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Generation of antibodies against disintegrin and cysteine-rich domains by DNA immunization: An approach to neutralize snake venom-induced haemorrhage

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ABSTRACT

Objective: To explore whether a DNA immunization approach targeting the major haemorrhage molecule of a prothrombin activator-like metalloproteinase from *Echis ocellatus* (*E. ocellatus*) venom could be conceived to inspire antibodies with more prominent specificity and equal adequacy to current conventional antivenoms systems.

Methods: The isolated DNA *EoMP-6* was used as the template for PCR amplification using the EoDC-2-specific forward and reverse primers. A PCR product of approximately 700 bp was obtained and cloned into pSecTag-B expression vector where anti-EoDC-2 antibodies were generated and analysed for their efficacy to neutralise local haemorrhage *in vitro* and *in vivo*.

Results: Our results suggest that the generated anti-EoDC-2 showed a remarkable efficacy by (a) interfering with the interaction of the recombinant disintegrin "EoDC-2" isolated from the *E. ocellatus* as well as other viper species to the $\alpha_2\beta_1$-integrins on platelets; (b) complete inhibition of the catalytic site of the metalloproteinase molecules *in vitro* using an adaptation antibody zymography assay. Furthermore, it has a polyspecific potential and constitutively expressed significant inhibition by cross-reaction and neutralised venom-induced local haemorrhage exerted by different viper species *in vivo*. The potential characteristic of EoDC-2 against one part (the non-catalytic domain) as opposed to the whole molecule to neutralise its haemorrhagic activity is of crucial importance as it represents a novel approach with greater immunological specificity and fewer hazards, if any, than conventional systems of antivenom production, by exposure large animals that usually being used for the current antivenom production to a less injurious than expression of the whole molecule containing the catalytic metalloprotease domain. Hence, we report for the first time that our preliminary results hold a promising future for antivenom development.

Conclusions: Antibodies generated against the *E. ocellatus* venom prothrombin activator-like metalloprotease and disintegrin-cysteine-rich domains modulated and inhibited the catalytic activity both *in vitro* and *in vivo* of venom metalloproteinase disintegrin cysteine rich molecules. Thus, generating of venom specific-toxin antibodies by DNA immunization offer a more rational treatment of snake envenoming than conventional antivenom.

1. Introduction

Envenoming resulting from snakebites remains the most neglected public health issue in many countries, particularly in tropical and subtropical countries [1]. *Echis ocellatus* (*E. ocellatus*) is the most ample and medically important snake species in West Africa and is thought to be accountable for more snakebite deaths worldwide than any other snake [2]. The exact frequency of snakebites hard to decide and is frequently underestimated, but in some zones of the Nigerian savannahs, victims of *E. ocellatus* envenoming may occupy more than 10% of hospital beds [2]. In the Benue valley of Nigeria, for example, the estimated incidence is 497 per

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100 000 populations per year with 10%–20% untreated mortality [3]. Furthermore, in Northern Nigeria, *E. ocellatus* is accountable for 95% of all envenoming by snakes [4] causing hundreds of deaths annually.

Local effects of *Echis* viper envenoming apart of haemorrhage include swelling, pain, blistering, and which in extreme cases, may lead to necrosis, permanent deformity, and even amputation of the affected limb [5]. Systemic effects include potentially lethal consumption coagulopathy, haemorrhage and hypovolaemic shock [6]. The only effective treatment is the administration of conventional antivenoms [7] that suffer from shortages imposed by the mode of preparation. Antivenoms are prepared by purifying the sera of large animals, typically horses, hyperimmunized with either individual or a range of venoms [8]. Since venoms contain numerous molecules, only some of which are toxic, antivenoms raised against these molecules consist of numerous antibodies with no known therapeutic functions [4]. Furthermore, because the toxicity of a venom molecule is unrelated to its immunogenic potential, the most potent antibodies in antivenoms are not necessarily targeted to the most pathogenic molecules [9]. In addition, an antivenom production system with less dependence upon snake collection, venom extraction and maintenance to give venoms for immunization would decrease the hazards as well as costs of conventional procedures.

To explore whether a DNA immunization approach targeting the major haemorrhage molecule of a prothrombin activator-like metalloproteinase from *E. ocellatus* venom could be conceived to inspire antibodies with more prominent specificity and equal adequacy to current conventional antivenoms systems. The notably T helper 2-type polarized immune response accomplished by GeneGun DNA delivery technique over intramuscular injection of DNA [10,11] was exploited here to advance antibody initiation against a toxin present in the venom of *E. ocellatus*. We utilized DNA encoding the carboxyl-disintegrin and cysteine-rich (DC) domains (EoDC-2) of *EoMP-6* (GenBank accession number: AY261531), a prothrombin activator-like metalloproteinase in the venom of *E. ocellatus* for the DNA immunization [12].

EoDC-2 of the *EoMP-6* possesses a 'DCD' collagen receptor binding motif in the disintegrin domain and the highly conserved cysteine scaffold present in the cysteine-rich domain similar to that of other viper species [13]. The rationale of utilizing the DC domains instead of the entire molecule was that expression of EoDC-2 in mammalian cells was thought to be less injurious to the host than expression of the whole molecule containing the catalytic metalloprotease domain. Furthermore, it was thought that, antibody bound to the DC domain, may counteract substrate binding [14] and/or catalytic function [15] of the whole molecule, thus achieving the objective. So the experimental design strategies of this research study were (i) to prepare the EoDC-2 DNA immunization construct (shown in Figure 1) (ii) to determine, using ELISA, the seroconversion efficiency of EoDC-2 delivered intradermally into mice or by GeneGun delivery or intramuscularly (iii) to assess, *in vitro*, the cross reactivity of antibody raised by EoDC-2 immunization to analogous molecules in venoms of other *Echis* species by immunoblotting and zymography assays, and (iv) to analyse, *in vivo*, the venom-neutralizing efficacy of antibody rose by EoDC-2 immunization.

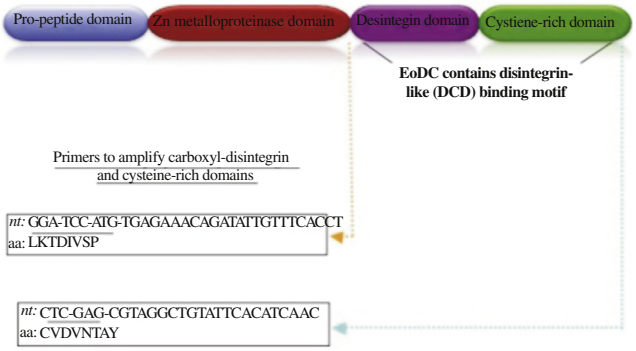

Figure 1. Schematic diagram demonstrating PCR primers design. Underlined nucleotides represent restriction digest sites of BamHI and XhoI, respectively.

2. Materials and methods

2.1. Isolation and analysis of DC domains from EoMP-6 clone

The DC domain of *EoMP-6* encoding a novel *E. ocellatus* prothrombin activator [12] was amplified by the PCR using primers complementary to nucleotides 890–912 (5′ primer) and nucleotides 1523–1545 (3′ primer). The amplicon was subcloned into the TA plasmid DNA cloning vector (pCR2.1-TOPO; Invitrogen, Groningen, The Netherlands) to produce a plasmid construct that was then transformed into a chemically competent *Escherichia coli* (TOP10F′, Invitrogen). The construct was then extracted (Mini-spin prep kit, Qiagen, Hilden, Germany) and digested with *Bam*HI and *Xho*I at 37 °C to select constructs with inserts of the predicted size for DNA sequencing. DNA sequencing was carried out by the dideoxy-nucleotide chain-termination method in a Beckman Coulter CEQTM2000 XL DNA analysis system. Only one clone showing an open reading frame identical to the DC domain of *EoMP-6* was selected. The EoDC-2/TOPO clone was digested with *Bam*HI and *XhoI* and the EoDC-2 insert was electrophoretically isolated from TOPO in order to be cloned into the mammalian expression vector pSecTag-B (Invitrogen, Netherlands) as described below.

2.2. Plasmid construction and clone isolation

2.2.1. The pSecTag-B DNA immunization plasmid clone construction

The amplified (EoDC-2) product was ligated into the mammalian expression plasmid vector pSecTag-B (Invitrogen, Netherlands) to produce immunization plasmid construct. The pSecTag-B plasmid vector has all the required components for successful protein expression from DNA [16,17]. The required quantity of the purified PCR product required for the ligation reaction was determined by the following equation which illustrates the conversion of molar ratio to mass ratios for both pSecTag-B plasmid vector = 5.2 kb plasmid and D-C = 700 kb insert DNA fragment.

DNA fragment insert (ng) = Vector (ng) × Size of insert (kb)/Size of vector (kb)

Due to the fact that pSecTag-B does not support blue/white selection several clones were selected randomly. Clones were grown in Lysogeny broth culture medium overnight and the

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