2008)
	Murine	Hybridoma cells (*in vivo* hybridoma cultivation/ascite)			(Perez et al., 1984)
					(Clot-Faybesse et al., 1999)
					(Frauches et al., 2013)
					(Li et al., 1993)
					(Alvarenga et al., 2005)
		Hybridoma cells (*in vitro* hybridoma cultivation)			(Jia et al., 2000)
		Hybridoma cells			(Charpentier et al., 1990)
IgG1	Murine	Hybdridoma cells (*in vivo* hybridoma cultivation/ascite)		Licea et al. describe a Fab fragment derived from the IgG originally produced by Zamudio et al.	(Licea et al., 1996; Zamudio et al., 1992)
					(Masathien et al., 1994, p. 3, p. 3)
			Titres: $1:10^5$–$1:10^6$		(Lomonte et al., 1992; Lomonte and Kahan, 1988)
			10.8 mg/mL		(Iddon et al., 1988)
		Hybridoma cells (*in vitro* hybridoma cultivation)			(Schneider et al., 2014)
			1/1024 for whole venom		(Fernandes et al., 2010)
			The greatest dilutions were of the order 10^4-10^5		(Tanjoni et al., 2003a, 2003b)
					(Bahraoui et al., 1988)

(*continued on next page*)

Table 6 (*continued*)

Format	Origin	Expression system	Yield	Note	Reference
IgG2	Murine	Hybridoma cells (*in vitro* hybridoma cultivation)	1/1024 for whole venom		(Fernandes et al., 2010)
IgG2a	Murine	Hybridoma cells (*in vivo* hybridoma cultivation/ascite)			(Stiles et al., 1994) (Trémeau et al., 1986)
IgG2b	Murine	Hybridoma cells (*in vivo* hybridoma cultivation/ascite)			(Stiles et al., 1994) (Masathien et al., 1994, p. 3, p. 3)
IgM	Murine	Hybridoma cells (*in vitro* hybridoma cultivation)	1/1024 for whole venom		(Fernandes et al., 2010)
		Hybdridoma cells (*in vivo* hybridoma cultivation/ascite)			(Masathien et al., 1994, p. 3, p. 3)
Ig	Murine	Hybridoma cells (*in vivo* hybridoma cultivation/ascite)	2 mg/mouse		(Boulain et al., 1982)
		Hybridoma cells (*in vitro* hybridoma cultivation)			(Dias-Lopes et al., 2014)

shake flasks after optimization (Kipriyanov et al., 1997). By comparison, Sletta et al. obtained a much higher yield of 1.2 g/L for the same scFv after optimization by using bioreactor production (Kipriyanov et al., 1997; Sletta et al., 2004). Nevertheless, bioreactor production may not generally be superior to shake flask production, and examples of high scFv expression yields when using shake flasks also exist. For instance, Gaciarz and colleagues were able to obtain yields of 240 mg/L for an scFv by shake flask cultivation of *E. coli* (Gaciarz et al., 2016).

6.3. Fabs are typically expressed in E. coli

Within antivenom research, Fabs have primarily been produced in *E. coli* strains (Table 6). Many of these strains have been engineered to circumvent problems inherent to expression of mammalian proteins in prokaryotic cells. As an example, *E. coli* strains have been modified to compensate for the limited availability of tRNAs corresponding to codons infrequently used in prokaryotes, but frequently used in eukaryotes. Bugli et al. tested such an *E. coli* strain and found that increasing the intracellular availability of tRNAs with anticodons for AGG, AGA, AUA, CUA, CCC, and GGA also increased yields of their Fab directed against alpha-latrotoxin from the venom of *L. tredecimguttatus* (Mediterranean black widow) from 0.5 mg/L to 1.5 mg/L (Bugli et al., 2008).

Optimization of growth media and additives, timing and duration of induction, concentration of reactants used for induction, and other parameters may dramatically increase antibody expression yields (Kipriyanov et al., 1997; Selisko et al., 2004; Ukkonen et al., 2013). Although still in the lower range of yields, this is demonstrated by a study of a Fab capable of neutralizing whole venom antigens of the *C. noxius* scorpion, in which Fab yields were increased by a factor of 20 (from 0.05 mg/L to 1 mg/L) through optimization of addition of sucrose to the medium, temperature and timing of induction, and concentration of the induction agent (Selisko et al., 2004). In the same study, Selisko and colleagues also found that lowering the temperature of induction in their case had a profound positive impact on the yield of biologically active protein, as this reduced the number of insoluble, cytoplasmic aggregates (Selisko et al., 2004). Conversely, however, Aubrey et al. found that inducing expression at low temperatures resulted in extensive cytoplasmic aggregation and low Fab yields (Aubrey et al., 2004). This demonstrates that the temperature of induction is of paramount importance for correct folding, but that the optimal temperature may be different from case to case.

Similar to scFvs, Fabs are often localized to the periplasm to promote disulphide bond formation and ameliorate aggregations (Aubrey et al., 2004; Bugli et al., 2008; Selisko et al., 2004). An alternative solution to periplasmic expression from outside the field of antivenom is introduction of enzymes (e.g. protein disulphide isomerase) facilitating disulphide bond formation in the cytoplasm, as used by Gaciarz and colleagues for Fab expression (Gaciarz et al., 2016). Thus, it is important to consider in which cellular space the Fab fragment should be localized to achieve the highest possible yield.

6.4. Diabodies and V_HHs are expressed in E. coli

E. coli is also a widely employed expression host for diabodies (Aubrey et al., 2003; di Tommaso et al., 2012; Rodríguez-Rodríguez et al., 2012) and V_HHs (Abderrazek et al., 2009; Chavanayarn et al., 2012; Hmila et al., 2012, 2010, 2008; Richard et al., 2013; Stewart et al., 2007), which similarly to scFvs and Fabs are often targeted to the periplasm to promote disulphide bond formation and proper folding (Abderrazek et al., 2009; Aubrey et al., 2003; di Tommaso et al., 2012; Hmila et al., 2010, 2008; Richard et al., 2013). Diabodies and V_HHs have been developed against toxins from snakes and, to a slightly greater extent, scorpions, whereas to the best of our knowledge, no diabodies nor V_HHs have been directed towards spider toxins. Specifically, three V_HH studies all focused on two different *N. kaouthia* (cobra) toxins (Chavanayarn et al., 2012; Richard et al., 2013; Stewart et al., 2007), while one study describes diabodies directed against *C. noxius* (scorpion) venom antigens (Rodríguez-Rodríguez et al., 2012), and six studies (two diabody studies and four V_HH studies) were all concerned with antibodies directed against AahI and AahII toxins from *A. australis hector* (scorpion) venom (Abderrazek et al., 2009; Aubrey et al., 2003; di Tommaso et al., 2012; Hmila et al., 2012, 2010, 2008).

6.5. IgGs are expressed in mammalian hybridoma cell lines within antivenom R&D

Although aglycosylated IgGs have been produced in *E. coli* cells (Frenzel et al., 2013), a much more commonly employed expression organism for IgGs for research use is hybridoma cells. Hybridomas are generated by fusion of antibody-producing, mammalian, B lymphocytes (typically murine cells) from immunized animals and an immortalized cell line of choice. Hybridomas thus present advantages and disadvantages, making them suited for R&D purposes, but less suited for large-scale production. As their most relevant feature, they are immortalized and capable of antibody production. Antibody expression in hybridoma cells has been extensively used within the field of antivenoms, as illustrated by Table 6, especially for the IgG format, partially due to the difficulty of expressing functional versions of the IgG format in prokaryotes. In 2008, Morine and colleagues produced two IgGs capable of neutralizing both the haemorrhagic and proteolytic activities of the

snake venom metalloproteinase Hr1a (Morine et al., 2008). These IgGs were produced by hybridomas cultivated *in vitro* and harvested from the culture supernatant (Morine et al., 2008). Others have followed similar procedures for expression of toxin-neutralizing IgGs (Bahraoui et al., 1988; Jia et al., 2000). Another approach entails *in vivo* production and harvest of IgGs from ascitic fluids (Alvarenga et al., 2005, 2003; Boulain et al., 1982; Clot-Faybesse et al., 1999; Frauches et al., 2013; Licea et al., 1996; Li et al., 1993; Lomonte et al., 1992; Lomonte and Kahan, 1988; Masathien et al., 1994; Perez et al., 1984; Stiles et al., 1994; Trémeau et al., 1986; Zamudio et al., 1992). Several reasons for favouring this approach exist for research purposes. Some hybridoma cell lines do not grow well *in vitro*, and purification of IgAs, IgMs, and IgG3s from *in vitro* cultures may result in denaturation and consequent loss of activity (Ward et al., 1999). Thus, if high antibody concentrations and activity levels are needed for preliminary studies and a small degree of impurity is permissible, growing hybridomas inside the peritoneal cavity of mice may be preferable to cultivation in conventional medium for research application (Ward et al., 1999). Hybridomas cultured *in vitro* have in some cases been shown to produce alternatively glycosylated IgGs relative to those produced by hybridomas *in vivo,* affecting their antigen-binding capacities (Ward et al., 1999). Thus, it may be important to investigate glycosylation patterns when going from *in vitro* to *in vivo*.

Although hybridomas have historically been used extensively for expression of antibodies within many fields, these cell lines have several restraints for upscaling. These restraints include poorly defined nutrient needs of these cell types, accumulation of toxic metabolites, high oxygen demand, and fragility of the cells (Randerson, 1985). The problem of chromosomal instability is also inherent to long-term expression in many cell lines, such as hybridomas, non-secreting murine myeloma (NS0) cells, and human embryonic kidney (HEK) cells, and overgrowth by nonproducing cells constitutes another potential problem (Randerson, 1985).

6.6. Organisms well suited for large-scale production of antibodies and antibody fragments

Antibodies and antibody fragments are the fastest growing class of biopharmaceuticals (Pucca et al., 2011a,b). Most of the organisms described above are suited for R&D purposes, but have their limitations when it comes to large-scale production. These limitations include the propensity for producing endotoxins and the restricted number of formats that can be produced in *E. coli,* and the low cost-efficiency and difficulty of upscaling for hybridoma cell lines.

From a quantitative perspective, microbial cell lines, and *E. coli* lines in particular, are responsible for the production of the majority of approved biotherapeutics (Walsh, 2014). However, they are not responsible for the production of the majority of approved therapeutic antibodies, which may be due to the inability of microbial cell lines to provide correct human glycosylation of antibodies (Ecker et al., 2015; Walsh, 2014). Furthermore, microbial cell lines often attain low yields due to incorrect folding and formation of aggregates (Chadd and Chamow, 2001). Another disadvantage of *E. coli* and other gram-negative bacteria is that they produce endotoxins, which may compromise safety, if they are not properly removed. While efforts have been made to produce endotoxin-free *E. coli* strains for recombinant protein production (Mamat et al., 2015), no antibodies produced in *E. coli* have been approved by the Food and Drug Administration (FDA) or European Medicines Agency (EMA) since 2009 and 2008, respectively (ACTIP, 2017).

For production of therapeutic antibodies, mammalian cell lines are often chosen as the expression organism (Berlec and Strukelj, 2013; Wurm, 2004). Mammalian cell lines were responsible for production of 95% of approved therapeutic antibodies in 2013 (Jäger et al., 2013) and for the production of 29 out of 30 (96.7%) approved therapeutic antibodies in 2014 (Walsh, 2014). By comparison, *E. coli* was only responsible for the production of one of these antibodies in 2014 (Walsh, 2014). One of the popular mammalian expression hosts for therapeutic proteins is the Chinese Hamster Ovary (CHO) cell. In 2014, CHO cells alone were responsible for the production of 35.5% of all approved biotherapeutics (Walsh, 2014). Although CHO cells are the most commonly used mammalian cell lines for IgG production, other cell lines (e.g. NS0, HEK, and hybridoma lines) are also used (Chadd and Chamow, 2001; Frenzel et al., 2013). Fig. 8 shows a schematic representation of mammalian (CHO) cell production of IgGs.

Finally, antibodies have also been expressed in other gram-negative bacteria (in addition to *E. coli*), gram-positive bacteria, various yeast strains, fungi, protozoa, insect cells, additional mammalian cell lines, transgenic plants, and even transgenic animals (Chadd and Chamow, 2001; Frenzel et al., 2013). Recently, a recombinant antivenom made in transgenic plants expressing various camelid antibodies against toxins of the venom of *Bothrops asper* was described (Julve Parreño et al., 2017). Several of the aforementioned production hosts are in use for large-scale production of biotherapeutics (Walsh, 2014), while others are still in the process of procedure optimization for future large-scale production. Given their regulatory success and the efforts put into strain development and genetic engineering in other fields, it seems likely, though, that the CHO cell will be the main expression organism for antibodies in most therapeutic areas – particularly full IgGs.

6.7. Practical considerations for production of recombinant antivenoms

In addition to production cost, factors to consider when choosing a manufacturing strategy for (mixtures of) antibodies and antibody fragments for recombinant antivenoms, include i) the therapeutic benefits of the specific antibody format (different formats have different PK-PD and are suitable for different purposes), ii) the importance of (proper) glycosylation, iii) ease of purification,

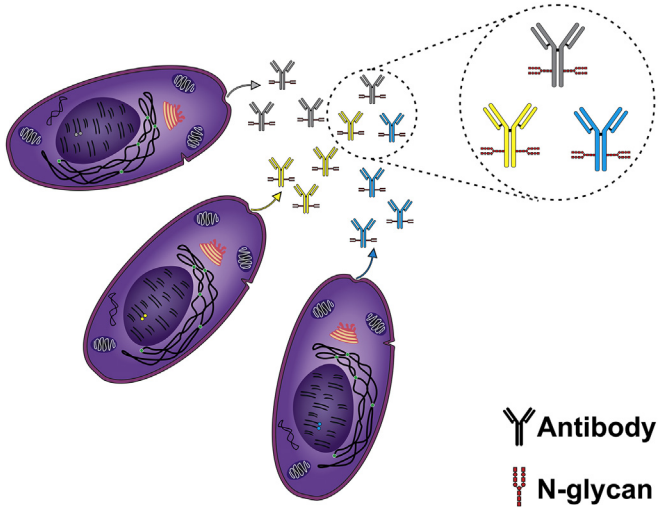

Fig. 8. Schematic representation of three different CHO cells expressing three different glycosylated IgGs. The mammalian cell line contains the necessary cellular components to produce properly folded and glycosylated IgGs. It has been proposed that co-culturing such cell lines could be used for the production of recombinant antivenom based on oligoclonal mixtures of (human) IgGs (Laustsen et al., 2017).

iv) history of regulatory approval, and v) availability of genetic tools for development of production strains, such as CRISPR/Cas systems (clustered regularly interspaced short palindromic repeats). Considering these factors, CHO cells or other mammalian cells may possibly be the best choice for large-scale production of recombinant antivenoms based on more complex antibody formats, such as IgG (Walsh, 2014; Wright and Morrison, 1997). In regards to cost of treatment, it has been suggested that using CHO cells for oligoclonal expression of mixtures of recombinant human IgGs could provide an entire treatment against a typical snakebite envenoming for as little as USD 30–350 (Laustsen et al., 2017, 2016b). This compares favourably with prices described by Harrison et al., who report a current market price of an antivenom vial in Kenya ranging from USD 47.9 to USD 315 (depending on the product), considering that the treatment of a snakebite case usually requires several vials (Harrison et al., 2017).

7. Targeting toxins of different toxicokinetic profiles and sites of action

Animal venoms contain cocktails of toxins with a wide range of biological activities and variable toxicokinetic profiles. Some toxins, like elapid and scorpion neurotoxins, are low molecular mass proteins with a large volume of distribution, which allows them to rapidly reach systemic distribution and access extravascular targets in the peripheral nervous system (Fig.7D) . Other toxins, such as high molecular mass metalloproteinases and serine proteinases, have a lower volume of distribution, and many of them act systemically within the vasculature, generating hemorrhage and coagulation disorders. Still, some toxins, particularly PLA_2s and metalloproteinases, generate local tissue damage at the site of injection before reaching a systemic distribution. Other venomous animals that cause local tissue damage include brown spiders (*Loxosceles* spp.), whose venom can induce dermonecrotic lesions, although systemic manifestations are also observed, including acute kidney injury (Chaim et al., 2006). Thus, these different toxicokinetic scenarios and the consequent profile of toxicity associated with the various types of toxins demand a detailed consideration when designing the most effective antibody format for neutralization. Locally acting toxins are possibly better neutralized by Fabs, scFvs, or V_HHs, as these fragments better reach and neutralize toxins in deep tissue compartments compared to IgGs (Fig. 7D), which largely remain within blood vessels. Unfortunately, biodistribution studies involving these fragments and their use as antivenoms are scarce. However, other studies involving anti-tumor antibodies have already demonstrated their rapid and efficient tissue penetration, in which scFvs exhibited fast and high penetration in the tumor mass, while Fabs demonstrated intermediate tissue penetration in comparison to IgGs (Yokota et al., 1992). In contrast, an *in vivo* study using mice envenomed with *B. asper* venom, demonstrated that IgG and $F(ab')_2$ were in fact capable of reaching muscle tissue, although the researchers pointed out that the observed antibody accumulation could be a result of venom-induced microvascular alterations, which could increase the antibodies extravasation (León et al., 2001). Interestingly, no differences in the ability to neutralize local tissue damage between IgG, $F(ab')_2$, and Fab antivenoms were observed, probably owing to the effects of tissue damage on antivenom PK (León et al., 2000, 1997). Thus, antivenom PK is affected by the pathological changes induced by venoms in the tissues, and this must be considered when discussing the best antibody format for a given type of envenoming.

Systemically acting toxins are known to induce systemic toxic effects, including neuromuscular blockade, bleeding, coagulopathies, acute kidney injury, and cardiovascular shock, among others (Gutiérrez et al., 2017a). Neurotoxins represent a relevant example, since they need to reach extravascular targets in the peripheral nervous system to exert their actions. Venoms from scorpions, spiders and elapid snakes are rich sources of such neurotoxins (Del Brutto, 2013; Escoubas et al., 2000; Kini and Doley, 2010; Laustsen et al., 2016a, 2016c). The best antibody format to treat systemically acting toxins may be one that enables rapid diffusion to the tissues to bind and neutralize toxins that have reached systemic tissue targets (see section 3). On the other hand, the long half-life of the IgG format provides prolonged protection from toxins remaining in the circulatory system, such as high molecular mass metalloproteinases and serine proteinases, or toxins escaping the bite site at late stages of envenoming, which is beneficial in cases where toxins leak from the bite wound over the course of days. In these circumstances, the prolonged half-life of IgGs ensures that toxins remaining in the circulatory system or getting access to the circulatory system at later time periods would be bound and neutralized. Thus, the optimal antibody format has to be analyzed on a case by case basis, and it is likely that formulations that combine high and low molecular mass formats may be the optimal solution in many cases (Gutiérrez et al., 2003).

Toxin neutralization has generally been considered to take place when a toxin is bound by the variable region of an antibody. Therefore, antivenoms used in passive immunotherapy are frequently prepared using Fab/$F(ab')_2$ formats to limit immunogenicity and the risk of serum sickness. However, with the possibility of using monoclonal human antibodies, the Fc region has gained renewed interest (Laustsen et al., 2017; Richard et al., 2013), as it dramatically increases antibody plasma half-life. The attached Fc domain also enables the interaction with Fc-receptors found on immune cells, a feature that is particularly important for clearance mechanisms. Additionally, from a biophysical perspective, the Fc domain folds independently and can improve the solubility and stability of the antibody molecule (Kontermann, 2011; Nimmerjahn and Ravetch, 2008). Use of the human Fc domain of novel monoclonal toxin-targeting antibodies thus deserves further investigation – particularly for targeting systemically acting toxins.

8. Conclusions and predictions

With the renewed focus on snakebite as a neglected tropical disease by the WHO (Gutiérrez et al., 2017a), a hope emerges that research efforts within novel envenoming therapies will be intensified. This may not only contribute to the development of a new generation of antivenoms for treating envenomed snakebite victims, but it may also pave the way for novel antivenoms against envenomings by other animals. In the field of antivenom, antibody technologies have been introduced several decades ago, although with very limited efforts compared to the fields of oncology, autoimmune diseases, and infectious diseases. Despite its nascent state, research within monoclonal antibodies against animal toxins is thus well-positioned to harness the developments from these other fields that have made major progress in antibody discovery technologies, antibody engineering approaches, and antibody manufacturing.

Based on what is known from the field of antivenom research itself and general knowledge on monoclonal antibodies, it seems likely that different antibody formats may be applicable for different types of envenomings. An urgent need exists for targeting locally acting toxins with better efficacy within snakebite envenomings (Gutiérrez et al., 2017a). However, improvements in monoclonal human IgG discovery and development also open a door for improved therapies targeting systemically acting toxins.

Generally, a trend in antivenom research seems to present itself as a move away from the use of immunization, hybridoma technology, and murine antibodies towards phage display technology and human and camelid antibodies instead (Laustsen, n. d.; Roncolato et al., 2015). One possible prediction may be that combinatorial approaches merging (novel) immunization techniques and phage display may be introduced into the field of antivenom R&D, as transgenic animals engineered to contain the human antibody repertoire become more widely available to academia. This would allow researchers to obtain human antibody mRNA from immunized transgenic animals and use this mRNA to construct affinity matured fully human antibody phage display libraries. In turn, such libraries could be employed in a high-throughput fashion for discovery of a multitude of novel toxin-targeting human antibodies. As auxiliary tools for guiding antivenom development, novel approaches within determination of antibody cross-reactivity may accelerate development of novel antivenoms. Particularly promising technologies include antivenomics, which may provide a holistic view of the toxin-capturing abilities of antibodies, and high-density peptide microarray technology, which can provide amino acid level resolution of epitope-paratope interactions between toxins and antibodies (Engmark et al., 2017b, 2016). Finally, it is possible that other display technologies (e.g. mammalian display (Bowers et al., 2014; Ho and Pastan, 2009)) and novel binding protein formats, such as DARPins (designed ankyrin repeat proteins) (Rasool et al., 2016; Stumpp et al., 2008), Armadillo repeat proteins (Varadamsetty et al., 2012), Affitins (Béhar et al., 2016; Correa et al., 2014; Pacheco et al., 2014), Adhirons (Tiede et al., 2014), Anticalins (Schiefner and Skerra, 2015), and various other protein scaffolds (Simeon and Chen, 2017) may find their way into the field of antivenom development.

Conflicts of interest

The authors declare no conflict of interest.

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