Antivenomic Assessment of the Immunological Reactivity of EchिTAб-Plus-ICP, an Antivenom for the Treatment of Snakebite Envenoming in Sub-Saharan Africa

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Abstract. The immunoreactivity of EchिTAб-Plus-ICP, an antivenom developed for the treatment of snakebite envenoming in sub-Saharan Africa, to venoms of seven Echिs and Bitиs species, was assessed by “antivenomics.” This proteomic approach is based on the ability of an antivenom to immunodeplete homologous or heterologous venom proteins. Our results show an extensive cross-reactivity of this antivenom against all Eчиs and Bitиs venoms studied, as revealed by the complete immunodepletion of the majority of venom components, including metalloproteinases, serine proteinases, C-type lectin-like proteins, some phospholipases A₂, and L-amino acid oxidase. However, some phospholipases A₂, disintegrins and proteinase inhibitors were immunodepleted to only a partial extent. These results support the hypothesis that immunizing horses with a mixture of the venoms of Eчиs ocellatus, Bitиs arietанс, and Naja nigricollis generates antibodies capable of recognizing the majority of components of medically-relevant homologous and heterologous viperid venoms of the genera Bitиs and Eчиs from sub-Saharan Africa.

INTRODUCTION

Snake envenoming represents a highly neglected public health problem in sub-Saharan Africa, where the number of snakebite cases have been estimated to be as high as 1,000,000 and annual fatalities may range between 3,500 and 32,000. In addition, unknown numbers of patients, as many as 36% in one community-based study, are left with permanent physical or psychological sequelae, an undocumented and largely forgotten aspect of this pathology. Bird-derived antivenom constitutes only the validated therapy for snakebite envenoming. However, there is a current crisis in antivenom supply to sub-Saharan Africa, because of multiple causes that include lack of commercial incentives for manufacturers, deficient purchasing systems, ignorance of true antivenom requirements, high costs of some available products, loss of confidence of antivenom therapeutic efficacy, and safety caused by the marketing of ineffective products and inadequate regulatory systems.

The seriousness of this problem has prompted a number of initiatives, fostered by the World Health Organization (WHO), to confront this serious health issue. Several manufacturers have responded developing antivenoms for sub-Saharan Africa. Thus, in addition to laboratories traditionally producing antivenoms for Africa, such as EgyVac (Egypt), Sanofi-Pasteur (France), and South African Vaccine Producers (South Africa), other manufacturers have recently developed new antivenoms for this region, e.g., MicroPharm (UK), Instituto Bioclon (Mexico), Instituto Clodomiro Picado (Costa Rica), and Instituto Butantan (Brazil) (Dias-da-Silva W, personal communication). However, there was a large heterogeneity in the design and composition of the venoms used in the immunization mixtures to prepare the above antivenoms, an issue complicated by the complexity of sub-Saharan herpetofauna and by the diversity of African snake venom proteomes (“venoms”), including intraspecies venom variability in those species with a wide geographical distribution. Thus, the selection of venom mixtures appropriate for raising an immune response with wide cross-reactivity against many snake venoms in sub-Saharan Africa is an important task that should be approached initially through a rigorous analysis of the cross-reactivity of antivenoms against the medically most important snake venoms from this region. In the end, however, antivenom safety and efficacy have to be demonstrated in clinical trials.

The study of cross-neutralization of venoms by antivenoms is classically performed, at the preclinical level, by assessing the ability of a particular antivenom to neutralize the most important and clinically relevant toxicological activities of snake venoms using standard laboratory tests in experimental animals. In the case of viperid snake venoms, which inflict the highest toll of envenoming in sub-Saharan Africa, preclinical analysis of the neutralizing efficacy of antivenoms should include the neutralization of lethal, hemorrhagic, coagulant, defibrinogenating, and necrotizing effects. In the case of EchिTAб-Plus-ICP antivenom, produced by immunizing horses with a mixture of the venoms of Eчиs ocellatus, Bitиs arietans, and Naja nigricollis from Nigeria, preclinical analyses have already shown its effectiveness in the neutralization not only of these three venoms, but also of the venoms of other Eчиs saw-scaled viper species (Eчиs leucogaster, Eчиs pyramidalium leakeyi) and other Bitиs viper species (Bitиs gabonica, Bitиs rhinoceros, and Bitиs nasicornis). Moreover, this antivenom proved clinically effective and safe in a powerful trial performed in Nigeria in patients systemically envenomed by E. ocellatus.

In addition to preclinical neutralization tests, a complementary approach to assess the immunoreactivity of antivenoms against individual venom components has been recently developed. It is known as “antivenomics” and is based on the application of proteomic analytical tools for studying venoms and antivenoms. Venom and antivenom protein complexes are then immunoprecipitated by the addition of anti-equine IgG. The supernatant, which contains the non-immunoprecipitated venom components, is analyzed by proteomic techniques and its composition is compared with the chromatographic pattern.
of the native venom. In this way, venom components escaping immunodepletion by the antivenom can be identified easily and unambiguously. In this work, we have applied an antivenomic approach to assess the cross-reactivity of EchiTAb-Plus-ICP toward the venoms of seven species of sub-Saharan snakes of the family Viperidae. Results provide evidence of a high degree of cross-reactivity of this antivenom against all venoms tested, but also identified a small number of venom proteins that were only partially immunodepleted by the antivenom.

MATERIALS AND METHODS

Venoms and antivenom. The venoms of the following vipersid snakes were used: Echis ocellatus (Nigeria), E. leucogaster (Mali), E. pyramidalum leakeyi (Kenya), Bitis arietans arietans (from Ghana and Nigeria), B. gabonica gabonica, B. rhinoceros, and B. nasicornis. The venom of B. rhinoceros was a gift from César Olmos Jiménez (Entomo Zoo Fauna Arcana, S.L., Cullera, Valencia, Spain), and the venoms of B. gabonica gabonica and B. nasicornis were obtained from Latoxan (Valence, France). The other venoms were from specimens kept at the herpetarium of the Liverpool School of Tropical Medicine, and correspond to venoms pooled from several adult specimens. All venoms were lyophilized and stored at −20°C until used.

The polyspecific EchisTAb-Plus-ICP antivenom was manufactured by caprylic acid fractionation of the plasma of four horses that had been immunized with a mixture (at a weight ratio of 1:1:1.33) of the venoms of horses that had been immunized with a mixture (at a weight ratio of 1:1:1.33) of the venoms of Bitis arietans arietans (from Ghana and Nigeria), B. gabonica gabonica, B. rhinoceros, and B. nasicornis. The venom of B. rhinoceros was a gift from César Olmos Jiménez (Entomo Zoo Fauna Arcana, S.L., Cullera, Valencia, Spain), and the venoms of B. gabonica gabonica and B. nasicornis were obtained from Latoxan (Valence, France). The other venoms were from specimens kept at the herpetarium of the Liverpool School of Tropical Medicine, and correspond to venoms pooled from several adult specimens. All venoms were lyophilized and stored at −20°C until used.

The polyspecific EchisTAb-Plus-ICP antivenom was manufactured by caprylic acid fractionation of the plasma of four horses that had been immunized with a mixture (at a weight ratio of 1:1:1.33) of the venoms of Echis ocellatus, Bitis arietans, and Naja nigricollis collected from Nigeria. The particular antivenom batch used in this preclinical study (Batch 4260308PALQ) was formulated to have the following composition: protein concentration 69.6 g/L, sodium chloride 7.6 g/L, phenol 1.86 g/L, and pH 6.78. The antivenom batch passed all the quality control requirements at the Quality Control Laboratory of Instituto Clodomiro Picado.

Antivenomics: Immunodepletion of venom proteins by the polyspecific EchisTAb-Plus-ICP antivenom. We have coined the term “antivenomics” to describe our proteomic protocol for identifying venom proteins bearing epitopes reactive with an antivenom. Briefly, two milligrams of whole venom were dissolved in 70 μL of 20 mM phosphate buffer, pH 7.0, mixed with 4 μg of purified polyvalent antivenom IgGs, and incubated with gentle stirring for 1 hr at 37°C. The IgG concentration was determined spectrophotometrically using an extinction coefficient (ε) of 1.4 for a 1 mg/mL IgG concentration at 280 nm using a 1 cm light pathlength cuvette. Thereafter, 6 mg of rabbit anti-horse IgG antisera (Sigma, Tres Cantos, Madrid, Spain) in 350 μL of 20 mM phosphate buffer, pH 7.0, were added, and the mixture was incubated for another 1 hr at 37°C. Immunocomplexes were precipitated by centrifugation at 13,000 rpm for 30 min in an Eppendorf centrifuge (Eppendorf Ibérica, Madrid, Spain) and the supernatant was submitted to reverse-phase separation.

Isolation and characterization of venom proteins. For reverse-phase high-performance liquid chromatography (HPLC) separations, 2 mg of crude, lyophilized venom or its immunodepleted fraction were separated using an ETTAN LC HPLC system (GE Healthcare, Uppsala, Sweden) and a Lichrosphere RP100 C8 column (250 mm × 4 mm, 5 μm particle size) eluted at 1 mL/min with a linear gradient of 0.1% TFA in water (solution A) and acetonitrile (solution B) (5% B for 10 min, followed by 5–15% B over 20 min, 15–45% B over 120 min, and 45–70% B over 20 min). Protein detection was at 215 nm and peaks were collected manually and dried in a Speed-Vac (Savant, Thermo Electron, Madrid, Spain). Isolated protein fractions were subjected to N-terminal sequence analysis (using a Procise instrument, Applied Biosystems, Foster City, CA) following the manufacturer’s instructions. Amino acid sequence similarity searches were performed against the available databanks using the BLAST program implemented in the WU-BLAST2 search engine at http://www.bork.embl-heidelberg.de. The molecular masses of the purified proteins were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (on 12% or 15% polyacrylamide gels), and by electrospray ionization (ESI) mass spectrometry using an Applied Biosystems QTrap 2000 mass spectrometer operated in Enhanced Multiple Charge mode in the range m/z 600–1,700.

Proteins exhibiting a blocked N-terminus were excised from Coomassie Brilliant Blue-stained SDS-PAGE gels and subjected to automated reduction with DTT and alkylation with iodoacetamide, and in-gel digestion with sequencing grade porcine pancreas trypsin (Promega, Madrid, Spain) using a ProGest digestor (Genomic Solutions Inc., Cambridgeshire, United Kingdom) following the manufacturer’s instructions. The 0.65 μL of the tryptic peptide mixtures (from a total volume of ∼20 μL) were spotted onto a MALDI-TOF sample holder, mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in 70% acetonitrile containing 0.1% TFA, air-dried, and analyzed with an Applied Biosystems Voyager-DE Pro MALDI-TOF mass spectrometer, operated in delayed extraction and reflector modes.

For peptide sequencing, the protein digest mixture was loaded onto a nanospray capillary column and subjected to ESI mass spectrometric analysis using a QTrap 2000 mass spectrometer (Applied Biosystems) equipped with a nanospray source (Procutana, Denmark). Doubly- or triply-charged ions of selected peptides from the MALDI-TOF mass fingerprint spectra were analyzed in Enhanced Resolution MS mode and the monoisotopic ions were fragmented using the Enhanced Product Ion tool with Q3 trapping. Enhanced Resolution was performed at 250 amu/s across the entire mass range. Settings for MS/MS experiments were as follows: Q1- unit resolution; Q1-to-Q2 collision energy ~30–40 eV; Q3 entry barrier ~8V; LIT (linear ion trap) Q3 fill time ~250 ms; and Q3 scan rate ~1,000 amu/s. Collision-induced dissociation (CID) spectra were interpreted manually or using a licensed version of the MASCOT program (http://www.matrixscience.com) against a private database containing 1,083 viperid protein sequences deposited in the SwissProt/TREMBL database (UniProtKB/Swiss-Prot Release 56.7 of 20-Jan-2009; http://us.expasy.org/sprot/) plus the previously assigned peptide ion sequences from snake venomics projects carried out in our laboratory22–28,30,31,36–43 MS/MS mass tolerance was set to ±0.6 Da. Carbamidomethyl cysteine and oxidation of methionine were fixed and variable modifications, respectively.

RESULTS

Figures 1 and 2 display reverse-phase HPLC separation patterns of the venoms sampled, along with HPLC chromatograms of the supernatants obtained after subjecting each venom to
immunoprecipitation by the EchiTAb-Plus-ICP antivenom. Detailed proteomic characterization of the venoms of *E. ocellatus*, *B. arietans*, *B. gabonica*, *B. rhinoceros*, and *B. nasicornis* has been reported previously.\textsuperscript{21,31,43} According to their antivenomic immunoreactivity profile, venom proteins were classified as C-toxins (completely immunodepleted toxins), P-toxins (partly immunodepleted toxins), and N-toxins (non-immunodepleted toxins).\textsuperscript{39} Our results show that the vast majority of venom components, in all venoms tested, are C-toxins (Figures 1 and 2). Based on data accrued from previous proteomic analyses of these venoms, these C-toxins comprise mainly P-I and P-III snake venom metalloproteinases (SVMPs), serine
Figure 2. Immunodepletion of venom proteins from Bitis taxa by the polyspecific EchiTAb-Plus-ICP antivenom. Panels B, D, F, H, and J show, respectively, reverse-phase separations of the proteins recovered after incubation of the crude venoms of (A) B. arietans (from Ghana), (C) B. arietans (Nigeria), (E) B. nasicornis, (G) B. rhinoceros, and (I) B. gabonica gabonica with the polyspecific EchiTAb-Plus-ICP antivenom, followed by rabbit anti-horse IgG antiserum and immunoprecipitation. The inset shows SDS-PAGE analyses of β-mercaptoethanol-reduced fractions labeled as in the respective chromatograms. Protein fraction numbering is as in Table 1.
proteinases, C-type lectin-like proteins, and some phospholipases A₁ (PLA₂s), as well as minor components such as L-amino acid oxidase (LAAO) and cysteine-ruch secretory proteins (CRISPs). Only a small number of Echis and Bitis venom components corresponded to P-toxins (Figures 1 and 2). These proteins were identified by proteomic analysis to be primarily PLA₂s, disintegrins and proteinase inhibitors. There were no toxins identified as N-toxins, i.e., not being recognized by the antivenom at all (Figures 1 and 2).

**DISCUSSION**

Proteomic techniques such as “venomics” and “antivenomics” are valuable tools for analyzing the complex composition of snake venoms and the immunological reactivity of antivenom antibodies against specific venom components. These proteomic approaches represent potential tools for designing suitable venom mixtures to be used in immunization protocols for generating monospecific and polyspecific antivenoms exhibiting a wide profile of cross-reactivity. In the case of sub-Saharan Africa, this is of particular significance because of the diversity of snake species and the large complexity of venom proteomes. Thus, the question of which is the simplest combination of venoms that would generate an antivenom capable of neutralizing the largest possible number of viperid snake venoms in sub-Saharan Africa is highly relevant in the context of current efforts being performed by manufacturers to produce antivenoms for this region.

EchiTAb-Plus-ICP antivenom is generated by immunizing horses with a mixture of the venoms of *Echis ocellatus*, *Bitis arietans*, and *Naja nigricollis* from Nigeria. Our present antivenomics results show that EchiTAb-Plus-ICP completely immunodepleted the vast majority of components from the sampled venoms, and partially immunodepleted some additional components. The results also show that all components in all Echis and Bitis venoms investigated were either completely or partially immunodepleted, and there were no components that were not reactive with the antivenom antibodies. However, keep in mind that immunodepletion does not necessarily imply therapeutic neutralization of these venoms and, therefore, antivenomic analysis has to be complemented by preclinical neutralization studies, followed by appropriate clinical trials. In this respect, preclinical studies with the venoms analyzed in this work performed with EchiTAb-Plus-ICP showed that this antivenom neutralizes the most clinically relevant toxic (lethal, hemorrhagic, coagulant, and necrotizing) activities of these venoms, and also of the venoms of other medically important sub-Saharan African species, i.e., *E. leucogaster*, *E. pyramidum leakeyi*, *B. gabonica*, *B. rhinoceros*, and *B. nasicornis*. We conclude, therefore, that partially immunodepleted toxins may not compromise the in vivo neutralizing efficacy of EchiTAb-Plus-ICP antivenom.

The complete immunodepletion of SVMPs and serine proteinases is particularly relevant, because these proteinases are abundant in the venomes of African *Echis* and *Bitis* species and are responsible for some of the most serious pathophysiological manifestations of envenoming by species of these genera. The SVMPs comprise 30–66% of the venom proteins of *Echis* and *Bitis* species, whereas serine proteinases represent 20–25% of the venom proteins of *Bitis* species, and only 2% of the venom proteins of *E. ocellatus*. The SVMPs, particularly P-III SVMPs, induce local and systemic hemorrhage, and severe hemostatic disturbances resulting from prothrombin activation. A number of hemorrhagic and procoagulant SVMPs have been isolated and characterized from the venoms of *Echis* species. Serine proteinases, in turn, exert a number of activities, such as defibrinogenation caused by the action of thrombin-like enzymes. Bleeding and coagulopathy are the most important systemic pathophysiological manifestations of these envenomings, creating the risk of provoking hemorrhagic strokes, haemodynamic disturbances, cardiovascular collapse, and disseminated intravascular coagulation. Thus, the effective immunodepletion of SVMPs and serine proteinases by the EchiTAb-Plus-ICP antivenom correlates well with our earlier reports of the preclinical venom-neutralizing effectiveness of this antivenom against these important African vipers (using assays of venom-induced lethality, hemorrhage, and coagulation and its clinical efficacy in treating human victims of *E. ocellatus* envenoming).

The partial immunological reactivity to some PLA₂s, disintegrins, and proteinase inhibitors revealed the presence of low affinity or low abundance of antibodies against these components in the antivenom. Previous studies on antivenoms that target a number of venoms of Central and South American viper species showed evidence that PLA₂s and other low-molecular-mass components were poorly immunogenic, thus resulting in the generation of low-affinity antibodies.
properly assess the therapeutic consequences of this observation, it is necessary to study the potential relevance of these proteins as toxins, a subject that deserves further study. The percentage of PLA₂s in the characterized venom proteomes of Bitis and Echis venoms ranges from 4.3%, in the case of *B. arietans*, to 20.1% for *B. nasicornis*. Some PLA₂s in snake venoms are toxic and exert various pathophysiological effects, such as neurotoxic, myotoxic, hypotensive, and hemostasis-perturbing activities. Other PLA₂s, particularly the acidic ones, are devoid of toxicity and are thought to be more likely to play a predominantly digestive function. In the case of *Echis* sp. and *Bitis* sp. venoms, a myotoxic Ser49 PLA₂ homologue was characterized from the venom of *E. carinatus sochureki*. and only partially immunodepleted, i.e., proteinase inhibitors, are also low molecular mass peptides whose main role is to

for novel strategies aimed at enhancing the immune response against venom PLA₂s. These might be based on chemical modification of antigens, the use of novel adjuvants and immunization schemes, or the introduction of recombinant toxins or DNA immunization strategies. The challenge will be to identify strategies that are immuno-potentiating without necessarily increasing the final cost of these antivenoms, which are destined for use in rural, subsistence farming communities in Africa.

Disintegrins, which were also only partially immunodepleted, comprised between 3.4% and 8.5% of the venoms of some *Echis* and *Bitis* species. Some disintegrins inhibit platelet aggregation, although their role in the pathophysiology of envenoming remains unclear. The observation that some disintegrins were not completely immunodepleted from Echis and Bitis venoms is probably attributable to their low molecular mass and structural compactness, making them poorly immunogenic. The other venom components that were poorly immunogenic. The other venom components that were only partially immunodepleted, i.e., proteinase inhibitors, are also low molecular mass peptides whose main role is to

### Table 1
Assignment of reverse-phase isolated proteins from the non-immunodepleted HPLC fractions of the venoms of *Echis ocellatus* (Figure 1B), *E. leucogaster* (Figure 1D), *E. pyramidum leakeyi* (Figure 1F), *B. arietans* (Ghana) (Figure 2B), *B. arietans* (Nigeria) (Figure 2D), *Bitis nasicornis* (Figure 2F), *B. rhinoceros* (Figure 2H), and *B. gabonica gabonica* (Figure 2I), to protein families by N-terminal Edman sequencing, mass spectrometry and collision-induced fragmentation by nESI-MS/MS of selected peptide ions from in-gel digested protein bands.

<table>
<thead>
<tr>
<th>HPLC fraction</th>
<th>N-terminal sequence</th>
<th>Molecular mass</th>
<th>% Of immunodepletion</th>
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<tbody>
<tr>
<td>Echis ocellatus</td>
<td>Eo-1 Blocked</td>
<td>444.1</td>
<td>444.1 1</td>
</tr>
<tr>
<td>Eo-2 DCESGPCDCAKFLKL</td>
<td>5494, 5592</td>
<td>Disintegrin ocellatus [Q3BER]</td>
<td>62</td>
</tr>
<tr>
<td>Eo-3 SVVELGKMIQETGKS</td>
<td>13825</td>
<td>PLA₁ [CAQ72890]</td>
<td>67</td>
</tr>
<tr>
<td>Eo-4 SVFECMTIEETGRSPF</td>
<td>13866</td>
<td>PLA₁ [CAQ72891]</td>
<td>45</td>
</tr>
<tr>
<td>Echis leucogaster</td>
<td>El-1 DCESGPCDCAKFLKL</td>
<td>5458</td>
<td>Disintegrin leucogastin B [P0C7A8]</td>
</tr>
<tr>
<td>El-2 Blocked</td>
<td>444.1 444.1 1</td>
<td>ZKW</td>
<td>Inhibitor of SVMP</td>
</tr>
<tr>
<td>El-3 DCASGPCDCAKFLKL</td>
<td>5426</td>
<td>Disintegrin leucogastin A [P0C7A7]</td>
<td>65</td>
</tr>
<tr>
<td>El-4 NLYQFGKMIKNTGK</td>
<td>14066</td>
<td>PLA₂</td>
<td>40</td>
</tr>
<tr>
<td>El-5 SVIELGKMIQLTNK</td>
<td>13696</td>
<td>PLA₂</td>
<td>25</td>
</tr>
<tr>
<td>Echis pyramidum leakeyi</td>
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<td>5555</td>
<td>Disintegrin pyramidin A [P0C6R7]</td>
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<td>PLA₁</td>
<td>30</td>
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<tr>
<td>Bitis arietans arietans (Ghana)</td>
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<td>8991</td>
<td>Disintegrin bitistatin D1 [P17497]</td>
</tr>
<tr>
<td>BaG-2 Blocked</td>
<td>6942 396.2 2</td>
<td>TPEECR</td>
<td>Kunitz-type inhibitor</td>
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<tr>
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<td>PLA₂</td>
<td>68</td>
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<tr>
<td>Bitis rhinoceros</td>
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<tr>
<td>Br-2 NANAAPCPPDTCK</td>
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<td>62</td>
</tr>
<tr>
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<td>80</td>
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<td>Br-4 SLEEFAKMIKIT</td>
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<td>Bitis gabonica gabonica</td>
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<td>Kunitz-type inhibitor [Q6T6T5]</td>
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<tr>
<td>Bg-2 NANAAPCPPDTCK</td>
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<td>Disintegrin Bitsigabonin-1 [Q6T6T3]</td>
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</tr>
<tr>
<td>Bg-3 HLEQFQNMIDHVSRR</td>
<td>13967</td>
<td>PLA₂ [Q6T7B8]</td>
<td>68</td>
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Note: *N*, Be or Leu; *Z*, pyrrolidone carboxylic acid. Unless otherwise stated, for MS/MS analyses, cysteine residues were carbamidomethylated; molecular masses were determined by electrospray-ionization mass spectrometry or SDS-PAGE of reduced (~) samples; n.p. = non-peptidic material found.
control proteinase activity in the venom gland and are, consequently, not likely to play a significant role in envenoming.

In conclusion, the antivenomics approach used in this study might become a useful tool to assess the profile of immune reactivity of antivenoms against individual venom components, thus complementing preclinical studies on the neutralizing capability of antivenoms. Our immunochemical observations showed evidence that antibodies raised in horses against the venom of *E. ocellatus* and *B. arietans* are capable of recognizing all venom components in some of the viperid snake venoms of greatest medical importance in sub-Saharan Africa, further supporting the hypothesis that a pool of venoms of these two species constitutes a good immunizing mixture for the generation of antivenoms aimed at treating viperid envenomings in this region. The preclinical assessment of EchiTAb-Plus-ICP, both in its neutralization of toxic activities and its antivenomic profile, strongly suggests that this antivenom may be effective for the treatment of envenomings by a number of *Bitis* and *Echis* species in sub-Saharan Africa, a hypothesis that must be addressed by clinical trials.

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