A NEW IMMUNOCHEMISTRY PROCESS THAT TRANSFORM A NON-IMMUNOGENIC CROTAMINE-LIKE ANTIGEN FROM RATTLESNAKE (*Crotalus durissus cumanensis*) VENOM, IN IMMUNOGENIC TO PRODUCE ANTI-CROTAMINE-LIKE ANTIBODIES

UN NUEVO PROCESO INMUNOQUÍMICO QUE CONVIERTE CROTAMINA DEL VENENO DE SERPIENTE DE CASCABEL (Crotalus durissus cumanensis), UN ANTÍGENO NO-INMUNOGÉNICO, EN INMUNOGÉNICO PARA PRODUCIR ANTICUERPOS ANTI-CROTAMINA

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ABSTRACT

The making of antibodies in animals can be demanding due to that several antigens, mostly of low molecular masses, provoke imperceptible immune response or are even totally non-immunogenic. The transformation of non-immunogenic molecules into effective antigens represent an important immunological tasks. The crotamine from the rattlesnake *Crotalus durissus cumanensis* snake venom was purified by a Mono S HR 10/10 chromatography column and used to immunise C57/B mice, after to be polymerised with glutaraldehyde. The murine polyclonal antibodies directed against native crotamine-like (NCL) treated with glutaraldehyde and their product crotamine-like polymer (CLP) were generated by immunisation injecting CLP via lymph node cells. These antibodies were capable of detecting CLP in an enzyme-linked immunosorbent assay. The SDS-PAGE of NCL and CLP showed bands of molecular masses ~ 3 kDa and ~18 kDa, respectively. These results offer evidence that the polyclonal antibodies recognise specific putative original and post-polymerisation epitopes on the CLP molecule, which were maintained following the process of polymerisation. The results are discussed in relation to the preservation of a functional post-polymerisation epitopes on CLP.

Key words: *Crotalus durissus cumanensis;* crotamine-like; glutaraldehyde; polyclonal antibody; polymerisation; venom

RESUMEN

La producción de anticuerpos en animales puede ser una actividad ardua, debido a que muchos antígenos, principalmente los de baja masa molecular, provocan una respuesta inmune imperceptible o aún son totalmente no-inmunogénicos. La transformación de una molécula no inmunogénica, en un antígeno efectivo representa un importante reto inmunológico. La crotamina obtenida del veneno de la serpiente de cascabel (*Crotalus durissus cumanensis*) fue purificada a través de una columna de cromatografía Mono S HR 10/10 (Biorad, EUA) y usada para inmunizar ratones de la cepa C57/B, luego de ser polimerizada con glutaraldehido. Los anticuerpos policlonales dirigidos contra la crotamina nativa tratada con glutaraldehido, y su producto el polímero obtenido de la crotamina (CLP) se lograron mediante inmunización vía ganglios linfáticos con polímeros de CLP. Esos anticuerpos policlonales fueron capaces de detectar el CLP, en un ensayo de ELISA. Los perfiles de migración (SDS-PAGE) de la crotamina nativa y la CLP mostraron bandas de masa molecular ~ 3 kDa y ~18 kDa, respectivamente. Estos resultados ofrecen evidencia de que los anticuerpos policlonales reconocen epítopes específicos originales y posteriores a la polimerización en la molécula de CLP, que se mantuvieron luego del proceso de polimerización. Los efectos se discuten en relación con la preservación de epítopes funcionales post-polimerización en CLP.

Palabras clave: *Crotalus durissus cumanensis*; crotamina-similar; glutaraldehido; anticuerpo policlonal; polimerización; veneno

INTRODUCTION

The study of antivenomic methodology about the immunoreactivity of *Crotalus* antivenoms against subspecies of rattlesnakes showed that many *Crotalus* antivenoms lack of recognising and binding competent antibodies against crotamine [5]. Based on the classical knowledge that less than 10 kilodalton (kDa) molecular mass proteins are not good immunogenic molecules, it is though that crotamine (~4.8 kDa in average) must be an insignificant immunogenic in horses (*Equus ferus caballus*)

The transformation of non-immunogenic molecules into effective antigens remains important immunological tasks. The proposal of transforming derived-glutaraldehyde precipitated crotamine into effective antigens are both basic and applied provocative defiance. Here, it is supplied information on glutaraldehyde molecule, mainly its mode action on proteins. Glutaraldehyde is a di-aldehyde composed of five highly reactive carbons, which has been isolated as oil and stored as an aqueous solution. Its storage forms a carbohydrate, pyrans (oxines) and polymers mixtures [4]. From the chemical point of view, it is a very reactive product, which polymerises in water and acidic aqueous solutions are stable. In alkaline medium, the reactivity is higher, in this environment, at room temperature, reacts rapidly with amino terminals of proteins and produce insoluble cross-linked aggregates. In correspondence with the conditions here described, yield covalently linked soluble protein oligomers and polymers were obtained. Glutaraldehyde is well known for its ability to react with proteins and this process is valid to a wide range of proteins, and by slight variation in the reaction conditions, soluble polymers in the molecular mass range from 1×10^4 x 100^4 were produced.

Crotamine is a basic~ 42-amino-acid polypeptide with an isoelectric point of 10.3 and a ~ 4.8 kDa molecular mass, originally isolated from the venom of the South American rattlesnake *Crotalus durissus terrificus* [11]. This toxin is regarded as a potential cell-infiltrating vehicle competent of gathering into replicating cells [19]. Here, it has been purified a ~3 kDa molecule with crotamine properties from *Crotalus durissus cumanensis, in order to be used* in all the experiments.

Currently, glutaraldehyde has been used for producing insoluble protein aggregates [2, 16], and insoluble products of some enzymes, e.g. carboxypeptidase [28], trypsin [13], papain [17, 26] and catalase [31], maintaining its enzymatic activity. This project intended to report the effect of molecular size on immunogenicity of proteins, which have been investigating procedures planned to produce high-molecular mass protein aggregates, via intermolecular cross-bridges. To this purpose, it was studied the reaction of glutaraldehyde with the crotamine-like non-immunogenic protein, in order to make a crotamine-like polymer (CLP), using a special lymph node via, to get an immunological response.

MATERIALS AND METHODS

Reagents

Glutaraldehyde (25%), bovine serum albumin (crystalline), acrylamide, methylene-bis-acrylamide and NNN'N'-tetramethylene-diamine, ammonium persulphate, trichloroacetic acid, sodium dodecyl sulphate and Coomassie Brilliant Blue R-250, Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) (GIBCO, USA), Trypan blue solution, Dimethyl sulfoxide (DMSO), Goat anti-mouse IgG conjugated with alkaline phosphatase and alkaline phosphatase substrate (pNPP) were all purchased from Sigma/Aldrich, USA. All stock reagents were stored as recommended by the manufacturer.

Snake and venom collection

Pool from six female and male adults of the rattlesnake (*Crotalus durissus cumanensis*) (CDC) captured in Santa Teresa del Tuy Town (Miranda State, Venezuela) and housed at the Tropical Medicine Institute of the Universidad Central de Venezuela (Caracas, Venezuela) were used in the experiments. Venom was extracted by allowing the snake to bite into a Parafilm® extended over a disposable plastic cup. The venom sample was centrifuged (Beckman Avanti 30, USA) at 5000 G for 10 minutes (min), and filtered through 0.45 micrometres (µm) filter. The venom was frozen at -90 °C (Thermo Scientific™ Revco™ UxF, USA). and then lyophilised.

Mice

C57/B inbred strains of mice (*Mus musculus*) between 18 and 22 grams (g), bred under specific healthy conditions were obtained from the Animal House of the Instituto Venezolano de Investigaciones Cientificas (IVIC)(Venezuela).

Purification of crotamine-like

Isolation and purification of (CDC) crotamine-like was carried out by one chromatographic step. Crude venom (30 milligrams (mg) by protein estimation) was diluted to 1.0 millilitres (mL of 50 millimol (mM) Tris–HCl buffer, pH 8.2, and then applied on a Mono S HR 10/10 (GE Healthcare Biosciences Ltd, USA) column equilibrated with same buffer. Attached proteins were eluted with a 0–1 M NaCl linear gradient in equal buffer over 60 minutes (min) at a flow rate of 1.5 mililiters (mL)/min. Proteins were monitored at 280 nm. The chromatogram displayed 8 fractions, which were identified agreeing to their elution (FIG. 1). Clear spastic hind-limb paralysis action was apparent in fraction 5, which was dialysed against water at 4^0 C, lyophilised and stored at -20°C (Frigidaire FGVU21F8QF Vertical Freezer, USA) until used. This fraction was chosen for the experimental assays.

Protein concentration

The CLP concentration was spectrophotometrically (Beckman, USA) calculated by assuming that 1 unit of absorbance/centimetres (cm) of path length at 280 nm corresponds to 1 milligram (mg) protein/mL [35].

Concentration of the crotamine-like under polymerisation

Equal volumes of *Cdc* crotamine-like (2 mL) at 5 mg/mL in phosphate saline buffer ,(PBS) pH 7.4, were treated with constant proportions (by weight) of glutaraldehyde at 2.5, 6.25 and 12.5% (2 mL); as a negative control PBS was added to the crotamine-like without glutaraldehyde. Reaction procedures were as follow: products were diluted to 5mg/mL; the crotamine-like and the glutaraldehyde solutions were mixed on a vortex mixer

(SI™ Vortex-Genie™ 2T, Scientific Industries, USA). Mixing was sustained for 1 min and the solutions were incubated (Shel Lab General Purpose Incubator, Global Lab, CA, USA) at 5^0C for 24 hours (h). All samples turned yellow after 10 min of addition of glutaraldehyde. Then, the mixtures were dialysed during 24 h at 6^0C, against several changes of 0.01 M PBS, pH 7.4, until yellow colour disappeared; the yields were checked and the molecular mass of crotamine-like and CLP solutions were determined using a 12.5% SDS-PAGE as described below.

Polyacrylamide gel electrophoresis (SDS-PAGE)

Twenty micrograms (ug) of crotamine-like and CLP were run on a 12.5% SDS-PAGE under non-reduced conditions. The gel was stained with Comassie brilliant Blue R-250 for 1 h.

Testing CLP on mice

In order to know if the polymer conserved the crotamine neurotoxic activity (posterior limb paralysis), three mice were intravenously injected with CLP and observed for this posterior limb paralysis. Three mice were also injected with the native crotamine-like as positive control.

Immunisation

Five male C57/B mice were immunised with CLP by injections at day (d) 0, in the lower hock of right limb (50 ug / 50 microliters (μg)/50 microliters (μL) of a 1:1 emulsion of Freund's complete adjuvant and the CLP dissolved in PBS, pH 7.4.

One injections using incomplete Freund's adjuvant were given after 4 d in the same place. Afterward, with 4 d' intervals the immunisation was carried out for three more times with CLP dissolved in PBS in the same location. Developed immunisation process, mice blood was obtained by cardiac puncture, collected, centrifuged and serum samples frozen at -20°C; at this point all surviving mice were ethically euthanised by neck dislocation and used to feed snakes.

ELISA test for specific antibodies

The ELISA [25] was implemented using antibodies against both CLP and native crotamine-like, which were used as antigens capturing and detecting antibody, respectively. The optimal activity of these antibodies was determined comparing with a negative- and positive-control serum samples in each plate.

Polystyrene microplate 96 wells (Nunc, USA) were sensitised overnight at room temperature with both native crotamine-like and CLP (10 μg/well in PBS, pH 7.4). For each step, 100 μL/well was added unless mentioned otherwise. A normal mice serum was used as negative control and immunised mice sera as experimental sample (1:200). Remaining binding sites were blocked by incubation with 1% Bovine serum albumin (BSA) in PBS for 1 h. The total volume of the well was kept constant (100/μL). The plates were exhaustively washed with PBS/Tween (2 min/wash), and unbound sites were blocked with 200 μL/well of 0.5% BSA diluted in PBS, pH 7.4 and 0.05% Tween 20 diluted in milli Q water. After 2 h incubation at 37°C, the plates were emptied by suction. Diluted anti-mouse serum (1:200) was added and the plates were incubated for 1 h at 37°C. After thorough washing as described above, horse-radish (HR) peroxidase-conjugated goat anti-mouse (10 µg/mL of PBS/Tween) was added. The plates were incubated for 1 h at 37°C and washed with PBS/Tween. The TMB (3,3',5,5'-tetramethyl-benzidine) was added and the plates were incubated for 1 h in the dark at room temperature. The enzyme reaction was blocked with 50 µL/well of 8 N H_2SO_4. The absorbance at 450nm wavelength (A_{450}) of the plates was read using a microplate ELISA reader (Bio-Rad, USA).

RESULTS AND DISCUSSION

Several proficient reagents for cross-linking proteins have been described [9], but in common, they have been utilised to generate intramolecular quite than intermolecular bridges. The information about the use of glutaraldehyde in biomedical research is vast. Several authors have described the basis for insoluble protein aggregates production [2,15,16]. Others had demonstrated that insoluble derivatives of numerous enzymes such as carboxypeptidase [28,29], trypsin [13] and catalase [31] could preserve significant enzymatic activity. Different authors [1, 26, 37] showed that it was possible to conjugate proteins and enzymes and coupling proteins to diverse matrices as well as evident cases, where soluble protein derivatives have been described [12,14]. The great affinity could be related to a significant success of configurational entropy after the polymerisation, which would be the case, if the folding and assembly processes are coupled.

In the present work, the crotamine-like from CDC venom was chromatographic isolated generating 8 peaks, where only the fraction 5 produced the classical spastic inferior limbs paralysis activity in mice (FIG. 1).
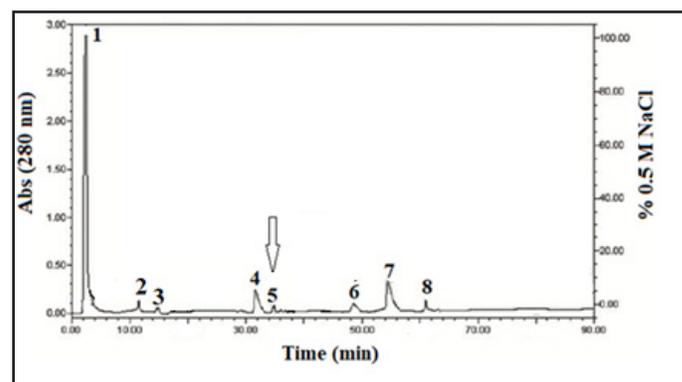

FIGURE.1. CATION EXCHANGE CROMATOGRAPHY OF RATTLESNAKE (_Crotalus durissus cumanensis_) VENOM. Crotamine (arrow). Absorbance at 280 nm. Elution with % 0.5 M NaCl linear gradient

To maintain natural protein conformation, guaranteeing the solubility of the secondary polymers by preservation of original hydrophilic amino acid residues, in the current work was required to produce cross-linked polymers with minimum amino acid substitution. With this objective, low glutaraldehyde concentrations were utilised, jointly with high protein concentrations, in order to make easy intermolecular reactivity. It was used crotamine-like protein concentration at 1.97 ± 0.02 mg/mL. In the past, many experimental studies had used high

concentrations of glutaraldehyde, which produced an extensive formation of derivatives, but without extensive crosslinking [2]. It has been proposed by Lee *et al.* [22] that crotamine is able to delivery molecules into mammalian cells without needing special receptors. Several crotamine isoforms have been described [10,21,25,27,30,32,34,36]. Crotamine hold three disulphide bridges [5] and the main crotamine folding is similar to many proposed as anti-bacterial defence peptides, which have been described to belong to defensins families (α-defensin, β-defensin and insect defensin) [7]. Although these molecules show a great structural diversity and a broad spectrum of activity, they have in common the ability to induce the permeation of liquids (by osmosis or diffusion) through the bacterial cytoplasmic membranes [6,8], as crotamine does in other cell membranes [19].

Immunoglobulins have the faculty to identify structures exclusively present on oligomeric assemblies [18,20]. The expression oligomers can, on the other hand, be applied to grouping ranging from a dimer to a much higher assembly [33]. Here, it was used 12% of polyacrylamide gel electrophoresis (SDS-PAGE) under non-reduced conditions of both native crotamine-like and CLP showed a molecular mass bands of ~3 and ~ 18 kDa, respectively, to show both proteins (FIG. 2).
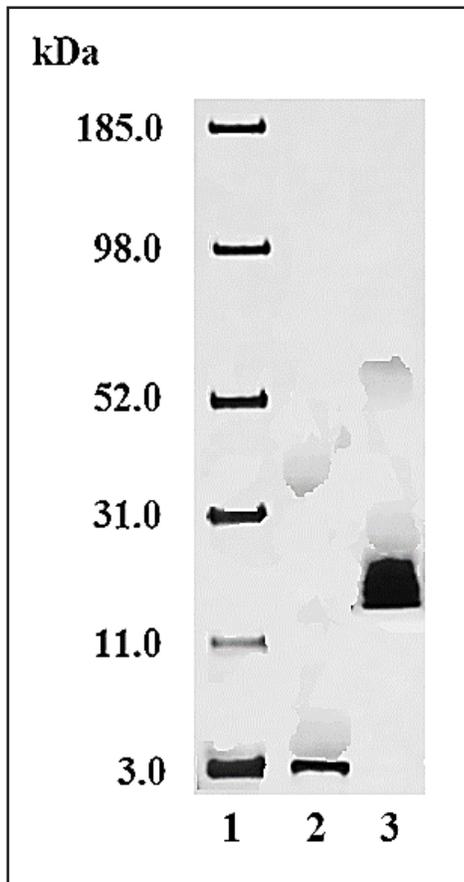

FIGURE. 2. SDS-PAGE ELECTROPHORESIS OF NATIVE AND CROTAMINE POLYMER. A SDS-PAGE (12.5 %) under non-reduced conditions of both native crotamine-like and crotamine polymer. (Lane 1): Molecular mass markers; (Lane 2): native crotamine-like ~3 kDa; (Lane 3) polymer ~ 18 kDa

Due to this intrinsic heterogeneity, and because it did not have *a previous* structural polymer crotamine information, the directed design of oligomer-specific antibodies was frequently randomly determined. They had a random probability distribution or pattern that may be analysed statistically, but may not be predicted specifically events. These limitations obstruct development in the field. Nevertheless, here, it is showed that the multivalent architecture of polymeric CLP, having multiple independent binding sites, can be used as a discriminating binder for an antibody, which is appropriate for the exposure of multiple epitopes, on the oligomeric and polymeric assemblies. This was successfully used for the immune system (through lymph node immunisation) that could recognise and build up an immune response of specific antibodies against CLP. As can be seen in TABLE 1, immunised mice sera reacted only with CLP, being capable of recognising the antigen in the ELISA assay. An immunisation was considered to be successful when an ELISA positive test with mouse anti-CLP mouse serum diluted at least 1:200 was obtained.

TABLE I
ELISA REACTIVITY OF NATIVE CDC CROTAMINE-LIKE AND CLP WITH IMMUNISED MICE POLYCLONAL ANTIBODIES. THE CONCENTRATIONS OF THE SAMPLES NATIVE AND POLYMER CLP WERE CALCULATED TO 5µg/ML

Normal	Mice serum (1:200)		0.055
	BSA		0.040
	No primary Ab		0.060
	No Secondary Ab		0.048
Controls	Medium Control		0.038
	Mice serum (1:200)	NCL	0.065
Immunised	Mice serum (1:200)	CLP	0.328

NCL: native crotamine-like. CLP: crotamine-like polymers

The polymers running above 18 kDa (crotamine-like monomers were ~4 kDa) showed that separation on the basis of size was achieved and fractions containing oligomers of particular molecular mass distribution were obtained, the progression throughout the fractions of large polymers from monomers was revealed (FIG. 2).

It is thought that the development of a technique that allows transforming a non-immunogenic molecule into an immunogenic one was performed by conjunction of both methods: the making of the glutaraldehyde polymers, but mainly because was used an innovative immunological technique via lymph node instead of spleen immunisation. As it is known, dendritic cells (Decs) play a crucial role in antigen presentation to CD4+ T cells, which open developed immune responses. Therefore, defining positive modulators of dendritic cell activation, to improve immune responses for low molecular masses antigens might be useful; Decs go through a route of differentiation identified as maturing. This advance considerably increases their ability for antigen handling and presentation [3]. The broad-spectrum facets of Decs progress are well empathised and comprise the redistribution of MHC-II molecules from the intracellular sections to the cell membrane, increased co-stimulatory molecules expression, such

as several cytokines (TNF-α and IL-12) [23,24].

It is believed that the immunisation pathway through the lymph node, which necessarily involved dendritic cells, makes the low molecular mass antigen well recognised and processed, as stated above. Thus, the both events, the production of a crotamine polymer and the intervention of dendritic cells, made the procedure successful.

This methodology would be of great importance in the field of immunology, not only for crotamine, but for other antigens of very low molecular masses. Probably, this original protocol of lymph node immunisation would permit a better immunological recognition and higher production of IgG, representing an alternative to the traditional protocol of splenic production, which generates a larger quantity of IgM.

The production of polyclonal antibodies anti- CLP is not only an original finding, as far as it is known, this is the first time that has been developed, allowing us to have a very useful tool in experimental processes, which could be carried out to track crotamine with labelled antibodies. This also opens an interesting field for the production of monoclonal antibodies, since it was demonstrated that there was an immunisation process and necessarily had lymphocytes recognising the antigen, which can be fused with myeloma cells, in order to obtain hybridomas producing monoclonal antibodies.

CONCLUSIONS

An initial aim of this project was to produce a large polymer of CLP through polymerisation, which was capable of inducing a humoral immune response by a protocol of lymph node immunisation, when introduced into C57/B inbred strains of mice. Successfully producing polyclonal antibodies immunologically tailored to crotamine, which has no precedent. Subsequent to constructing a large polymer through protein crosslinking, the ultimate goals of this endeavour was to produce polyclonal antibodies specific to CLP secreted by B-lymphocytes. In the future, the laboratory is planning to produce monoclonal antibodies using this immunisation protocol as a probe, in various tissues of vertebrates, and why not in invertebrates too.

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ETHICAL STATEMENT

Skilful workers did all the experimental proceedings regarding the use of live animals. The applicable regulations as well as institutional guidelines, according to protocols approved by by the Institute of Anatomy Ethical Committee of the Universidad Central de Venezuela, on 18 January 2019, under assurance number (#18-01-19). The research was carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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