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INCORPORATION OF NATIVE CROTOXIN INTO LIPOSOMES IN THE PROTECTIVE IMMUNISATION AGAINST THE VENOM OF *CROTALUS DURISSUS TERRIFICUS* (SOUTH-AMERICAN RATTLESNAKE)

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INTRODUCTION AND OBJECTIVES: The toxicity and the low immunogenicity, in some cases, of the venom/Freund's Adjuvant (FCA) emulsion, during the course of traditional methods of equine immunisation, have led to search for new immunisation systems in the production of commercial antivenoms. The incorporation of venoms into liposomes preserves antigenic sites with the advantages of increasing the immunogenicity and reducing the toxicity. The incorporation of *Crotalus durissus terrificus* (Cdt) whole venom together with lipopolisaccharide immunostimulant (LPS) into liposomes using the Reverse-phase evaporation vesicles (REVs), has been already evaluated on experimental animals successfully. However, when this preparation was tested in sheep or horses, it was either toxic or unable to elict antibody response. The general aim of this study is to evaluate different methods of preparing liposomes incorporating crotoxin (neurotoxic fraction from Cdt venom), select a preparation regarding the toxicity, stability and antigen entrapment and evaluate the adjuvant effects of liposome incorporation on the immunogenicity of native crotoxin in experimental animals.

MATERIAL AND METHODS: Native crotoxin was incorporated within liposomes using Dehydration-rehydration vesicles (DRVs) in the absence (DRV) or presence of LPS (DRV/LPS) or REVs methods or was emulsified with FCA. Crotoxin treated with $Os0_4$ (osmicated crotoxin) was incorporated into REVs liposomes treated with $Os0_4$ (osmicated REVs). Stability of membranes was evaluated in the presence of trealose by detecting the leakage of crotoxin after storage. Female mice (18-20 g) were immunised (s.c. route) with DRVs preparations or FCA and bled routinely during a period of 70 days. Blood samples were analysed using Enzyme linked immunosorbent assay (ELISA) to estimate antibody levels and the protective effect was evaluated by subsequent challenge with 8 LD_{50} of Cdt venom at the end of immunisation period.

RESULTS: DRV preparation was less toxic than REVs and FCA: intravenous injections of DRV preparation incorporating more than 90 LD_{50} of crotoxin do not cause animals' harm. The release of crotoxin after storage of DRV preparation in presence of trealose was inferior to 10%. Anticrotoxin production was dose dependent (the inoculation of different groups of mice with 6 or 20 or 70 or 350 µg of encapsulated crotoxin/20 g mice, increases proportionally antibody level). Booster injections (same dosade as primer) also increase antibody production. However, neutralization of lethal effects of Cdt venom, was the same for animals immunised with 20 or 70 µg/20 g mice (3 survivors out of 8). Preparations containing immunostimulants (DRV/LPS and DRV + $AlOH_3$) have an important effect of increasing antibody production to a level with a correspondent increase in protective effects. After the immunisation with DRVs/LPS preparations (3 doses of 20 µg of encapsulated crotoxin/20 g mice) the immune response protects 7 out of 8 animals to challenge with Cdt venom.

CONCLUSION: The DRVs preparation incorporating native crotoxin was less toxic than others preparations including FCA emulsion. DRVs preparations have shown to be stable in the presence of trealose. DRV/LPS preparations had adjuvant effect and the neutralization of lethal effects of Cdt venom was even higher than that of FCA emulsion. The DRV/LPS preparation is able to substitute the FCA system in animals' immunisation and therefore a putative candidate to vaccines against snake venoms.

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DETECTION AND MEASUREMENT BY IMMUNOASSAYS OF Crotalus durissus terrificus VENOM AND PURIFIED TOXINS IN MICE SERA.

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Snake bites were a severe public health problem in our country, which therapy was based mainly on immunotherapy with specific antisera. The most important and lethal accident was caused by South American rattlesnake, *C. d. terrificus*. Despite its higher efficiency, the specificity of antiserum required a correct diagnosis for adequate therapy, often demanding the use of less specific and potent polyvalent antisera, in the absence of clues for a correct medical diagnosis. Another problem was the measurement of injected venom, which would be a prognostic factor. To solve those diagnostic problems, we developed and stardardized two immunoassays for the detection and measurement of venoms and toxins, comparing immunoenzymatic or immunoradiometric approaches.

Both assays were developed on plastics microplates adsorbed with an anticrotalic horse antisera, which were deleted from its Fc domain. After blocking and washing, quantified venom, purified crotoxin, other snake venoms or sera from experimentally injected mice were applied on those wells, to combine with the specific antiserum. The bound antigen was reacted with a secondary antibody, obtained from rabbits immunized with irradiated *C.d.terrificus* venom, that presented highest titer and specificity, as determined by Western Blot and routine immunoassay. The bound secondary antibody was detected by two ways. For the immunoenzymatic detection(ELISA), we use a horseradish peroxidase conjugated anti-rabbit IgG, the development with o-phenylenediamine and hydrogen peroxyde, with optical density measurement on microplate reader. For immunoradiometric assay(IRMA), the secondary antibody was reacted with iodinated Protein A from *S.aureus*, and the remaining radioactivity detected on Gamma counter. The sensitivity of ELISA was 0.01 ng antigen/ml, ten times higher than IRMA, which presents only 0.1 ng/ml, but intertest variation precludes the use of this test on quantitative analysis. The IRMA, despite its lower sensitivity, presents a good correlation with added antigen, allowing its use on quantitative analysis. Both tests presented no cross reaction with venoms from other snakes from Brazilian area, mainly from *Bothrops* genera.

The above data show that IRMA and ELISA assay here presented could be used in the diagnosis and measurement of antigen in sera from snake bite patients with diagnostic problems.

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Venom characterization among Tityus serrulatus and Tityus bahiensis scorpions.

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Scorpion venoms have powerful toxic effects and the treatment of human scorpion envenomation is a difficult problem requiring an extensive knowledge of the venom components. The aim of this project is to clarify the Immunological cross-reactivity among the venom of Tityus serrulatus and Tityus bahiensis scorpions. It has been showed that antibodies against T. serrulatus crude venom is able to neutralize the lethal effects of T. serrulatus and also T. bahiensis venom. However T. bahiensis anti-venom is not able to neutralize the toxic effects of the T. serrulatus venom.

The venom of both scorpion showed a strong cross-reactivity with anti-T. serrulatus or anti- T. bahiensis rabbit policlonal antibodies as revealed by western blotting analysis and ELISA. The cross-reactivity against anti-alpha-type toxin (TsTx I) and anti-beta-type toxin (TsTx II) antibodies (toxins purified from T. serrulatus venom) were specifically analysed. Anti-beta-type toxin antibodies recognize the main beta-type toxin TsTx II (TS VII) from T. serrulatus venom and was able to detectd a strong signal (western blotting) in the T. bahiensis venom. By ELISA the presense of beta-type and alfa-type toxins was also demonstrated.

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Identification and characterization of different scorpion proteins sharing similar epitops with the non toxic protein TsNTxP.

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Scorpion venoms contain several basic proteins that are responsible for the neurotoxic effects of the venom. A non toxic protein (TsNTxp) from the scorpion Tityus serrulatus venom was isolated and characterized. TsNTxp showed to be an efficient immunogen where anti-TsNTxp antibodies were able to recognise and neutralize the effect of different toxins from the Tityus serrulatus and Tityus bahienses venom. To understand the effect of anti-TsNTxp antibodies and to establish structure/function relationships of different scorpion toxins, we decided to compare the antigenic cross-reactivity among different proteins of T. serrulatus venom and analyze the cDNA and amino acid sequence of all venom proteins recognized by anti-TsNTxp antibodies. We prepared an unidirectional lambda ZAP cDNA library from the venom glands and the screening was carried out using the anti-TsNTxp antibodies. From this screening we have detected several positive signals and the clones are currently being characterized.

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VACCINATION AGAINST KALA-AZAR WITH THE FML-VACCINE. COMPARATIVE ANALYSIS OF THE HEMOLYTIC AND ADJUVANT POTENTIAL OF ***Periandra mediterranea***, ***Smilax officinalis*** AND COMERCIAL SAPONINS.

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Kala-azar is a frequently lethal disease whose severe aspects were related to the progressive supression of the cellular immune response. No vaccine against Kala-azar is available at present. Recently, we described the FML antigen of ***Leishmania donovani***, as a complex glycoproteic fraction. The FML vaccine, using saponin from comercial origin (Riedel De Haen), developed a significant protective effect increasing the specific splenocyte proliferation and antibody response and reducing the parasitic load in the CB hamsters and Balb/c mice. In Swiss Albino mice this saponin and Alumina were more potent than the Freund's Incomplete Adjuvant, both through the intraperitoneal and the subcutaneous immunization routes. An indesirable hemolytic effect has been described for several saponins. In this work we comparatively analyzed the haemolytic and adjuvant activities of comercial Riedel De Haen saponin and of purified and chemically characterized saponins obtained from ***Periandra mediterranea and Smilax officinalis*** The Haemolytic dose 50 (HD50) was determined using 0.5% human normal blood red cell, incubated for 30 min at 37^0C with increasing concentrations of saponin in saline and further centrifuged at 1000 rpm for 10 min. Supernatants were assayed for the presence of free Haemogloblin by screening at 412nm. Saline and distilled water were included as minimal and maximal haemolytic controls. The saponin concentration inducing 50% of the maximum hemolysis was considered the HD_{50}. We could demostrate an HD50 of 19.2 ug/ml for ***Smilax Officinalis***, 17.5 ug/ml for Riedel De Haen's while no hemolysis was detected for the saponin of ***Periandra mediterranea*** even at 100ug/ml. The adjuvant effect of the saponin of ***Periandra mediterranea***, was further assayed in SW females, immunized with three weekly doses of the FML (150µg) and 100ug of saponin, either through the intraperitoneal or the subcutaneous route. Saline- and saponin treated animals were included as controls. Seven days after the third antigen injection, the anti-FML antibody levels were assayed in all the vaccinated groups. The response was higher in the IP group (score 1.614) than in the SC one (1.154). Comparing with the previous studies using comercial saponin (Riedel de Haen), Freund Adjuvant and Alumina, the use saponin of ***Periandra mediterranea*** as adjuvant showed no hemolytic effect and a signifficant induction in antibody synthesis.Support:FNS,CNPQ,FINEP,CEPG-UFRJ.