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Crotalic Venom Fraction as Promoter of the Transfection Mechanism of a Genic Vaccine (Naked DNA) Against Rabies

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Abstract: The AL27 crotalic fraction is a lectin recognized by cell receptors and then introduced into the cytoplasm. In the present research a new transfection promoter agent for a non viral expression vector was used, using as a model a plasmid expressing the gene that codifies for the G glycoprotein of the rabies virus. The objective was to evaluate the ability of AL27 to increase the rabies plasmid transfection in *in vitro* and *in vivo* conditions. The AL27 fraction was isolated from the venom of *Agkistrodon piscivorus* by HPLC-RP. AL27 maintained its recognition by sialic acid receptors up to a titer of 64 HU. The pC38 plasmid was selected from a panel of 18 clones constructed by inserting the gene that codifies for glycoprotein G of rabies strain HQIMSS99 into the commercial pCI-neo expression vector. *In vitro* results showed that pC38, with or without AL27, transfected CHO cells under the calcium-phosphate system and using Confocal Microscopy (CM) increased fluorescence (+++) was observed with the mixture pC38/AL27, than with pC38 alone (++). Flow cytometry (FC) revealed expression percentages of 27.78 and 38.66% for pC38 and pC38/AL27, respectively. The *in vivo* experiment was performed using three groups of BALB/C mice: group 1 was inoculated with pC38 in the tibial muscle; group 2 with pC38 and AL27 and group 3, negative control, with only PBS. Tissues showed the highest expression by CM (+++) when using pC38/AL27. Antibodies detected ranged from 0.75-1.2 international units (IU) mL^{-1} for pC38 and from 1.1-1.27 IU mL^{-1} for pC38/AL27. Results showed that AL27 promoted cell entrance mechanisms, generating a better expression level of the gene that codifies for the G glycoprotein of the rabies virus and consequently enhancing the stimulation of the immune response.

Key words: Hemoagglutinins, lectins, plasmids, *Agkistrodon piscivorus*

INTRODUCTION

One of the main difficulties that genic therapy and vaccines confront is the need to use DNA releasing systems that are capable of surmounting poor pharmacokinetics, limited distribution and low efficiency entrance of DNA into the cell (Judge *et al*., 2006). These systems consist of vectors that are in charge of transporting and collaborating in the expression of the genetic material. Currently there are viral and non-viral transference vectors (McKnight and Tjian, 1986). The main viral vectors are retrovirus, adenovirus, adeno-associated virus, herpes virus and lentivirus (Judge *et al*., 2006). Within the non-viral vectors or systems are liposomes (Judge *et al*., 2006), transfection promoting sequences (plasmids) (Raki *et al*., 2006), cytokines (interleukin IL-6, IL-12) (Spitz *et al*., 2002), electroporation (Spitz *et al*., 2002; Khan and Draghia-Akli, 2005) or bombardment with gold particles (Donelly *et al*., 1997). Plasmids are effective in their capacity for expression but generally, *in vivo*, they require the addition of another release system due to their poor transformation efficiency. The development of new viral and/or synthetic systems that combine with the best elements of the current array of vectors has been suggested (Harris and Lemoine, 1996). The crotalic fraction AL27 is a lectin with haemoagglutination properties that is characterized by being recognized and transported into the cell through sialic acid type cellular receptors (Sharon and Lis, 1972). It has been isolated from the venom of *Agkistrodon piscivorus* (Viperidae family, Crotalinae subfamily). Its use has been focused on the determination of human blood

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groups through agglutination tests (Sharon and Lis, 1972). There is no background on the use of lectins (in this case AL27), as agents for facilitating transfection by non-viral expression vectors in genic vaccines. Nevertheless, type C which are isolated from plants (concanavaline A) (Hayakawa *et al*., 1995) and those present in dendritic cells are known to have affinity for viral antigens (Cambi *et al*., 2004). It is considered that if the plasmid carrier of genes that code for glycoprotein G of the rabies virus is administered at the same time that crotalic lectin AL27, which functions as a transfection promoter, the plasmid could be transported and penetrate into the cell. Another possible mechanism for entry of the plasmid could be that in which the AL27 fraction functioned with a reversible myotoxic activity favoring the entry and expression of the plasmid similar to the process described for cardiotoxins and bupivacaine (Gutiérrez and Cerdas, 1984; Hasseett and Whitton, 1986; Warren and Vogel, 1986; Jin *et al*., 2004). It is known that an injection of DNA into muscle that has been previously treated with cardiotoxin or bupivacaine increases the number of cells that express the antigen and increases the immune response. In this study the expression of exogenous DNA is mediated directly in regenerating cells or by the entry and expression of DNA by immune cells recruited into the damaged tissue (Hasseett and Whitton, 1986). The purpose of this research was to assess crotalic fraction AL27 as a possible facilitating agent of transfection of non-viral expression vectors susceptible to be used in therapy or genic vaccines. Therefore, a rabies plasmid bearing the gene that code for glycoprotein G of the rabies virus was selected as the experimental model.

MATERIALS AND METHODS

Tissue culture: Cell lines used were CHO (Chinese hamster ovary) and BHK-21 (Syrian baby hamster kidney). Both lines were sowed at a concentration of 2×10^5 cells mL^{-1}. The former were replicated in F12 HAM culture media while the latter in MEM Glasgow media. Both media were supplemented with 10% bovine fetal serum (BFS) and a mixture of penicillin-streptomycin at a proportion of 10000 UI/μg/mL (Cumming, 1995).

Viral strains: Two rabies virus strains were used. The first was the PV (Pasteur virus) reference strain with a titer of 10^{-5} $TCID_{50\%}$ (Tissue Culture Infections $Dose_{50\%}$), destined for serological diagnosis and the second was a national strain isolated from the follow-up of a human rabies case caused by a haematophagus bat known as HQIMSS99, with a titer of 10^{-3} $TCID_{50\%}$ destined for cloning and construction of the rabies plasmid.

PV strain was replicated by inoculation in 100% confluent single-layer BHK with 0.5 mL of viral suspension and incubated at 37°C for 1 h. The viral suspension was decanted and the single-layer was maintained in SFB free culture media for 48 h at 37°C. Viral release was done in 3 cycles of freezing/thawing at -70°C/ambient temperature. This was clarified by centrifuging at 3500 × G for 10 min and concentrated in a refrigerated centrifuge at 12000 × G for 1 h (Cumming, 1995). Strain HQIMSS99 was replicated by inoculating 0.3 mL of viral suspension in mouse brain and the gene was amplified from brain tissue.

Vaccine plasmid: Using reverse transcriptase polymerase chain reaction (RT-PCR) the gene that codes for protein G of the HQIMSS99 rabies virus was cloned. This was inserted into the Xba1 restriction site of the pCI-neo commercial expression vector. The resulting construct was replicated in *E. coli* (DH 10b) competent cells. Both products were incubated in ice for 30 min followed by 2 min at 42°C, then 1 min in ice and sowed for 24 h at 37°C in solid Luria Bertani (LB) media. Eighteen different clones were isolated and assessed (numbers 8, 11, 14, 15, 17, 18, 19, 20, 21, 22, 23, 25, 26, 29, 34, 38, 39 and 52). Clones and the selected plasmid were replicated once more in *E. coli* DH 10b using LB broth maintaining constant stirring at 300 rpm for 12-16 h at 37°C. The bacterial package was obtained by centrifuging at 6000 × G at 4°C for 15 min (Alonso and Castro, 2001; Schorr *et al*., 1999).

Purification was carried out with a modified alkaline lyses protocol in an anionic interchange column with a Qiagen commercial kit (Schorr *et al*., 1999; Anonymous, 2000; Birnboim and Doly, 1979). The process consisted in dissolving the bacterial package in a buffer solution (50 mM Tris-HCl, 10 mM EDTA, pH 8.0) with RNase and a lysis buffer (200 mM NaOH, 1% SDS) was added and mixed gently for 5 min at environment temperature. A neutralizing buffer was added (3.0 M potassium acetate), incubated in ice for 20 min and centrifuged at 20000×G for 45 min at 4°C. The supernatant was loaded into an anionic exchange equilibrated column (750 mM NaCl, 50 mM MOPS, 15% isopropanol, 0.15% triton, pH 7.0). DNA was clarified with a washing buffer (1.0 M NaCl, 50 mM MOPS, 15% isopropanol, 0.15% triton, pH 7.0) and eluted with a buffer solution (1.25 g NaCl, 50mM Tris-Cl, 15% isopropanol, pH 8.5). DNA was precipitated using isopropanol, centrifuged at 15000 × G for 30 min at 4°C and washed with 70% ethanol centrifuging at 5000 × G for 10 min. DNA was then dried at 55°C for 5 min and suspended in 10 μL of sterile water and stored at -70°C.

Verification of plasmid purity was carried out using electrophoresis (Laboratory Practices, 2002) of 1%

agarose gels in TBE buffer solution (45 mM Tris-borate, 1 mM EDTA, pH 8.0) at 30 mA, 80 V for 60 min. The molecular size marker that was used ranged from 8454-702 base pairs (bp). DNA concentration was determined through spectrophotometer using Hoechst's solution in 1×TNE buffer (0.2 M NaCl, 10 mM Tris-Cl, 1 mM EDTA, pH 7.0) and a 260 nm wavelength.

Isolation of the crotalic fraction: The AL27 crotalic fraction was obtained from lyophilized venom of *Agkistrodon piscivorus* using reverse phase high-pressure liquid chromatography (HPLC-RP) (Alvarado *et al*., 2004; VYDAC, 1995; Mancin *et al*., 1998) with a silica C_{18} gel and 50 μL injector. The venom was diluted at a rate of 10 mg mL^{-1} of HPLC grade water. The solution was filtered using a 0.22 μm pore size membrane. Dissolution and separation of polypeptides was carried out with 0.1% trifluoroacetic acid (TFA) in HPLC grade water (Solvent A) and 0.1% acetonitrile (ACN) in TFA (Solvent B) as an organic modifier. The elution gradient started at an initial proportion of 30% solvent B over solvent A for 10 min at a 1 mL min^{-1} flow rate. Absorbance was measured at a 280 nm wavelength. The protein obtained was concentrated by lyophilizing and then suspended in saline solution (0.15 M NaCl, pH 7.2) for its use.

Preservation of agglutinating activity of the fraction was determined using a Hemoagglutination (HA) (Sharon and Lis, 1972; Alvarado *et al*., 2004) test with 2.5% rabbit red blood cells in saline solution. The fraction was diluted from 1:2 up to 1:256 and the Hemoagglutination Units (HU) in the test were defined as the reciprocal of the final dilution in which agglutination was present (Gartner *et al*., 1980).

***In vitro* transfection:** In order to assess the expression of the 18 clones that were constructed, as well as that which was selected (Plasmid pC38) *in vitro* transfections were carried out using the calcium-phosphate method (Sambrook *et al*., 2001; Bulletin No. MB-315, SIGMA, 1997). Each clone was inoculated in CHO cell lines developed to an 80% confluence using a 5 μL dose at 1 μg μL^{-1} diluted in 6 μL of 2.4 M $CaCl_2$ and diluted to 60 μL with molecular biology grade water using gentle pipetting. A similar volume of 2X Hepes was added a drop at a time, mixing gently for 2-4 sec and incubating for 20 min at environment temperature. In clone pC38 inoculations were carried out in duplicate, one single-layer culture followed the normal transfection procedure while a second single-layer culture had the AL27 fraction added at a dose of 1.062 μg mL^{-1}. CHO cells treated with calcium-phosphate were used as controls. Incubation in the transfection media lasted for 16 h, which was then followed by a change into fresh media and further incubation at 37°C for 48 h. Evaluation of transfection efficiency was carried out with Confocal microscopy (CM) and Flow Cytometry (FC).

In order to carry out CM observation (Jallet *et al*., 1999) single-layers were fixed with 80% acetone in water and stained with polyclonal anti-glycoprotein G antibodies obtained by inoculating rabbits with a recombinant vaccine and with one for human use developed in Vero cells. The secondary antibody used was rabbit anti-IgG conjugated with fluorescein. Both steps were incubated using a humid chamber at 37°C for 30 min and washed with PBS. Samples were read using a 488 nm wavelength and 63 or 40 μm magnifications in a confocal microscope.

In order to carry out FC quantitative determination (Swartzendruber *et al*., 1985; Bogh and Duling, 1993) transfected cells were released by exposing them to trypsin-versene and suspended in PBS buffer with 0.1% sodium azide, 5 mM EDTA and 2% SFB. These were then stained with polyclonal antibodies and anti-IgG in a similar fashion to what was described above. Samples were incubated at 8°C for 1 h with gentle stirring. Cells were centrifuged at 3500 × G for 10 min and suspended in 1% paraformaldehyde v/w in PBS at 37°C. Counts of transfected cells were carried out through logarithmic expression and 10000 event counts.

In vivo transfection

Assessment of clones: Three week old BALB/C mice were used, distributed in 8 groups with 3 mice each. Each group was inoculated in the tibial muscle with clones 6, 8, 11, 14, 15, 20, 38 and 39 at a dose of 40 μg 0.1 mL^{-1} of sterile PBS (Helater, 2000). Blood samples were taken from each group 30 days after inoculation by distal severance of the tail after they had been anesthetized with Xylazine hydrochloride (20 μg mL^{-1}) and Ketamine chloride (500 mg mL^{-1}) diluted 1:3 with sterile PBS and dosed at 0.3 mL per mouse. Serum was obtained by centrifuging blood samples at 1500 × G for 10 min and a pool was formed for each group.

Determination of the immune response was carried out with western blot using the PV reference strain that was replicated in BHK cells and semi-purified by centrifugation at 20000 × G for 2 h in a 20% sucrose support. These were concentrated to no less than 50 μg mL. Samples were run in a 12% polyacrylamide gel at 15 and 50 mA for 10-45 min. An internal size marker weighing from 201-6.600 kDa was used. Transfer was carried out in a 0.2 μm pore nitrocellulose membrane run at 40 mA for 1 h (16, BIO-RAD. Mini-Protean II.

Electrotransfer Cell. Instruction Manual). Membranes were blocked with 3% bovine serum albumin, incubated with stirring using serum diluted to 1:40 with PBS-Tween 20 at 37°C for 2 h and then with mouse anti-IgG conjugated with peroxidase, diluted to 1:2000, at 37°C for 1 h. Diaminobenzidine (20 mg diaminobenzidine, 50 mL 50 mM H_3PO_4, 50 µL 30% hydrogen peroxide) was used as chromogen and incubated for 10 min with stirring. The reaction was stopped using distilled water and membranes were dried at environment temperature (Schwimmbeck *et al*., 1990).

Assessment of the selected clone (pC38) with or without fraction AL27: Three groups of ten 3-week old BALB/C mice were used. The first group was inoculated with 40 µg of pC38 diluted in 0.1 mL of sterile PBS in the tibial muscle. The second group was inoculated in the same location and pC38 dose but with 1.062 µg mL^{-1} of fraction AL27 added, while the third group was the negative control ((Helater, 2000). A total of five mice from each group were sacrificed on the third day after inoculation in order to obtain their tissues and observe them in CM. The remaining mice were kept alive to obtain serum at 30, 45 and 60 days after inoculation following the protocol mentioned above to assess their immune response using the Rapid Fluorescent Focus Inhibition Test (RFFIT).

Biopsies of the tibial muscle of approximately 3 mm were obtained for CM, a "squash" was done on glassslides and fixed with 80% acetone. Staining was carried out with polyclonal antibodies and anti-IgG, similarly to what was described above (Jallet *et al*., 1999). Plasmid transfection efficiency in the tissues was analyzed by observing in a confocal microscope at 488 nm with 63 or 40 µm magnifications.

The RFFIT (Smith *et al*., 1996) was carried out in a 96-well plate in which serial dilutions of 1:5-1:265 were done. The PV strain was added (at a titer of 10^{-5}TCID$_{50\%}$) at a rate of 1:400 (± 20 fluorescent focus/field ± 7) and incubated for 1 h at 37°C. The serum/virus suspensions were transferred into another plate with single-layer 100% confluent BHK-21 cells and incubated at 37°C for 24 h. Well contents was fixed in 80% acetone for 30 min and stained with anti-rabies conjugate. The titer was expressed in International Units (IU) and a standard serum with 10 IU was used as reference (Schwimmbeck *et al*., 1990).

RESULTS

Clone selection: It was found in the assessment of expression capacity of *in vitro* transfection through MC analysis that of the 18 constructed plasmid clones (numbers 8, 11, 14, 15, 17, 18, 19, 20, 21, 22, 23, 25, 26, 29, 34, 38, 39 and 52) 5 of them had efficient expression, which corresponded to clones 8, 20, 38, 39 and 52 (Fig. 1). When these clones were quantitatively analyzed using FC transfection percentages reached 9.38, 10.63, 21.8, 22.61 and 26.18% for clone 52, 20, 39, 8 and 38, respectively. The latter was selected and labeled as "pC38" (Fig. 2).

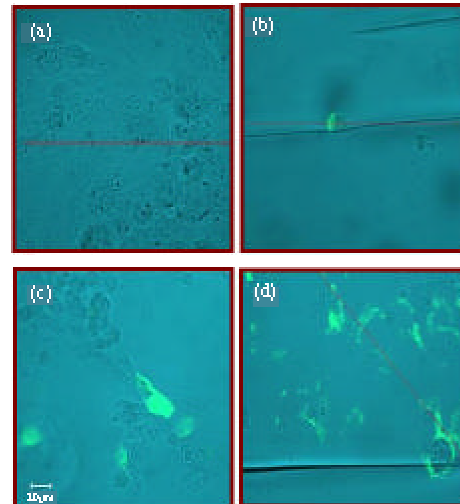

Fig. 1: Selection of rabies plasmid clones through confocal microscopy (CM). *In vitro* transfection of CHO cells with various national rabies plasmid clones and quantitative observation. Figure 1a shows the negative control inoculated with calcium-phosphate transfection media. Figure 1b shows a single-layer culture transfected with 5 µg of clone 20 demonstrating low expression efficiency manifested by fluorescence. Figures 1c and 1d correspond to transfections carried out under the same conditions and dosage with clones 8 and 38, respectively. Both show positive and favorable expression with clone 38 (pC38) exceeding in area and fluorescence intensity

Regarding *in vivo* selection of rabies clones the expression capacity of the rabies plasmid was determined according to the seroconversion efficiency when analyzing sera through western blot. Antigen-antibody recognition bands of 69 kDa with greater intensity were found in samples from mice inoculated with clones 8 and 38, while bands with lesser intensity were found in samples of sera with antibodies against clones 6, 20 and 39 (Fig. 3).

Once clone pC38 was selected, it was replicated and purified. A total of five lots were obtained with variable plasmid harvests ranging from 959-1237 ng mL^{-1}.

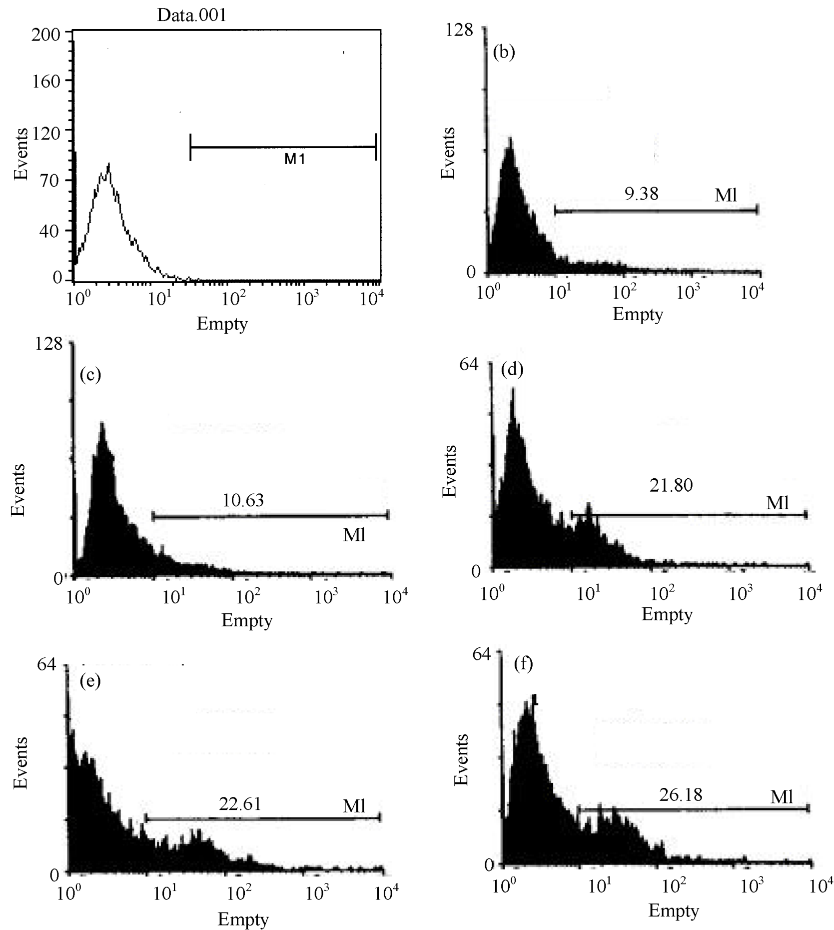

Fig. 2: Selection of rabies plasmid clones using Flow Cytometry (FC). Expression levels of various clones. *In vitro* transfection using the calcium-phosphate method in 80% confluent CHO cells inoculated with various rabies plasmid clones with a 5 μL dose at a 1 μg mL^{-1} concentration and assessing their efficiency by percentage quantification. Figure 2a shows negative control CHO cells treated only with calcium-phosphate solution. Figures 2b-2f show the expression levels obtained with clones 52, 20, 39, 8 and 38 (pC38) which had transfection percentages of 9.38, 10.63, 21.80, 22.61 and 26.18%, respectively

Crotalid fraction obtainment: The venom of *A. piscivorus* manifested 15 different polypeptides during the chromatography process for isolating the crotalid fraction, which migrated at elution times of 1.1', 1.39', 1.83', 2.11', 2.48', 2.72', 2.99', 3.62', 4.23', 4.92', 5.44', 6.04', 6.79', 7.32' and 8.28' (Fig. 4, shown as peaks), of which the one with elution time of 6.04' was the target polypeptide and was labeled "AL27". This fraction was collected at a proportion of 17 ng mL^{-1} from each 10 mg of venom subjected to the process.

In the biological activity tests of the 15 polypeptides and proteins that were collected only that which corresponded to peak 12 had the capacity to agglutinate rabbit red cells up to a dilution of 1:64 or 64 HU. The remaining polypeptides and the negative control were precipitated. The positive control consisted in complete *A. piscivorus* venom, which showed a marked agglutination activity (Fig. 5).

Fraction AL27 as promoter of the expression of the rabies plasmid: Through *in vitro* and *in vivo* transfection the capacity of AL27 to increase the expression levels of pC38 was determined. Figure 6 shows the *in vitro* results obtained through CM. The degree of fluorescence of inoculated single-layer cultures indicates a decreased or increased positive reaction, from plasmid only to plasmid plus crotalic fraction AL27. The transverse line in the figures indicates the passage of ultraviolet light on the transfected tissue and this information is translated into

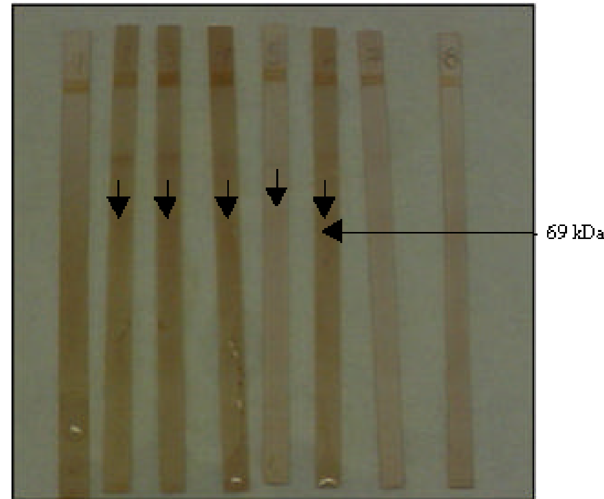

Fig. 3: *In vivo* selection of rabies clone. Detection of the immune response by western blot tests of samples from mice inoculated with 40 µg of various rabies plasmid clones (11, 8, 38, 6, 20, 39, 14 and 15) in the tibial muscle. Sera corresponding to mice inoculated with clones 8 and 38 show antigen-antibody recognition and/or seroconversion band of 69 kDa. Number 38 (pC38) was selected due to is higher recognition capacity. In sera of mice inoculated with clones 6, 20 and 39 there is a weak 69 kDa band. There is no recognition in mice sera inoculated with clones 11, 14 and 15

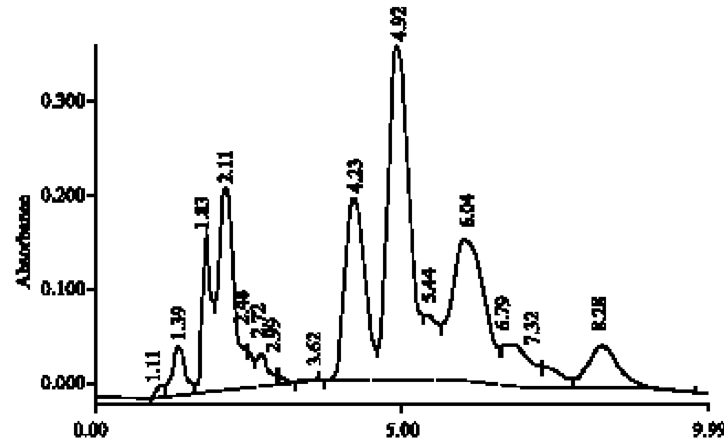

Fig. 4: Isolation of fraction AL27 from *Agkistrodon piscivorus* venom. Graph shows the 15 different polypeptides present in the venom of *A. piscivorus* which are manifested as peaks that correspond to elution times of each one during the process for obtaining fraction AL27 through reverse phase high-pressure liquid chromatography (HPLC.RP). Peak twelve, which has an elution time of 6.04', is the one corresponding to the fraction with agglutinating activity labeled AL27

graphs. In essence, it can be seen that the level of transfection by pC38 when inoculated on its own is less than when fraction AL27 is added.

The greater *in vitro* transfection capacity of the pC38/AL27 combination over pC38 on its own was also demonstrated with FC. Figure 7 shows that when the pC38 rabies plasmid was inoculated on its own single-layer cell cultures had a 27.78% transfection, while when fraction AL27 is added the level increases to 38.66%.

In the *in vivo* analysis, using CM, mouse tissues that had been inoculated with pC38/AL27 had cellular fibers more intensely transfected than when using pC38 on its own. Figure 8 shows the results obtained in this stage. The recognition and introduction of the plasmid

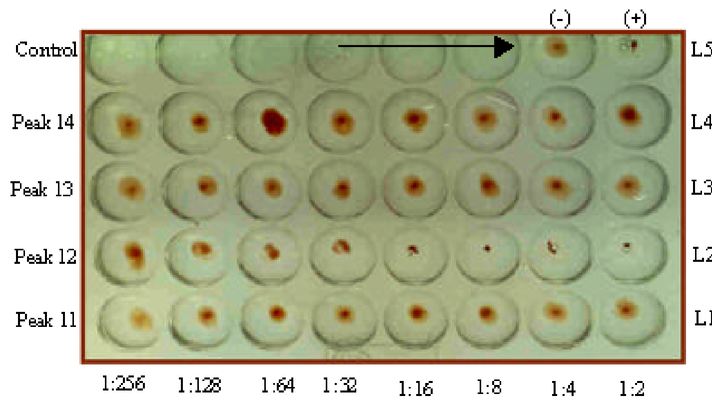

Fig. 5: Hemagglutinating activity of fraction AL27. Agglutination tests with fraction AL27 on rabbit red cells. Lines L1 to L4 correspond to polypeptides (peaks) 11, 12, 13 and 14 collected by HPLC-RP. Line L5, from right to left, contains positive (*A piscivorus*, 6.25 mg mL^{-1}) and negative control (saline solution). Dilution of the fractions goes from right to left (1:2 to 1:256). Line L2 (polypeptide or peak 12) shows agglutination up to a level of 64 HU (fraction AL27). Lines L1, L2 and L4 show precipitation of red cells

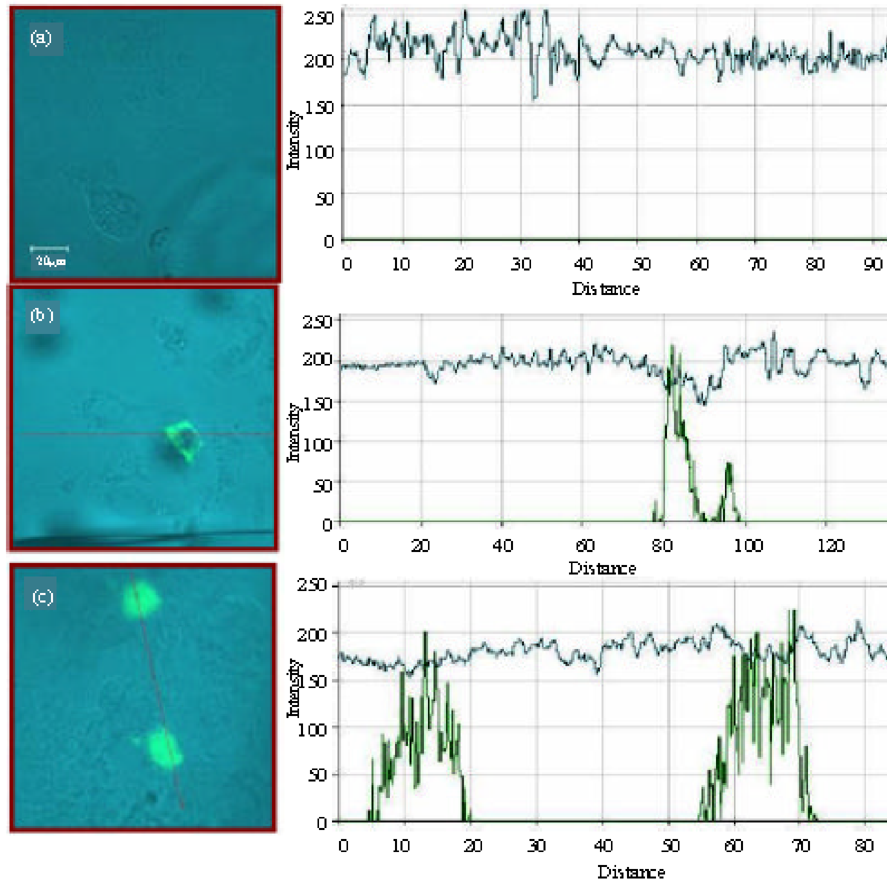

Fig. 6: *In vitro* expression of the pC38 rabies plasmid through CM. Transfection of CHO cells with the calcium-phosphate method with pC38 with or without added crotalic fraction AL27. Figure 6a shows negative control CHO cells, which were treated only with calcium-phosphate buffer and its corresponding graph without fluorescence. Figures 6b and 6c correspond to single-layer cultures inoculated with pC38 without fraction AL27 and with fraction AL27, respectively. CM shows transfection levels in the graphs to the right determined from fluorescence levels. Noteworthy is the use of the mixture pC38/AL27

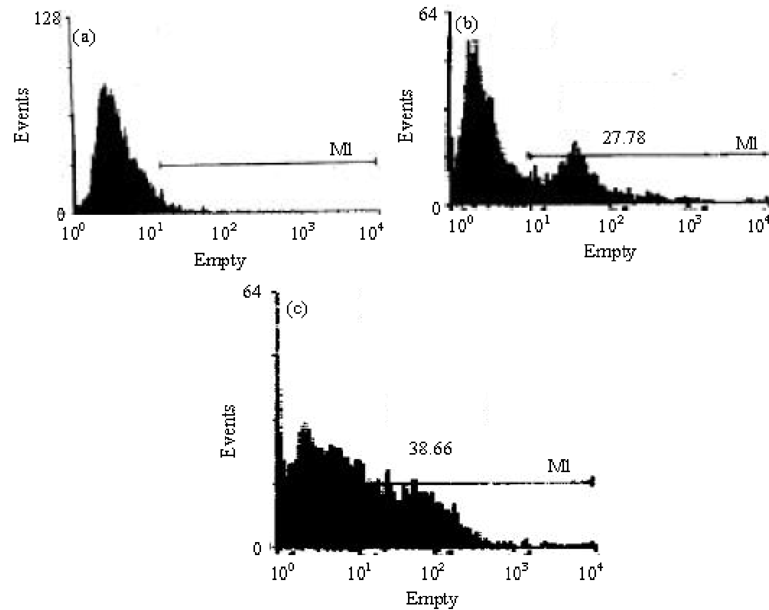

Fig. 7: *In vitro* expression of the pC38 rabies plasmid through FC. Increase in the expression efficiency of pC38 manifested as percentage of plasmid transfection. Figure 7a corresponds to the negative control CHO cells treated with calcium-phosphate buffer only. Figure 7b shows the use of only pC38, which generated a transfection percentage reaching 27.78%. Figure 7c on the other hand shows the same dose of pC38 with added crotalid fraction AL27, which increased the percentage to 38.66%

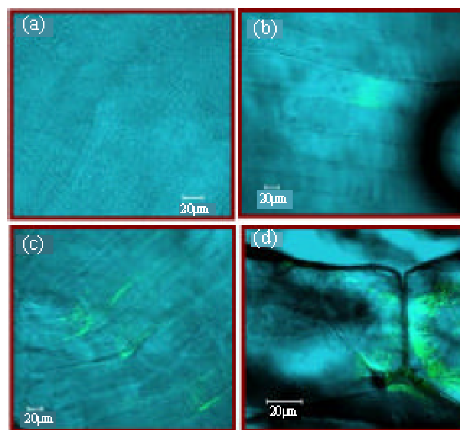

Fig. 8: *In vivo* expression of rabies plasmid through CM. Tissues from tibial muscle of mice inoculated with pC38 with or without added crotalic fraction AL27. Figure 8a shows the tissue from a negative control mouse. Figure 8b corresponds to the tissue inoculated only with pC38 demonstrating efficient expression. Figure 8c and 8d show tissues inoculated with the pC38/AL27 mixture showing a higher transfection capacity. Muscle fibers can be seen in Fig. 8c with abundant fluorescence, which indicated a higher transfection, while Fig. 8c is a close-up of the union between 2 cells which shows marked fluorescence at the membrane level

into muscle cells can be appreciated in a close-up of the muscular fiber joints due to their marked fluorescence (Fig. 8d).

Lastly, the *in vivo* assessment of the immune response stimulation by the pC38 plasmid, with crotalic fraction AL27 added or not, was detected with RFFIT and

the negative control had serum levels of 0.2, 0.2 and 0.3 IU mL^{-1} during the three serum samplings. Serum of mice immunized with only pC38 showed an increasing seroconversion of 0.75, 0.9 and 1.2 IU mL^{-1} at days 30, 45 and 60 after vaccination, respectively. Those that were inoculated with the pC38/AL27 mixture produced antibody levels that reached 1.1 and 1.27 IU mL^{-1} at 45 and 60 days post-vaccination, increasing slightly over time. It must be noted that WHO requires a minimum of 0.5 IU mL^{-1}. This result indicates that the plasmid not only is recognized and introduced by muscle cells, but that it is also is expressed and presented to the immune system.

DISCUSSION

The results obtained in this study demonstrate the effectiveness of using crotalid fraction AL27 as an agent for promoting transfection of the non-viral expression vector in a rabies genic vaccine. To our knowledge there is no background related to the use of this agent as a promoter of transfection by external genes, therefore no comparative analysis can be attempted. Nevertheless, there is research using other chemical agents such as bupivacaine and biological agents such as proteins isolated from snake venom like cardiotoxin, that have a similar action and have been previously used as transfection promoting agents in genic vaccines. In fact, research related to these products were the motive for selecting a lectin of the *Agkistrodon piscivorus* snake (AL27) and propose its use as a transfection promoter for non-viral expression vectors selecting at the same time a genic rabies vaccine (rabies plasmid pC38) as the experimental model.

It was thought that one of the possible mechanisms of action of crotalic fraction AL27 would be similar to that of bupivacaine, which acts by destroying muscle fibers causing infiltration by macrophages and proliferation of myoblast precursor cells that fuse and form syncytia (multinuclear giant cells) giving origin to a new viable myofiber (Hasseett and Whitton, 1996; Jin *et al*., 2004; Nichols *et al*., 1995). It is speculated that the momentary damage that is produced in membranes would be that which facilitates penetration of the plasmid that carries the gene of interest, in this case pC38, which is then followed by an increase in its expression and corresponding immune response.

In this study, the increased fluorescence of muscle tissue inoculated with plasmid pC38 plus fraction AL27, show in Fig. 8c and 8d, agree with the reports by Thomason and Booth (1990), who found an increase in expression of *E. coli* β-galactosidase genes within mice cell fibers in regeneration after treatment with bupivacaine and described a diffuse expression within the fibers and focal expression within sarcolemma. Furthermore, Jin *et al*. (2004) found that previous treatment of muscle tissue with bupivacaine increases genic expression between 30-50 times which in turn increases the immune response.

Another possible mechanism of action of fraction AL27 could be similar to that of cardiotoxin which is present in elapid snakes. In these, there is myotoxic activity, which is described as the ability to break the plasmatic membrane and alter its permeability regulatory function allowing the entrance of ions and macromolecules, as well as dilating the sarcoplasmic reticulum, generating changes in mitochondria and disorganizing myofibers (Gutiérrez and Cerdas, 1984; Manjunatha and Evans, 1990). Its use as transfection promoter in genic vaccines against rabies has resulted in contrasting results. While, Ray *et al*. (1997) reported that intramuscular inoculation of cardiotoxin 5 days before plasmid DNA is inoculated increases the mean antibody titer, Bahloul *et al*. (1998) when administering cardiotoxin four days before plasmids found no favorable effect in Th cell or neutralizing antibody production.

In this study it was considered that crotalic fraction AL27, similar to cardiotoxin, could alter muscle cell permeability favoring the entry of the plasmid and subsequent DNA expression. This was carried out with the notion that AL27 would not cause damage since Borkow *et al*. (1994), while evaluating the effect of crotalic venom on *in vitro* endothelial cells found that after the cell functions were altered due to the myotoxic effect of the venom there is a regeneration process which maintains cell viability and replication capacity. Furthermore, in a previous study carried out by the authors in which fraction AL27 was first isolated from the venom of *Agkistrodon piscivorus* permanence of cell viability was demonstrated *in vitro* and *in vivo* together with no damage to muscle tissues after treatment (Alvarado *et al*., 2004).

A further third possible mechanism of action of fraction AL27 could be associated to the recognition and union of crotalic lectin, a haemagglutinin, with cellular sialic acid receptors thus acting in this case as a carrying agent of the rabies plasmid. This is supported by other studies in which C-type lectins, which are calcium dependant, show a high affinity for this type of receptors (Drickamer, 1988; Drickamer, 1999) and their union sites, which are formed by sugars, facilitate their entry into the cell (Manjunatha and Evans, 1990).

Independently of the cellular mechanism that is triggered, fraction AL27 managed to act as a promoting agent for transfection of non-viral expression vectors by

contributing to the expression of the gene that code for glycoprotein G of the rabies virus both in *in vitro* as well as *in vivo* conditions. This makes it an element that can be used to improve the efficacy of genic vaccines and therapy. Nevertheless, there are still several factors to be solved, such as corroborating the mechanism of action triggered in the host cell by fraction AL27, sequencing studies of this protein for its large scale production and the assessment of the biological agent in large species.

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