



Immunological and allergenic responses induced by latex fractions of *Calotropis procera* (Ait.) R.Br.

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Abstract

Immunological and allergenic responses against the latex of *Calotropis procera* were investigated in mice by oral and subcutaneous routes. The latex was fractionated according to water solubility and molecular size of its components. The fractions were named as non-dialyzable latex (NDL) corresponding to the major latex proteins, dialyzable latex (DL) corresponding to low molecular size substances and rubber latex (RL) which was highly insoluble in water. Anti-sera against these fractions were assayed for total IgG and IgA titration by ELISA and IgE and IgG₁ were quantified by passive cutaneous anaphylaxis (PCA) in rats and mice, respectively. None of the fractions induced antibodies level increases when mice received latex fractions by oral route and thus, did not develop allergy. Nonetheless, anti-sera of mice sensitized with NDL and RL by subcutaneous route displayed considerable immunological response while DL did not. IgG level augmented consistently against NDL and RL while IgA response was detected only to NDL. NDL and RL induced very strong PCA reactions suggesting that both fractions would contain latex substances involved in allergy. Furthermore, protein analysis of NDL and RL suggests that RL still retain residual proteins abundantly found in NDL that could explain its similar allergenic effect. No IgG₁ reaction was detected in any of the anti-sera tested. According to the results, the proteins of latex of *Calotropis procera* can provoke allergy by subcutaneous route. The NDL has previously shown to display anti-inflammatory and analgesic activities by intraperitoneal injection. It should be relevant to determine whether NDL could induce such activities when assayed by oral route since it was ineffective to induce allergy by this way.

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1. Introduction

Adverse reactions to proteins containing-rubber has long been recognized in the form of contact dermatitis (Sussman et al., 2002). Likewise, undesired allergenic reactions have been well documented to occur in health care workers due to excessive exposure to manufactured latex-derived products such as gloves. More than 99% of the manufactured products made of natural rubber have the latex from the rubber tree (*Hevea brasiliensis*) as the main chemical constituent. Hypersensitivity reactions

against latex may occur shortly after allergen exposure and it is well established that very small amounts of latex proteins still remaining in rubber manufactured products are responsible for allergenic reactions (Yeang et al., 2002).

More than 12,000 plant species are latex producers and latex fluids are frequently reported to possess relevant biological activities and applications. Recently, the plant *Calotropis procera* has received special attention because of distinct relevant activities found to be present in its latex (Arya and Kumar, 2005; Choedon et al., 2006; Ramos et al., 2006a).

The plant *Calotropis procera* is a non-cultivated shrub of geographic distribution covering Asia, Africa and Northeast of South America. The medicinal uses of the plant are well documented in the poorest regions of India and many of the curative properties of the plant have been reported (Iqbal et

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al., 2005). Similar to *Hevea brasiliensis*, *Calotropis procera* produces appreciable amount of endogenous latex that can be easily collected from the youngest leaves when they are damaged. Pharmacological properties of the latex include pro- and anti-inflammatory, antinociceptive, antipyretic and antidiarrhoeal activities among others (Kumar et al., 2001; Larhsini et al., 2002). We have estimated that rubber fraction and soluble proteins comprises 84% and 9% of the dry matter of latex of *Calotropis procera*, respectively. Our recent results suggest that a soluble protein fraction purified from the poly-isoprene fraction exhibits anti-inflammatory and analgesic properties while strong inflammatory reaction is induced by another latex fraction (Alencar et al., 2004, 2006; Soares et al., 2005). It would be thus interesting to investigate the immunological properties and allergenic effects of these fractions since the whole latex of *Calotropis procera* has been previously reported to possess strong inflammatory action (Shivkar and Kumar, 2004), to induce permanent endothelial cell injury after contact (Al-Mezaine et al., 2005), and it can cause dermatitis (Handa et al., 1984). In this study the whole latex of *Calotropis procera* was fractionated in three distinct fractions. These fractions were evaluated to their ability of stimulating specific antibodies synthesis in mice by both oral and subcutaneous routes of administration.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA) fraction V, Tween 20, Goat anti-mouse IgA (α -chain specific) alkaline phosphatase conjugated, Rabbit anti-mouse IgG (whole molecule) alkaline phosphatase conjugated and *p*-nitrophenyl phosphate disodium were purchased from Sigma Chemical Co., St. Louis (USA). Microtitration plates (U96-POLYSORP SURFACE-NUNC-IMMUNO PLATE) were from Nunc Brand Products, Nalge Nunc International, Denmark. Other chemical were of analytical grade.

2.2. Botanical material

The latex of *Calotropis procera* (Ait.) R.Br. (Asclepiadaceae) was collected of plants growing wildy in the vicinity of Fortaleza-CE, Brazil and the plant exsiccate (sample specimen no. 32663) as authenticated by Prof. Edson Paula Nunes, was deposited at the Prisco Bezerra Herbarium of the Universidade Federal do Ceará, Brazil.

2.3. Animals

Animals were obtained from the Central Animal House of the Universidade Federal do Ceará, Brazil where they were produced. Twenty-one-day-old mice (Swiss) were maintained in laboratory cages with free access to water and commercial food until reached 25–30 g under light periods of 12 h and $27^{\circ}\text{C} \pm 2$ in appropriate sanitary conditions before using in experiments of sensitization. To develop PCA reactions, 6-month-old rats weighting 500–600 g and 6-month-old mice weighting 35–40 g

were received from the animal house a week before using and were maintained under similar conditions as above. All experiments involving animals were conducted in accordance to the rules of the Ethic Committee for animal experimentation of the Universidade Federal do Ceará and essentially as determined by international rules.

2.4. Latex preparation

The crude latex of non-cultivated and healthy plants was collected in distilled water (ratio 1:1) in plastic tubes that were shaken gently, closed and maintained at environmental temperature ($25\text{--}28^{\circ}\text{C}$) until handled in the laboratory. The samples were initially submitted to centrifugation at 25°C during 10 min in a bench centrifuge. The precipitated rubber was separated of the soluble phase that was submitted to dialysis against water using membranes of 8000 molecular weight cut-off. During the first hour of dialysis the volume of water used to dialysis was exactly the volume of latex material into dialysis tubes. This water of dialysis was pooled and named dialyzable latex (DL). After additional 60 h of continuous dialysis the membrane-retained material was newly centrifuged as before and the very clean and water soluble material was separated of the new pellet and named non-dialyzable latex (NDL). The new pellet was joined to that of the first centrifugation and named rubber latex (RL). The three latex fractions were thus freeze-dried and used in the further determinations.

2.5. Protein content

Protein content in latex samples used to induce antibodies synthesis was estimated according the method of Bradford (Bradford, 1976) using bovine serum albumin as reference.

2.6. Protein analysis

Protein profile of rubber fraction was evaluated by 12.5% polyacrylamide gel electrophoresis under denaturing conditions according to (Laemmli, 1970). Samples of the rubber fraction were first extracted with different solvents (distilled water, 1 M NaCl, 1N NaOH and 1N HCl) throughout one hour, centrifuged at $10,000 \times g$ and 10°C during 10 min. The soluble materials were submitted to dialysis and lyophilized prior to analyses. Samples of such materials, including the non-extracted rubber were dissolved in 0.0625 M Tris buffer pH 6.8 containing 1% SDS and 1% 2-mercaptoethanol. Insoluble materials were removed by light centrifugation as before and the soluble phase was submitted to electrophoresis after adding of $1 \mu\text{l}$ 5% bromophenol blue and 10 mg sucrose. Protein profiles of NDL and DL have been previously determined (Alencar et al., 2004; Ramos et al., 2006a).

2.7. Subcutaneous immunization

Groups of 15 2-month-old male Swiss mice weighting 25–30 g received subcutaneous injection of 100 μg of NDL, DL or RL in 0.5 ml of sterile saline containing 1 mg of aluminum

hydroxide as adjuvant. Booster injections were carried out on the 21st and 35th day after the primary doses. Mice were bled from the retro-orbital plexus just before first immunization (day 0) and at 7, 14, 21, 28, 35 and 42 days after first injection. For this, mice were first slightly sedated with 10% chloral hydrate in saline (25 μ l/10 g of body weight) by intraperitoneal injection. After collecting, sera were allowed to repose for 30 min at 37 °C and thus slightly centrifuged in a bench centrifuge during 10 min at 25 °C and the clean material stored at –20 °C until use.

2.8. Oral immunization

Groups of 15 2-month-old male Swiss mice weighting 25–30 g received oral doses of 5 mg/kg of NDL, DL or RL in 0.5 ml of sterile saline daily, until 10th day and additional boosters were given on 21st and 35th day following the same protocol by oral route. Anti-sera were recovered similar as before and animals handled essentially as described above.

2.9. Enzyme linked immunosorbent assay (ELISA)

ELISA was carried out in triplicates as previously reported using anti-NDL, anti-DL and anti-RL as primary antibodies (Ramos et al., 1996). Alterations in latex-specific IgA and IgG antibodies levels in sera of mice sensitized with latex fractions were estimated using goat anti-mouse IgA and rabbit anti-mouse IgG both conjugated to alkaline phosphatase as secondary antibodies, respectively. Experiments were developed in 96-well microplates and antigens were prepared at concentrations varying of 0.195–1600 μ g (NDL); 7.8–500 μ g (DL and RL) in all measurements. Pre-immune and 42 days sera were diluted in the ratio of 1:10,000 and secondary antibodies phosphatase alkaline conjugated were diluted to 1:2000. Kinetics of IgG synthesis in mice receiving NDL by subcutaneous immunization was followed using anti-sera of days 0, 7, 14, 21, 28, 35 and 42. Alkaline phosphatase reaction using the substrate *p*-nitrophenyl phosphate disodium (5 mg/ml) was stopped by adding of 50 μ l of 4N NaOH and absorbance was determined at 492 nm.

2.10. Antibody titrations by passive cutaneous anaphylaxis (PCA)

The IgG₁ antibodies were measured in pools of sera of immunized mice by PCA in 6-month-old Swiss mice using a 2 h latent period for skin sensitization as cited in (Melo et al., 1994). The PCA reactions were elicited by intravenous injections of the antigens *via* retro-orbital plexus. The antigens were prepared in 0.5% Evans blue in sterile saline to correspond to 250 μ g in 250 μ l. Control groups were formed by animals that received sterile saline or non-immune sera (day 0).

The titers of IgE antibodies were determined in pooled sera of mice by PCA in 6-month-old Wistar rats similar as in (Mota and Wong, 1969) using an 18 h latent period for skin sensitization. The PCA reactions were elicited by intravenous injections of the antigens *via* penis vein. The antigens were prepared similar as above using doses of 2 mg/ml. Controls were assayed as above.

Table 1
Protein content estimated in *Calotropis procera* latex fraction

Latex fractions	Protein content (mg/ml)
Whole latex	5.2
NDL	9.8
RL	2.2
DL	0.32

Data shown are mean of three independent experiments in all cases.

2.11. Statistical analyses

Statistical analyses were performed using one-way analysis of variance (ANOVA) to results of IgE titles of anti-sera of mice that received samples subcutaneously. Other results were shown as average of three different experiments.

3. Results

Three distinct fractions were prepared from the whole latex of *Calotropis procera*. Protein content of such fractions is shown in Table 1. The richest protein fraction named NDL has been characterized in previous works and exhibits great diversity of proteins (Alencar et al., 2004). The DL, poor in proteinaceous material has been shown to contain compounds implicated in the pro-inflammatory activity previously found in the whole latex (Alencar et al., 2006). The RL represents the rubber fraction of the latex that precipitates in aqueous medium. This fraction as well as products of their further extraction in water, acidic, basic and strength ionic solution was analyzed by electrophoresis as shown in Fig. 1. As observed in the figure various proteins can be still extracted of RL. However, the profiles of these newly extracted proteins were recently shown to match exactly with those in found in NDL despite the way they were

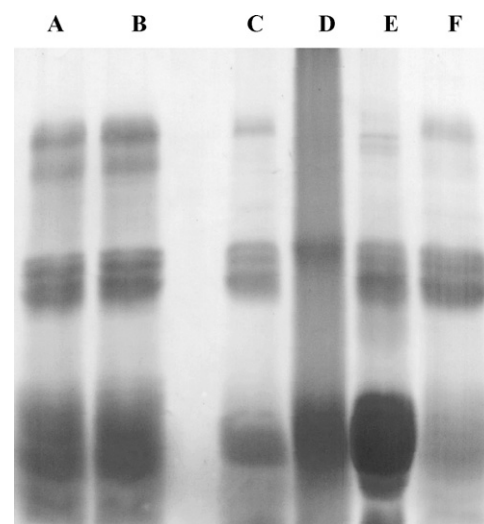


Fig. 1. Polyacrylamide gel electrophoresis (12.5%) pattern of RL fraction (A, B) and RL extracted with 1 M NaCl (C), 1N NaOH (D), 1N HCl (E) and distilled water (F). Twenty milligrams of each material were applied in all cases.

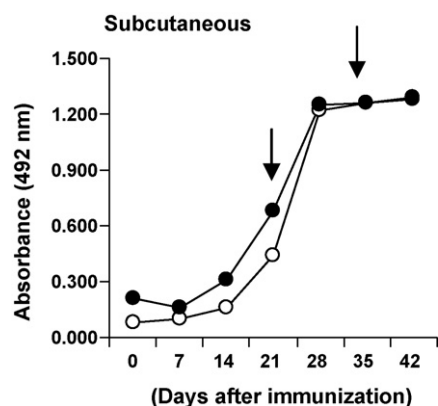


Fig. 2. Kinetics of *Calotropis procera* latex-specific serum antibody (IgG) response of mice in two different concentrations 8 µg/ml (○) and 500 µg/ml (●) after subcutaneous immunization with non-dialyzable latex (NDL) detected by ELISA. Arrows indicate the days of booster injections.

re-extracted (Alencar et al., 2006). Thus it is expected that proteins still found in the highly water insoluble RL fraction should represent soluble proteins naturally found in NDL. Probably they co-precipitate with rubber by imprisonment during the coagulation-like event involving the poly-isoprene chains that initiates immediately after collecting the latex. Such an event generates the rubber aspect of the material. Strong association of *Calotropis procera* latex proteins with rubber may be similar to that observed in manufactured products of latex from *Hevea* that still retain minor quantities of proteins even after chemical procedures applied to process the rubber. These resting were shown to be involved in allergy of *Hevea* latex derived products (Sussman et al., 2002). Accordingly, RL of *Calotropis procera* was investigated to determine its allergenic potential.

Subcutaneous injections of NDL at 0, 21st and 35th day after the primary doses induced a typical profile of IgG synthesis as shown in Fig. 2. Doses as lower as 8 µg/ml of NDL were enough to induce cross-reaction with primary antibodies present in sera. DL was fully ineffective while RL induced an IgG synthesis comparable to that of NDL (Fig. 3). Increment of IgA synthesis was observed in anti-sera of animals that received NDL but it was not detected in anti-sera of animals sensitized with DL and RL fractions (Fig. 4).

The fractions of latex of *Calotropis procera* were also tested to induce immunological stimulation in mice by oral route of sensitization. Only a discrete augmentation of IgG level was perceptive in anti-sera against NDL and RL while DL did not change IgG profile (Fig. 5). Apparently, IgA synthesis was not stimulated by any of the fractions when animals were sensitized by oral route with latex fractions (not shown). It should be mentioned that in the protocol designed for oral sensitization, animals received antigens daily until the 10th day and additional doses at days 21 and 35, even administered by oral route. Doses of RL corresponding to 10 mg/ml provoked animal death before 24 h after administration.

Possible allergenic effects induced by NDL, DL or RL fractions were investigated by PCA in rats and mice to estimate IgE and IgG₁ synthesis, respectively. The latex constituents were not able to induce synthesis of IgG₁ by neither oral nor sub-

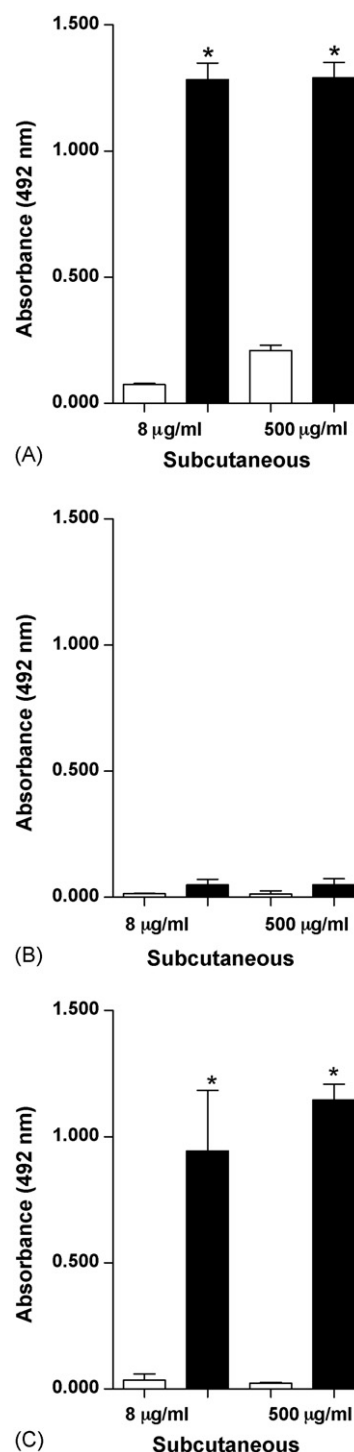
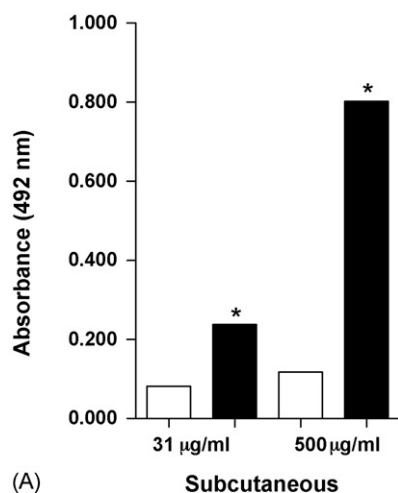
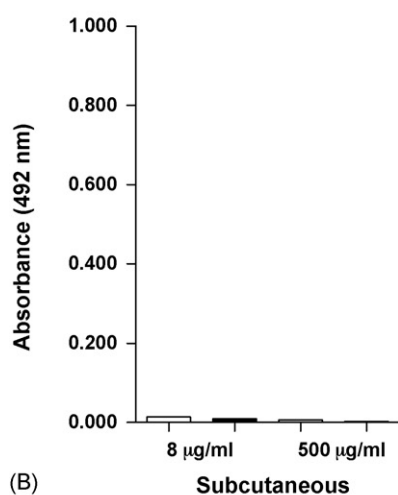


Fig. 3. *Calotropis procera* latex-specific serum antibody (IgG) response corresponding to 0 (○) and 42 (●) days after subcutaneous immunization of mice with: (A) non-dialyzable latex (NDL); (B) dialyzable latex (DL); (C) rubber latex (RL) detected by ELISA. * $P < 0.05$, significantly different from day 0.

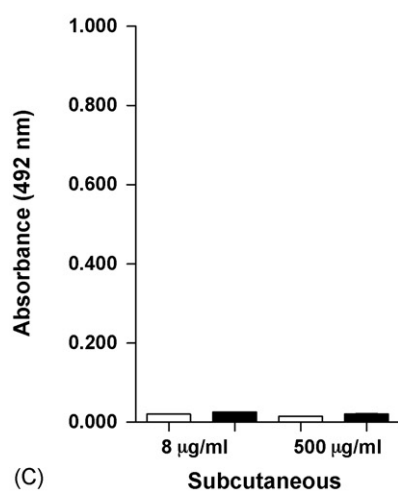
cutaneous route. However, as shown in Fig. 6 synthesis of IgE was estimated to occur in animals that received NDL and RL by subcutaneous stimulation. Such a response was not detected in anti-sera of animals that received the same samples by oral route, suggesting that latex constituents may induce a tolerance in animals receiving continuous doses of antigens (NDL/RL)



(A)

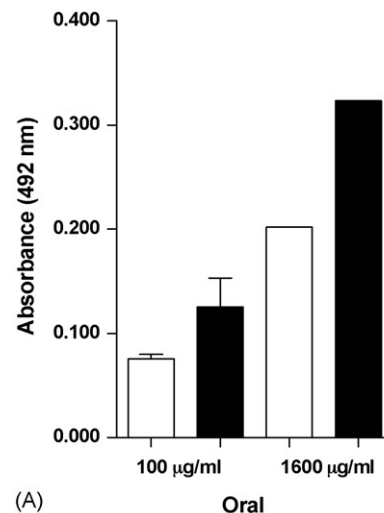


(B)

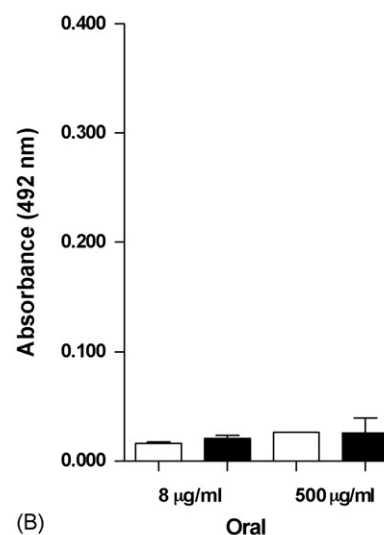


(C)

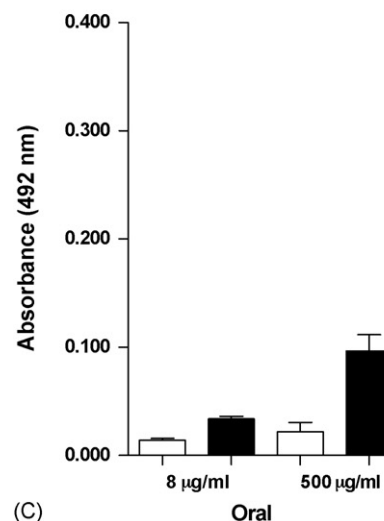
Fig. 4. *Calotropis procera* latex-specific serum antibody (IgA) response corresponding to 0 (○) and 42 (●) days after subcutaneous immunization of mice with: (A) non-dialyzable latex (NDL); (B) dialyzable latex (DL); (C) rubber latex (RL) detected by ELISA. * $P < 0.05$, significantly different from day 0.



(A)



(B)



(C)

Fig. 5. *Calotropis procera* latex-specific serum antibody (IgG) response corresponding to 0 (○) and 42 (●) days after oral immunization of mice with: (A) non-dialyzable latex (NDL); (B) dialyzable latex (DL); (C) rubber latex (RL) detected by ELISA. Values do not differ significantly ($P > 0.05$).

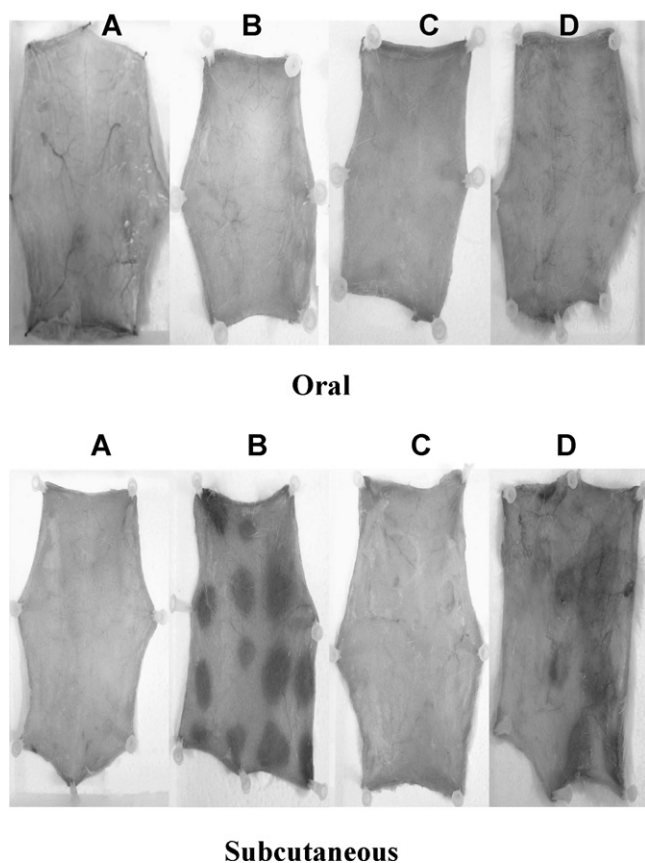


Fig. 6. Response of *Calotropis procera* latex-specific serum antibody (IgE) after oral (high) and subcutaneous (low) immunizations of mice as detected by PCA. (A) Serum obtained from pre-immune mice; (B) antiserum obtained from mice immunized with non-dialyzable latex (NDL); (C) antiserum obtained from mice immunized with dialyzable latex (DL); (D) antiserum obtained from mice immunized with rubber latex (RL). The PCA reaction was challenged by 2 mg of each respective latex fraction in 1 ml saline containing 0.5% Evans blue.

by oral route. As judged from the production of IgE, the allergenic capacity of NDL is not prominent when the stimulation is induced by oral route. Subcutaneous sensitisation, however, seemed to induce IgE synthesis more intensive against NDL than to RL (Fig. 7). As a rule, the latex proteins induce immunological response and animals receiving latex samples *via* subcutaneous were more sensitive to produce IgE.

4. Discussion

There is an increasing interest concerning the plant *Calotropis procera* due to innumerable relevant biological activities found in its vegetative tissues. It is easy to obtain *Calotropis procera* latex where the plant is found because of its extraordinary endogenous production. It would not be surprising *Calotropis procera* to produce more latex than *Hevea brasiliensis* in a comparative basis. Twenty milliliters of latex can be collected within 12 min. This certainly contributed to the exploitation of the latex for curative purposes. The plant seems to be deeply inserted in folk medicine in India. In Brazil poorest people use the crude latex mainly to combat skin infections by topic application. There is some information of people that

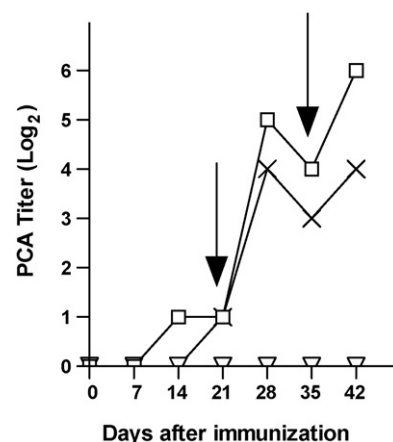


Fig. 7. IgE immune response to protein fractions of *Calotropis procera*. Swiss mice were immunized by subcutaneous route with 100 μ g of protein fractions: non-dialyzable latex (NDL) (□); dialyzable latex (DL) (▽); rubber latex (RL) (×) mixed with 1 mg of aluminum hydroxide in saline by injection. Boosters with each protein fraction are indicated by arrows. PCA reactions were challenged in rats for IgE with 2 mg of the corresponding protein fraction used for immunizing. All values refer to pooled sera of 15 mice and are means of three experiments. Differences between control and experimental groups are significant with values of $P < 0.05$.

ingest the crude latex diluted in water to combat hyperglycemic effects. Such usages may provoke unexpected effects by continuous latex exposure not yet identified. Rasik et al. (1999) reported that latex of *Calotropis procera* has healing potential on dermal wounds in Guinea pigs. However, adverse effects of latex of *Calotropis procera* have also been reported as cited before (Alencar et al., 2006). Furthermore, no information is available concerning possible allergenic effects caused by latex contact. We have accumulated efforts to fractionate the whole latex of *Calotropis procera* in attempt to separate interesting activities from undesired properties. The NDL, DL and RL fractions were obtained after executing an ordinary protocol based on centrifugation and dialysis that gave rise to a fraction (NDL) that anti-inflammatory and antinociceptive properties were joined (Alencar et al., 2004; Soares et al., 2005). Recently we have shown that both NDL and DL are also potent in prevent development of larvae of *Aedes aegypti*, the mosquito vector of Dengue virus transmission (Ramos et al., 2006a). Proteins of NDL were also shown to be devoid of acute toxicology and could not be found in fecal material of rats receiving doses by oral route for 35 days (Ramos et al., 2006b). Despite the relevance of these results, it is not known yet whether the latex of *Calotropis procera* displays adverse allergenic effects and if does, what could be the contribution of the latex proteins in such an undesired event. This question has motivated the present study as well the fact that the latex has been shown to exhibit anti-inflammatory and anti-cancer activity when tested by oral route in rats (Arya and Kumar, 2005; Choedon et al., 2006).

As it could be expected more expressive immunological responses were verified in animals that were sensitized by subcutaneous route. The DL fraction was unable to amplify antibodies synthesis. However, it has been already determined that all inflammatory potency of the whole latex of *Calotropis procera* is present in this fraction (Alencar et al., 2006). The DL fraction

is composed of salts and many other low molecular substances some of such already described (Seiber et al., 1982; Shivkar and Kumar, 2003). In addition we have determined the presence of peptides in DL although any further biochemical or functional characterization has been done yet. The very low concentration of such molecules in DL could certainly explain absence of detectable immunological response.

Even by subcutaneous route NDL as well as RL elicited IgE synthesis that evidences the presence of proteins in these fractions able to induce allergy. Nevertheless, this profile was not repeated in animals receiving NDL or RL by oral route, even considering that repetitive doses were initially done for 10 consecutive days. These findings provide evidences for an inducible tolerance acquired by mice receiving latex proteins by oral route. In a recent study, we have submitted groups of rats to receive doses of NDL by oral route during 35 consecutive days to evaluate sub-chronic effects. Surprisingly, white blood cell profile was altered in which lymphocytes were found to be augmented (unpublished results). Another previous study had already cited similar observation, although the whole latex was the sample assayed instead of the richest protein fraction of the latex (Dada et al., 2002). These results and those shown in Figs. 3A and 5A suggest that latex proteins may act as an immune stimulant. The fact that results found with RL fraction were fully similar to those determined to NDL reinforces the idea that immunological and allergenic responses displayed by RL should be attributed to the presence in RL of proteins naturally found in NDL.

The present study describes immunological and allergenic effects induced by latex fractions of *Calotropis procera* in mice. Allergenic effects could be detected in anti-sera of mice that were sensitized subcutaneously while such effects were not detected when the animals were treated by oral route. The fact that treatment of rats with latex fraction (NDL) by oral route did not induce detectable allergenic response ought to be considered as a relevant point. This opens the question if allergenic patients could be treated by receiving oral doses of latex in attempt to further develop tolerance when exposed to latex proteins. This exciting question will remain to be investigated.

Considering that latex is also used as topic agent, it remains to be investigated if this contact could induce undesired effects by long-term usage. Of similar relevance, skin reactions provoked by latex exposure should be investigated taken as sample the latex fractions described here. The anti-inflammatory and anti-cancer activities found to be present into the latex were already reported to be effective by oral administration of the latex (Arya and Kumar, 2005; Choedon et al., 2006).

5. Conclusion

The results presented here, regarding absence of allergenic effects when the latex proteins were administrated by oral route represent new relevant data to the use of latex constituents as putative drugs. Presently, anti-inflammatory and antinociceptive activities of the NDL fraction of the latex are being evaluated by oral route and will clarify the remaining question; if such activities will be retained when tested by oral route. Contributions

of different research groups have progressively supported the Ethnopharmacology of this species.

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