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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.

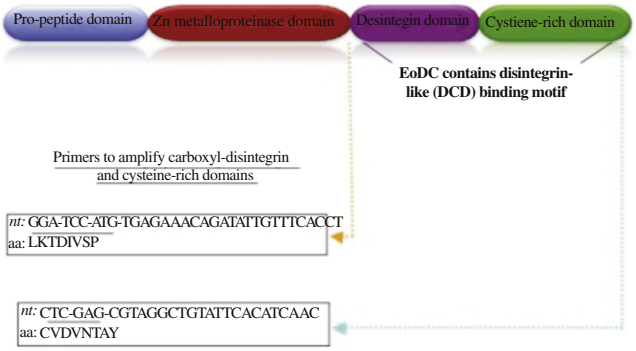

Figure 1. Schematic diagram demonstrating PCR primers design. Underlined nucleotides represent restriction digest sites of BamHI and XhoI, respectively.

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

extracted plasmid construct was digested with *Bam*HI and *Xho*I. Clones that showed appropriate insert sizes were sequenced using the T7 and BGH primers incorporated into pSecTag-B. The plasmid DNA immunization constructs were purified from large-scale *Escherichia coli* cultures using Qiagen Megaprep kits, according to manufacturer's instructions (Invitrogen, Netherlands). The purified DNA was stored at −20 °C until ready for use.

2.3. In vitro EoDC-2 protein expression

Before proceeding to DNA immunization, the ability of the pSecTag-B/EoDC-2 construct to express the EoDC-2 DNA fragment was confirmed *in vitro* using two different assays as described below. This was to ensure that all possible errors that may interfere with DNA immunization, particularly by the GeneGun, were eliminated.

2.3.1. In vitro translation of mammalian COS-7 with pSecTag-B/EoDC-2

Transient transfections of COS-7 (a mammalian fibroblast cell line kindly donated by Dr. Edwin de Vet, RFCGR, Cambridge, UK) were carried out to demonstrate both the transcription and translational validity of the EoDC-2/pSecTag-B immunization construct. Cells spread at a low density (40% per well) into 35 mm six-well plate (Nunc). Cells were then incubated overnight in a CO_2 incubator overnight at 37 °C. Culture medium was aspirated from each well and replaced by serum-free Dulbecco's modified Eagle medium. The following reaction mixture was assembled for each well: 100 μL serum-free medium, at room temperature, was pipetted into a 15-mL Falcon tube, 4 μL FuGEN6 (Roche, Indianapolis, USA) was then added to the centre of the serum-free media (avoiding contact with the sides of tube), gently mixed and incubated at room temperature for 5 min. One microgram of DNA was added as droplets into the tube, with gentle but continuous mixing. The reaction was incubated at room temperature for 30 min. Twenty microliters of the reaction mixture was spread as droplets into 2 wells. Wells were gently swirled and incubated in a CO_2 incubator overnight at 37 °C.

2.4. Analysis of EoDC-2 recombinant protein

2.4.1. Harvesting of recombinant protein

The supernatant of each well was harvested 72 h after transfection and transferred into a sterile 5-mL tube and stored immediately at −20 °C. Two millilitres of 0.5 mmol/L ethylene diamine tetraacetic acid was added to each well followed by incubation at room temperature for 10 min. This is to loss cells into a suspension phase. A volume of 2 mL of cold 10% trichloroacetic acid was added, followed by further incubation in ice for 30 min. Cells were centrifuged for 15 min at 15500 r/min at 4 °C, the supernatant discarded and 10 mL pre-chilled acetone were added to wash the cells, which were then centrifuged as above. The supernatant was discarded and the cell pellet was air dried, re-suspended in 2× protein loading buffer and stored at −20 °C. Analysis of the harvested protein was performed using standard one-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot assays.

2.4.2. One-dimensional SDS–PAGE

Recombinant EoDC-2 (100 μg/mL) were solubilized in SDS–PAGE loading buffer (2% SDS, 5% β-mercaptoethanol in 62 mm Tris–HCl, pH 6.8), boiled for 5 min and fractionated on a 15% SDS–PAGE gel along with low molecular weight markers (Bio-RAD) which consisted of phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (32 kDa), soybean trypsin inhibitor (21 kDa) and lysozyme (14 kDa). The gel was stained with Coomassie blue R-250 and destained. After electrophoresis, the gel was stained in 5% Coomassie blue for 15 min and destained with methanol: acetic acid: water (30%: 7%: 63%).

2.4.3. Immunoblotting

Proteins fractionated on a 15% SDS–PAGE gel were transferred to nitrocellulose paper and molecular weight markers visualized by reversible staining with Ponceau S. The nitrocellulose papers were blocked with 5% non-fat milk for 1 h at room temperature washed with Tris (0.01 mol/L, pH 8.5), saline (NaCl, 0.15 mol/L) and Tween 20 (0.1%) (TST) and incubated with 5% milk-diluted sera at 4 °C overnight. The nitrocellulose papers were washed three times with TST and incubated with horseradish peroxidase- or alkaline phosphatase-conjugated goat anti-mouse IgG, or anti-rabbit IgG (1:1000; Nordic, The Netherlands) at room temperature for 2 h. After washing off unbound secondary antibody, the specific antigen-bound antibody was visualized with the appropriate substrate buffer.

2.5. GeneGun immunization

2.5.1. Production of DNA-coated gold beads for GeneGun immunization

pSecTag-B/EoDC-2 DNA plasmid construct and the control pSecTag-B plasmid were precipitated onto 1.6-μm gold beads and loaded by addition of 1.0 mol/L $CaCl_2$ for 2 min followed by vigorous mixing and the beads were left to settle for 15 min at room temperature. The sample was centrifuged for 1 min and supernatant was removed without disturbing the pellet of gold beads. Three "1 min" spin washes with 700 μL of 100% ethanol were performed to remove the excess spermidine, $CaCl_2$ and H_2O. The quantity of gold powder and DNA was adjusted to provide immunization 'shots' of 1 μg DNA/0.5 mg gold powder per cm Tefzel tubing according to manufacturer's instructions.

2.5.2. Confirmation of DNA coated of the gold bead 'shots'

To verify DNA loading of the gold beads, single 1-cm 'shots' were selected at random from each preparation and placed in 1.5 Eppendorf tube containing 100% ethanol and vortexed vigorously for 2 min and then incubated overnight at room temperature. Shots were vortexed vigorously and centrifuged for 2 min at 13000 r/min to pellet the gold beads. The tubing and ethanol were removed and the coated gold bead centrifuged again for 1 min to remove residual ethanol. The pellet was air dried for 15 min and then re-suspended in 10 μL double distilled water and incubated at 55 °C for 5 min to re-suspend the DNA. Two microliter of 6× sample loading buffer was added and samples loaded on to a 0.7% agarose gel.

2.6. BALB/c mice

Male BALB/c mice of 8–10 week-old were purchased from Harlan Olac (UK) or Charles River Laboratories (UK) or were produced from breeding colonies in the Biomedical Services

Unit, University of Liverpool. All mice were housed in designated areas of the Biomedical Services Unit of the University of Liverpool. This study was carried out in strict according to National Institutes of Health policies outlined in the Guide for Care and Use of Laboratory Animals. All protocols for animal research were reviewed and approved by the Animal Research Ethics Committee, The University of Liverpool, Liverpool, UK.

2.7. DNA immunization of mice by GeneGun

The Helios GeneGun was attached by metal tubing to a pressurised helium cylinder and the pressure set at 350 kPa according to manufacturer instructions. Previous studies had shown that this level of pressure delivered the gold beads into the epidermal layer required for delivery of antigen to antigen-presenting cell and that DNA-transfected cells were shown to be transcriptionally active by RT-PCR for three days [18]. The 1 cm 'shots' were loaded into the cartridge supplied by the manufacture and inserted into the GeneGun. The abdomens of anaesthetized 8–10 week-old male BALB/c mice were shaved and each subjected to three 'shots' expelled under a burst of helium gas at 350 kPa into the epidermal layer using the Helios GeneGun (BioRad). Groups of 10 BALB/c mice were immunized with 3 μg of the pSecTag-B/EoDC-2 DNA construct or the plasmid vector alone. Immunizations were given on three occasions, two weeks apart and the sera examined 8 weeks later.

2.7.1. Intradermal and intramuscular injections of DNA

Two groups of 10 BALB/c mice each were immunized with (A) pSecTag-B/EoDC-2 DNA construct and (B) pSecTag-B only. DNA was adjusted to 100 μg in 50 μL double distilled water and 25 μL was injected *i.d.* into two sites on the back of anaesthetised mice or into the rectus femoris muscle of each hind leg *i.m.* of mice with a 25 g needle. A time course of 14 weeks was followed for immunizations, the first three was performed with two weeks intervals between immunizations with a further two immunizations given at four week intervals.

2.7.2. Collection of sera from immunized mice

The first three immunizations were performed at two-week intervals; with serum samples taken two weeks post immunization. The fourth and fifth immunizations were performed at four-week intervals. Sera collected at various intervals throughout the experiment by tail snip or, at the end of the experiment, by cardiac exsanguinations under terminal anaesthesia [19] from the DNA-immunized and non-immunized control animals were examined by ELISA to determine the titre of antibodies to *E. ocellatus* venom. The blood was incubated overnight at 4 °C to allow formation and contraction of the blood clot, which was then removed. Serum samples were then centrifuged at 13 000 r/min for 5 min. Serum was transferred into sterile 1.5 mL Eppendorf tube and stored at −20 °C. Subsequently, the sera from each group were serially diluted with phosphate-buffered saline and the titre determined. Serial dilutions of sera taken from mice 12 weeks after the final DNA immunization were tested by ELISA to determine levels of EoDC-2 specific IgG. The optical density (OD) values displayed were calculated by subtracting the OD value of blank from the OD value of each serum. Each line represents the mean OD of each group.

2.7.3. ELISA

Ninety-six-well plates (Maxisorp, NUNC, Denmark) were coated with 1 mg/mL of the harvested *E. ocellatus* venom EoDC-2 in coating buffer (0.015 mol/L Na_2CO_3, 0.035 mol/L $NaHCO_3$, 3 mmol/L NaN_3, pH 9.6) and left overnight at 4 °C. The plates were then washed three times with TST and blocked for 1 h with 5% fat-free dried milk (Carnation, Wirral, UK) in TST at 37 °C. Individual sera from immunized animals were diluted 1:500 with 5% milk and applied, in duplicate, to the plates overnight at 4 °C. The wells were then washed as above and 100 μL of a 1:1 000 dilution of goat anti-mouse or goat anti-rabbit alkaline phosphatase conjugate (Sigma, Poole, UK) was added. The plates were then incubated for 2 h at 37 °C before rewashing. Chromogenic substrate, 2,2′-azino-bis (2-ethylbenzthiazoline-6-sulphonic acid tablets, Sigma, Poole, UK), was then added and the plates developed at room temperature for 30 min in phosphate citrate buffer (pH 4.0) containing 0.015% hydrogen peroxide. The OD was read at 405 nm using a model 450 microplate reader (BioRad, Hemel Hempstead, UK).

2.8. Evaluation of anti-EoDC-2 antibodies

Due to the deduced amino acid sequence of EoDC-2 of the *EoMP-6* similarities with other clinically important vipers we presumed that the peaks in the EoDC-2 antigenic index profile (Figure 2) indicated numerous domains predicted to have a surface location and potential for antibody induction. The thin vertical lines (F–M) illustrate that many of the antigenic residues of EoDC-2 are shared by snake venom metalloproteinase (SVMP) of related vipers and that antibodies raised by EoDC-2 DNA immunization are likely to possess considerable cross-reactivity.

2.8.1. Mechanism of action of EoDC-2 inhibition on platelet aggregation using platelet aggregometry

One millilitre of blood was drawn via cardiac puncture from anesthetized mice (n = 5) with into a syringe containing 100 μL of sodium heparin solutions, resulting in a final heparin concentration of 15 USP/mL. Then 400 μL of the heparinized whole blood was mixed with the same volume of normal saline. Venom disintegrin-cysteine rich including EoDC-2 concentrations were adjusted by dilution with normal saline, and 0.1, 1.0, or 2.0 μg of EoDC-2 against adenosine diphosphate and 5, 50, or 100 μg of venom disintegrin-cysteine rich against collagen were added into each test cuvette and preincubated with magnetic stirring at 37 °C for 5 min. The same volume of ordinary saline was included as control. Agonists for platelet aggregometry were 20 μmol/L adenosine diphosphate or 5 μg/mL of collagen (Chronolog Corporation, Havertown, PA). Platelet aggregation activity was measured with an impedance procedure using a platelet Chrono-Log Lume-aggregometer.

2.8.2. Antibody zymography

To further investigate if the generated anti-EoDC-2 may neutralize the SVMPs we performed antibody zymography assay as previously described [20]. Briefly, we simply used anti-EoDC-2 or control EchiTAb™ antivenom preparations in place of water in the preparation of the zymograms, without further modification of the gel constituents or protocol. The total volume of the mini-gel was 5.6 mL. Therefore, to achieve the required dilution of antibody preparations of 1/10 (560 mL serum of EoDC-2 immunized mice, and 12 mg/mL EchiTAb™

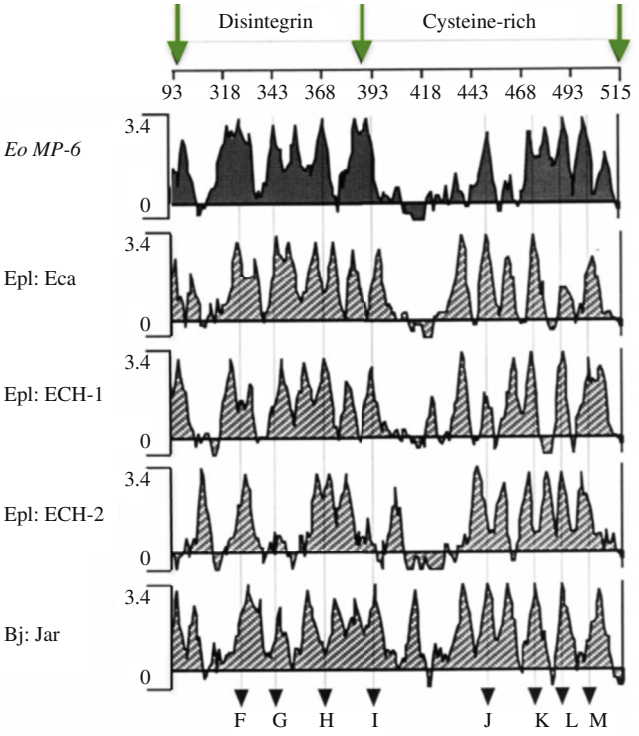

Figure 2. Jameson and Wolf antigenic profiles of *EoMP-6* and SVMPs from related vipers.
The structural organisation of SVMPs is depicted in the uppermost box. The large arrows distinguish the four main domains of the intact zymogen and indicate the amino acid sequences shown on the horizontal scale. The pro-peptide domains have been excluded from this analysis. The vertical scales represent comparative antigenic values. The thin vertical lines (F–M) are a subjective assignation of antigenic domains that exhibit the greatest phylogenetic conservation. Lines H and F correspond to the catalytic zinc binding, DCD and platelet-inhibitory motifs, respectively, as described in the text. Epl: Ecarin, Epl: Ech-1 and Epl: Ech-2–Ecarin and two other SVMPs from *Echis pyramidum leakeyi* (*E. pyramidum leakeyi*) venom; Bj: jar—Jararhagin from *Bothrops jararaca* (*B. jararaca*) venom.

antivenom), and 1/1 000 (anti-EoDC-2), we respectively substituted 560 mL and 5.6 mL of water with antibody. The total volume of water or water/antibody was 2.5 mL. Snake venom samples (5 mg/mL) were electrophoresed at 115 constant volts. After electrophoresis the gels were washed in 2.5% Triton X-100 for 30 min to remove SDS and then the gels were incubated in an activating buffer [50 mmol/L Tris–HCl (pH 8.0), 5 mmol/L $CaCl_2$, 10 ng NaN_3] at 37 °C for 18 h, which then stained in 5% Coomassie blue for 15 min and destined with methanol:acetic acid:water (30%:7%:63%).

2.8.3. In vivo evaluations of anti-EoDC-2 to neutralize venom-induced haemorrhage

In the first experiment in this section we used sera from the immunized and non-immunized control animals to assess their efficacy to neutralize the hemorrhagic activity of *Crotalus atrox* (*C. atrox*), *E. ocellatus* and *Bitis arietans* (*B. arietans*) venoms using an *in vivo* minimum hemorrhagic dose assay utilized to pre-clinically assess new antivenoms [20]. Fifty millilitres of sera taken 12 weeks after immunization from CD1 [Crl:CD1 (ICR) Swiss mice–Charles River Laboratories] mice and pre-immunizations serum from the control (immunized with the vector only) CD1 mice were mixed with an equal volume of venom (8, 6 and 10 mg; as previously identified to generate a lesion of 10 mm–1 mm minimum hemorrhagic dose), incubated at 37 °C for 30 min and then the mixtures injected intradermally, together with a negative control (phosphate buffered saline/venom) and/or positive controls using commercialized antivenoms (EciTab, SAIMR and CroFab antivenoms), into the dorsal skin of groups of four male CD1 mice. All animals were observed regularly over 24 h. At the end of the observation period, animals were sacrificed, and their skins were dissected. The size of each hemorrhagic lesion was recorded to examine the haemorrhage neutralization efficacy of the anti-EoDC-2.

3. Results

3.1. PCR amplification of DC domain of the prothrombin activator EoMP-6 and clone construction

The PCR amplification for the EoDC-2-specific forward and reverse primers (Figure 1) was successful in amplifying the carboxyl-DC domain of the *EoMP-6* prothrombin activator cDNA [12] giving a PCR product of approximately 700 bp and the clones containing inserts (named: EoDC-2) of the correct size were submitted for DNA sequencing. The clone that shows the right insert with a better open reading frame was selected to be sub-cloned into TOPO vector and inserted in frame the mammalian vector pSecTag-B.

3.2. Confirmation of the transcriptional and translational validity of EoDC-2/pSecTag-B construct plasmid

The transcriptional and translational of the EoDC-2/pSecTag-B DNA construct were successfully confirmed prior to DNA immunization by the detection of the 28 kDa EoDC-2 protein in SDS-PAGE and immunoblotting as illustrated by Figure 3. Results also showed that the EoDC-2 protein was secreted into the culture supernatant confirming the correct operation of the signal peptide sequence (Figure 3a, b) in contrast with the control (Figure 3c).

3.3. Responses of BALB/c mice immunized with pSecTag-B/EoDC-2 DNA

Different routes of DNA immunization were evaluated. The result of the time course ELISA to determine the relative contribution of each route of immunizations showed highest antibody titre (1:20 dilution) in responsive to snake venom (Figure 4). The results clearly demonstrated that GeneGun immunization was superior to intradermal immunization in terms of efficiency of seroconversion (Figure 5). Furthermore, we have observed that EoDC-2/pSecTag-B immunization elicited a highly heterogeneous response in mice (data not shown) (*i.e.*, some mice responded while others did not). Results of the time course of both GeneGun and intradermal immunizations with EoDC-2/pSecTag-B DNA antibody titers after the second immunization showed barely detectable deficiencies from the background. Thereafter, antibody titres doubled with each immunization in both the GeneGun and intradermal-immunized mice.

3.4. Cross-reactivity of the EoDC-2 antibody with venoms from Echis snakes of different geographical areas

The phylogenetic limits to the cross-reactivity of the EoDC-2 antibodies raised by GeneGun and intradermal were examined by probing immunoblots of venoms of a variety of *Echis* vipers

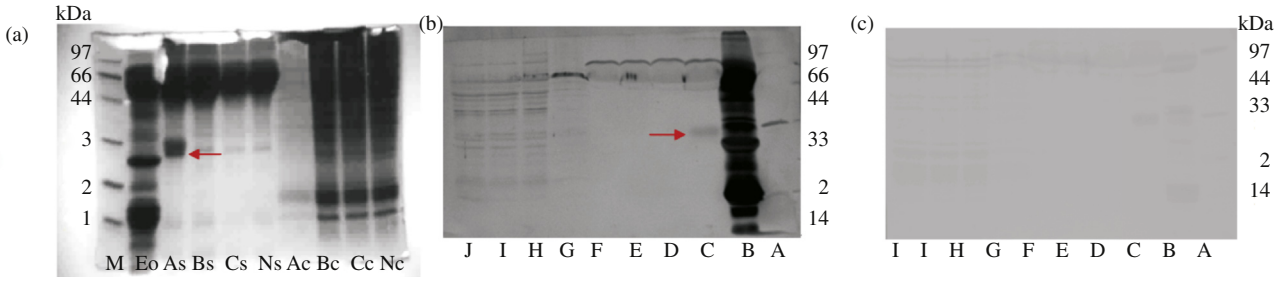

Figure 3. Examination of the cellular protein and culture supernatant of COS-7 cells transfected with EoDC-2/pSecTag-B by (a) 12% SDS-PAGE and immunoblotting with rabbit anti-*E. ocellatus* venom (b) and normal mice serum (c).

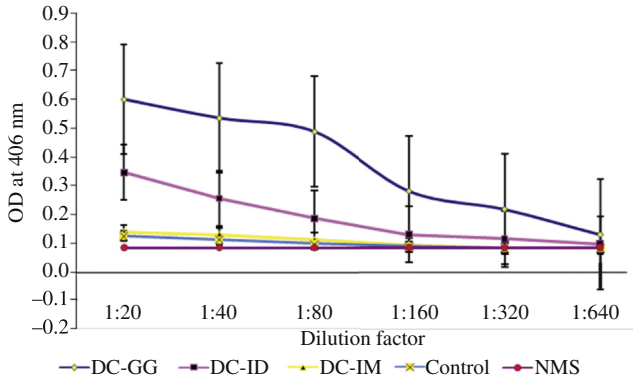

Figure 4. Antibody responses to *E. ocellatus* venom of the DNA immunized mice as determined by ELISA.
DC-GG: GeneGun; DC-ID: Intradermally; DC-IM: Intramuscularly; NMS: Normal mice serum.

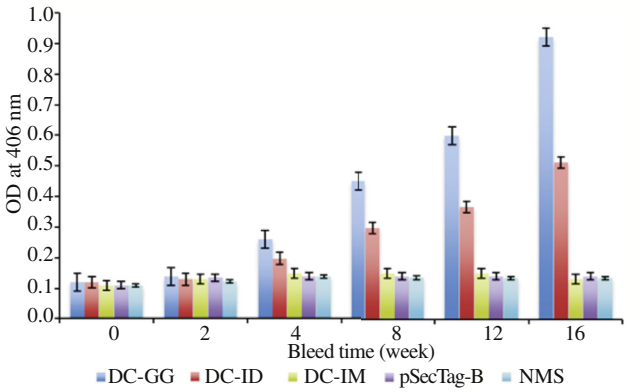

Figure 5. Antibody response to *E. ocellatus* venom of 1:20 diluted sera from the DNA immunised mice collected at intervals throughout the experiment.
DC-GG: GeneGun; DC-ID: Intradermally; DC-IM: Intramuscularly; NMS: Normal mice serum.

(Figure 6a, b). It can be seen that antibody generated by GeneGun (Figure 6a) was cross-reactive with high molecular weight bands (52 kDa) in all venoms, particularly those in *E. ocellatus* venom. As expected sera from mice immunized with EoDC-2 by the intradermal route (Figure 6b) were fainter than those from GeneGun-immunized mice. Sera from the intramuscular-immunized mice (Figure 6c) were as non-reactive as the control mice (Figure 6d, e). However, bands of venom proteins below 33 kDa have been recognised by all blots except in that where normal mouse serum was used. The EoDC-2-specific antibodies reacted to the 50–60 kDa molecules in the homologous *E. ocellatus* venom with an intensity matched by its reactivity with analogous bands in venoms of *Echis* vipers of various African origins. The immunological reactivity of the anti-EoDC-2 sera to components in *Echis* vipers from Iran (*Echis pyramidum*) and

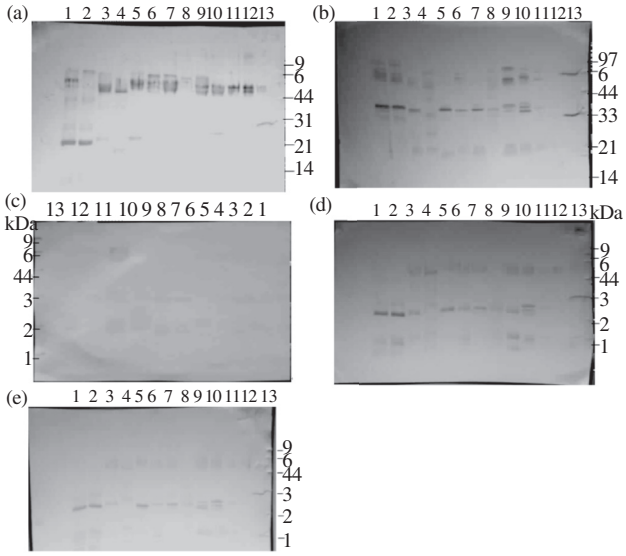

Figure 6. Reactivity of sera from EoDC-2 DNA immunized mice to components in venoms of various *Echis* species.
Identical immunoblots of venoms from [1–12, stated in text], were probed with sera from mice immunized with EoDC-2 by GeneGun (a), intradermal (b), intramuscular (c); immunoblots were probed with sera from mice immunized with pSecTag-B by GeneGun (c) and intradermal (d); whereas, immunoblot (e) was probed with sera from normal mice. Lane 13 is the low molecular weight marker (BiorRad, UK).

Pakistan [*Echis sochureki* (*E. sochureki*)] was considerably weaker than that of *E. ocellatus* and the other African vipers.

3.5. Effects of EoDC-2 on platelet aggregation

Platelet aggregometry showed that recombinant EoDC (10 μg/mL) has a strong inhibitory effect on platelet aggregation similar to that of other snake species in response to 5 μg/mL collagen (Figure 7). However, when the generated anti-EoDC-2 incubated at 37 °C with the recombinant EoDC and other snake venom disintegrins for 30 min at different serum dilutions (1/10, $1/10^2$, $1/10^3$ and $1/10^4$), from the immunized mice, platelets showed dose-dependent aggregation and reach to the level similar to that of the control (Figure 7). This indicates that binding of the EoDC-2 antibody to the EoDC-2 domain of *E. ocellatus* and other viper species showed high affinity to interfere with the interaction of this domain to the $\alpha_2\beta_1$-integrins on platelets and hence affects their aggregation (Figure 7).

3.6. Anti-EoDC-2 antibody zymography

The results of evaluating whether blocking the DC domains in the SVMP may play a significant role in the activation of the

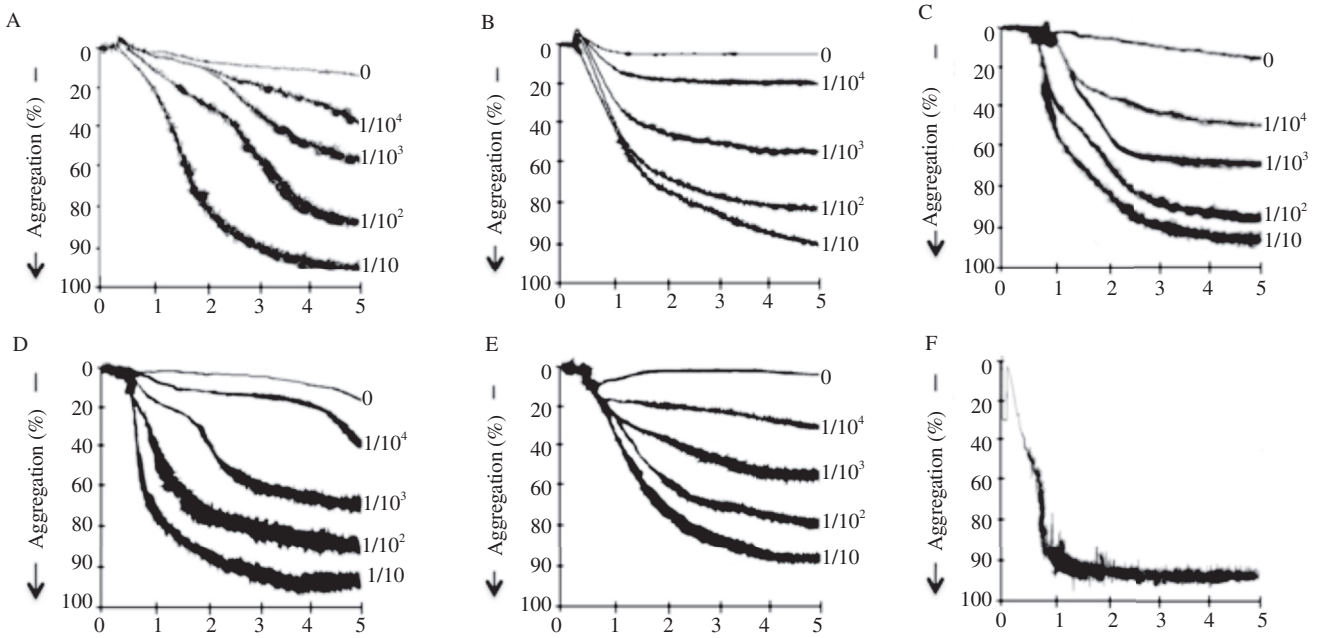

Figure 7. Efficacy of anti-EoDC-2 to neutralise the effect of disintegrin cysteine-rich molecules in venoms of *E. ocellatus* (EoDC-2) (A) and other snake species, B and C: Ecarin and two other SVMPs from *E. pyramidum leakeyi* venom; D: Ecarin; E: Jararhagin from *B. jararaca* venom; F: Normal mice serum used as a control; Platelet aggregometry shows dose-dependent inhibition of anti-EoDC-2 antibodies.

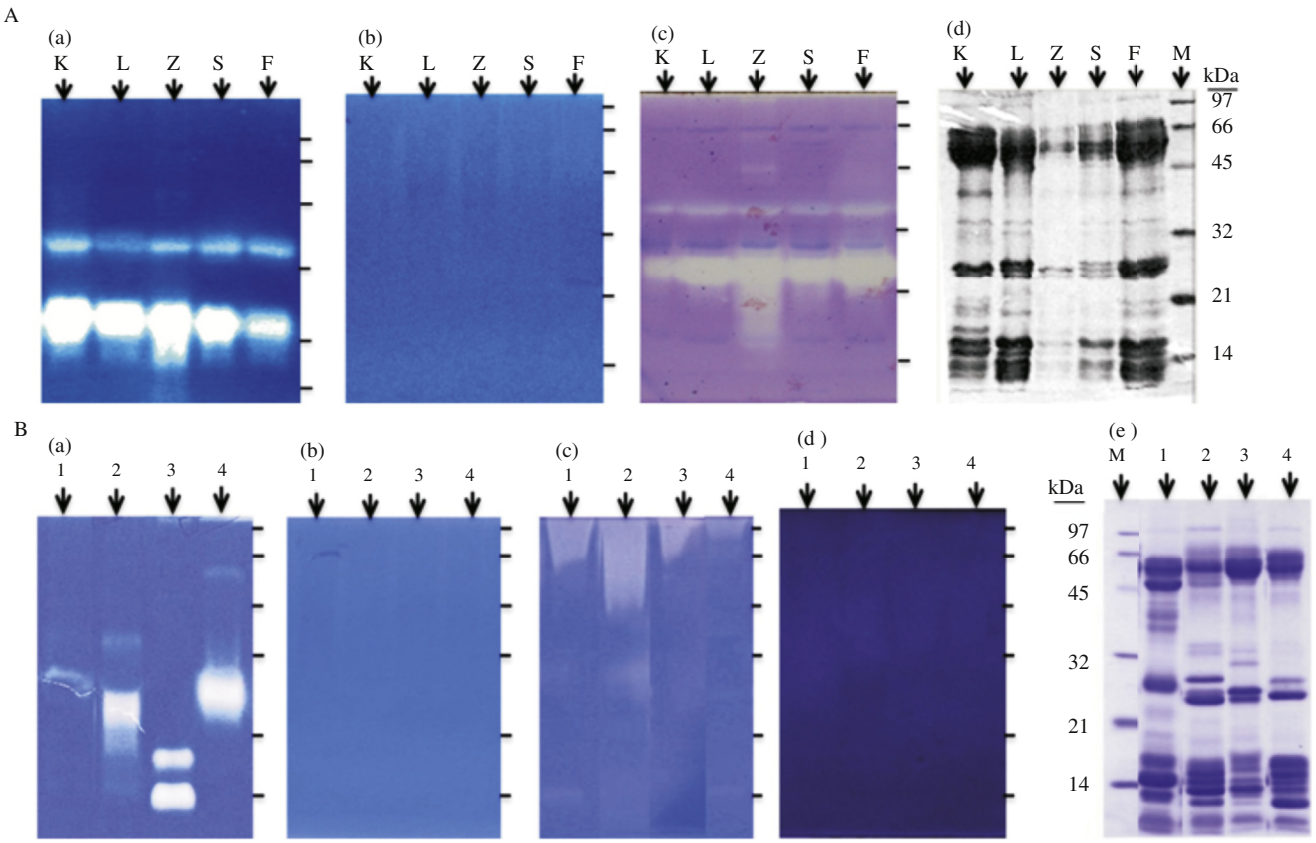

Figure 8. Detection of gelatinolytic activity by gelatin zymography depicting differences of biological samples of venoms from different sources.
A: Neutralisation of venom gelatinolytic proteases of *E. ocellatus* venoms from five different regions in Nigeria by anti-EoDC-2 antibody, Lanes K, L, Z, S, F represent Kaltungo, Langtang, Zamko, Saminako and Fanshin, respectively; B: Neutralisation of venom gelatinolytic proteases from four different viper species by anti-EoDC-2 antibody, Lanes 1–4 represents *E. pyramidum leakeyi*, *E. ocellatus*, *E. Sochureki*, *B. arietans*, respectively. Samples (5 mg/mL) were electrophoresed into the (b–e) zymogram 15% PAGE gels at 115 constant volts. After electrophoresis the gels were washed in 2.5% Triton X-100 for 30 min to remove SDS and then the gels were incubated in an activating buffer (50 mmol/L Tris–HCl [pH 8.0], 5 mmol/L $CaCl_2$, 10 ng NaN_3) at 37 °C for 18 h, which then stained in 5% Coomassie blue for 15 min and destained with methanol:acetic acid:water (30%:7%:63%). (c and d) Same as (b) except that anti-EoDC-2 (c) and EchiTAbTM antivenom (d) were added instead of distilled water at 1:10 dilution. (e) Same as (c) except that no anti-EoDC-2 was added at 1:1 000 dilution. (a) SDS PAGE gel was stained with Coomassie blue R-250 and destained. Low molecular weight markers (Bio-RAD) shown in (M) were phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (32 kDa), soybean trypsin inhibitor (21 kDa) and lysozyme (14 kDa). After electrophoresis, the gel was stained in 5% Coomassie blue for 15 min and destained with methanol:acetic acid:water (30%:7%:63%).

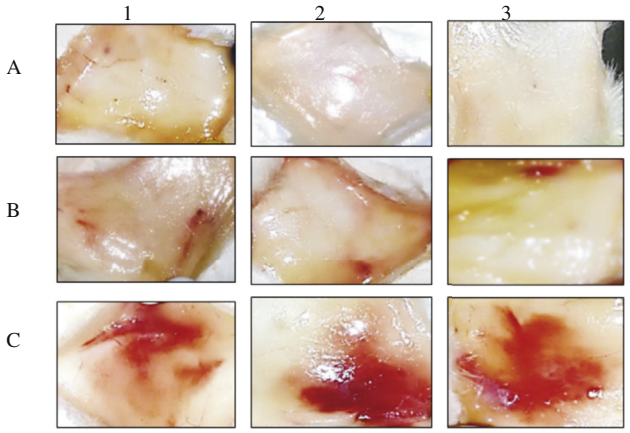

Figure 9. Efficacy of anti-EoDC in serum of EoDC DNA immunized mice to neutralised snake venom from three different viper species.
A: Group A1-3 (*C. atrox*, *E. ocellatus* and *B. arietans* venoms) respectively, incubated with serum of DNA immunized mice with pSecTag/DC construct; B: Group B1-3 (*C. atrox*, *E. ocellatus* and *B. arietans* venoms) incubated with EciTab, (2) SAIMR & (3) CroFab antivenom, respectively; C: Group C is the control group where *C. atrox*, *E. ocellatus* and *B. arietans* venoms were incubated with serum from mice immunized with pSecTag-B alone.

catalytic activity of the metalloproteinase were highly significant as the generated anti-EoDC-2 antibody totally block the gelatinase activity of metalloproteinase molecule of *E. ocellatus* EoMP-06 as well as cross-reactivity, and inhibit metalloproteinases of the same snake species from five different regions (Figure 8A). This was further confirmed by that anti-EoDC-2 showed a capacity to cross-react and suppress the proteolytic activity of metalloproteinase molecules in venoms of other viper species (Figure 8B). These results are correlated and in total agreement with the early prediction that the disintegrin-cysteine rich domain modulates the substrate specificity of venom metalloprotease, disintegrin, and cysteine-rich domains [21], and apparently enhances the hemorrhagic activity of SVMPs [22].

3.7. Evaluations of anti-EoDC-2 in vivo for neutralization efficacy of venom-induced haemorrhage

The results demonstrated that serum from mice, immunized with DNA construct encoding cDNA sequences of the DC domain of the snake venom metalloproteinase has a polyspecific potential antibody and constitutively expressed significant and complete neutralization of metalloproteinase by cross-reactivity and neutralized venom-induced haemorrhage from different snake viper species (Figure 9A) in contrast with the use of the three commercialised antivenoms (Figure 9B). We presented only the dorsal skin of three representative mice injected with (i) venom/serum from DNA immunized mice with pSecTag-B/EoDC-2 DNA construct (Figure 9A1–3), (ii) venom/antivenom from control of three commercialised antivenom (Figure 9B1–3) and (iii) venom/serum from immunized mice with pSecTag-B alone (Figure 9C1–3).

4. Discussion

The remarkable progress of DNA vaccination from the initial observation since 1992 that plasmids could be used to express exogenous DNA in mammalian cells and stimulate immune responses [23] to the current clinical trials [24] has largely been concerned with inducing protective cellular immune responses against intracellular bacteria, viruses and parasites [25]. In 2000 Harrison *et al.* [26], demonstrated that DNA immunization is a promising technique for antivenom production because it represents a system of generating toxin-specific antibodies.

The primary objective of this present investigation was to (i) use DNA immunization to generate specific antibodies to the major haemostasis-disruptive toxins in the venom of *E. ocellatus* and (ii) to define the route of administration favouring maximal induction of specific antibodies.

The distinctly T helper 2-type polarized immune response accomplished by GeneGun DNA route over intramuscular injection of DNA [27,28] was investigated in this study to promote antibody induction against a toxin present in the venom of *E. ocellatus*. We utilized DNA encoding the carboxyl-DC domains (EoDC-2) of *EoMP-6*, a prothrombin activator-like metalloproteinase in the venom of *E. ocellatus* for DNA immunization [12].

Based on the rational stated earlier, therefore, the experimental design strategies of this research study were mainly to assess (i) *in vitro* cross-reactivity of antibody raised by EoDC-2 immunization to analogous molecules in venoms of other *Echis* species by immunoblotting and zymography assays, and (ii) to analyse *in vivo* venom-neutralizing efficacy of EoDC-2 antibody raised by DNA immunization.

PCR amplification was successful in amplifying the carboxyl-DC domain (approximately 700 bp) of the *EoMP-6* prothrombin activator cDNA [12]. The PCR product was subcloned into TOPO vector and inserted in frame of the mammalian vector pSecTag-B which then been used in the DNA immunization. The assay of the transcriptional and translational of the EoDC-2/pSecTag-B DNA construct were successfully confirmed that all the possible errors that may interfere with DNA immunization will not encountered and that was illustrated by the detection of the 28 kDa EoDC-2 protein was secreted into the culture supernatant confirming the correct operation of the signal peptide sequence as illustrated by Figure 3.

Different routes of DNA immunization were evaluated. The results of the EoDC-2/pSecTag-B DNA immunization clearly demonstrated that GeneGun immunization was superior to intradermal immunization in terms of efficiency of seroconversion. The inability of the mice immunized with EoDC-2/pSecTag-B by intramuscular injection to raise anti-EoDC-2 antibodies was unexpected. The plasmid used was from the same stock used to unsuccessfully immunize mice by the GeneGun and intradermal routes, indicating that the plasmid was not at fault. This suggests that the route of delivery was the problem. Harrison *et al.* [26] observed that mice immunized with JD9 DNA (the DC domain of an analogous SVMP from the venom of *Bothrops jararaca*) by intramuscular raised markedly weaker antibody titers than mice immunized by GeneGun. Together, these results indicate that intramuscular DNA immunization may not appropriate for raising antibodies.

We have observed that EoDC-2/pSecTag-B immunization elicited a highly heterogenous response in mice (data not shown) (*i.e.*, some mice responded while others did not). DNA immunization studies have previously provided evidence for differential transfection efficiencies in individual mice as reflected by variable levels of expression of the encoded protein [29]. The variation in the antibody response of individual mice within each group may reflect different rates of epidermal cell transfection following GeneGun and intradermal immunizations.

Results of the time course of both GeneGun and intradermal immunizations with EoDC-2/pSecTag-B DNA antibody titres after the second immunization showed barely detectable deficiencies from the background. Thereafter, antibody titres doubled with each immunization in both the GeneGun- and intradermal-immunized mice. This suggests that time intervals between boosts and/or DNA dosage needs to be investigated further with a view to improving the antibody titre and the antibody isotypic responses generated by the various routes of immunization.

We utilized DNA encoding the carboxyl-DC domain (EoDC-2) of *EoMP-6*, a prothrombin activator-like metalloproteinase in the venom of *E. ocellatus* for DNA immunization [12]. To our knowledge, this is one of the pioneer reports to describe novel cDNA encoding a disintegrin and cystiens-rich domains.

SVMPs from venoms of phylogenetically related vipers show extensive molecular sequence and structural similarities [20,30,31]. One of the fundamental hypothetical attractions of having this strategy *i.e.*, DNA immunization to produce antivenoms against potent molecules, is that the broad and extensive sequence and structural conservation of venom toxins can be exploited to produce antivenoms equipped of neutralizing the venoms of a wide variety of snakes. In this study the phylogenetic limits to the cross-reactivity of the EoDC-2 antibodies raised by GeneGun and intradermal were examined by probing immunoblots of venoms of a variety of *Echis* vipers (Figure 6). The EoDC-2-specific antibodies reacted to the 50–60 kDa molecules in the homologous *E. ocellatus* venom with an intensity matched by its reactivity with analogous bands in venoms of *Echis* vipers of various African origins.

The immunological reactivity of the anti-EoDC-2 sera to components in *Echis* vipers from Iran (*E. pyramidum*) and Pakistan (*E. sochureki*) was considerably weaker than that of the *E. ocellatus* and the other African viper.

Overall, GeneGun delivery of EoDC-2 DNA induced titres of total IgG greater than those achieved by the intradermal or intramuscular delivery routes. It has been suggested that direct transfection of epidermal dendritic cells results in their migration to drain lymph nodes where antigen presentation to the immune system occurs [32]. The lower IgG titres obtained by intradermal injection suggest that this delivery route is markedly less efficient than GeneGun in activating dendritic cells.

Binding of the EoDC-2 antibody to the EoDC-2 domain of *E. ocellatus* and other viper species showed high affinity to interfere with the interaction of this domain to the$\alpha\alpha_2\beta_1$-integrins on platelets and hence affects their aggregation (Figure 7) and by that contributed to neutralization of venom-induced catalytic activity (Figure 8a and b). Our results are correlated and in total agreement with the early prediction and findings that the DC domain modulates the substrate specificity of venom MDCs [33], and apparently enhance the hemorrhagic activity of SVMPs [22]. Therefore, these results suggest that EoDC-2-specific antibody has the potential to inhibit the enzymatic degradation of sub-endothelium by the *E. ocellatus* MP domain. It is conceivable that EoDC-2 antibody may operate in both modalities to inhibit venom hemorrhagic activity.

These preliminary results demonstrated for the first time that antivenom generated by DNA immunization against the DC domains of the snake venom metalloproteinase has a polyspecific potential and constitutively expressed inhibitor *in vivo* by cross-reactivity and neutralised venom-induced haemorrhage from different snake viper species. This was further confirmed in the conducted, experiment in which the absence of haemorrhage in mice injected with the mixture of the three venoms and sera from the DNA immunized mice was significant and surprising (Figure 9A) in contrast with the use of the three commercialised antivenoms (Figure 9B) and strongly suggested that anti-EoDC-2 has polyspecific potential to cross-react and inhibit of venom-induced haemorrhage from venoms of different viper species. The significant of this finding is based on the following: (i) the potential of having antivenom generated against one part (non-catalytic domain) within the whole potent molecule to neutralised its hemorrhagic activity of such potent molecules, (ii) although anti-EoDC-2 antibody was generated against *E. ocellatus* it shows a promising result and great potential to completely neutralised the hemorrhagic activity within venoms of other snake species involved in this study.

Furthermore, this study demonstrate for the first time that the generated anti-EoDC-2 showed a remarkable efficacy by interfering with the interaction of the recombinant disintegrin "EoDC-2" isolated from *E. ocellatus* as well as other viper species to the $\alpha_2\beta_1$-integrins on platelets. Moreover, in addition to inhibiting completely the catalytic site of the metalloproteinase molecules *in vitro* using an adaptation antibody zymography assay, it has a polyspecific potential and constitutively expressed significant inhibition by cross-reactivity and neutralising venom-induced haemorrhage from different snake viper species.

The significant of this finding based on that (i) although anti-EoDC-2 antibody was generated against *E. ocellatus* it shows another approach with great potential to neutralise the local hemorrhagic activity significantly induced by venoms of other snake species involved in this investigation; (ii) the potential of having antivenom generated against one part (the non-catalytic domain) as opposed to the whole molecule to neutralise its hemorrhagic 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 here that our preliminary results may hold a promising future for antivenom development.

Antibodies generated against the *E. ocellatus* venom prothrombin activator-like metalloprotease and more specific against its DC domains, named EoDC-2, prove to modulate and inhibit the catalytic activity both *in vitro* and *in vivo* of venom MDCs. These preliminary findings may have great potential for antivenom development in which anti-Eo-DC antibody, constitutively expressed inhibitor of viper venom-induced haemorrhage. Preclinical antivenom efficacy assays are required to be conducted in order to gain insight into the provision of these antibodies with the possibility of better advantages over the current equine and ovine antivenoms. This will have significant contribution to improve the treatment of systemic and necrosis effects that exerted by the saw scaled viper *E. ocellatus* envenoming.

Conflict of interest statement

I declare that I have no conflict of interest.

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