SPECIFIC HETEROLOGOUS F(ab')2 ANTIBODIES REVERT BLOOD INCOAGULABILITY RESULTING FROM ENVENOMING BY LONOMIA OBLIQUA CATERPILLARS


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Abstract. Contact with Lononmia obliqua caterpillars results in a bleeding syndrome characterized by hemorrhage and blood coagulation disturbances. Conventional therapy using antifibrinolytics or cryoprecipitates has been unable to treat pathophysiologic alterations. As antivenoms are effective therapy for treatment of victims of venomous animals, a process of manufacturing a specific antinomeric serum by immunizing horses with Lononmia caterpillar bristle extracts (LBE) was developed. Lononmia caterpillar bristle extracts exhibited several protein bands on SDS-PAGE, induced blood coagulation abnormalities and lethality in mice, and stimulated specific antibody production in horses. Sera obtained from immunized horses were rich in anti-LBE specific antibodies distributed among the horse IgG isotypes. These antibodies had the ability to recognize various LBE antigens as well as to neutralize their coagulopathy-inducing activity. The antivenom manufactured by the developed process was composed of purified and sterilized F(ab')2, with ED50 = 38.61 μL, potency = 0.29 mg / ml, and 95% confidence limit of potency 0.20–1.36.

INTRODUCTION

In the late 1980s, moth caterpillars classified as Lononmia obliqua were detected in orchard trees in the Brazilian states of Rio Grande do Sul, Santa Catarina and Paraná. Coincidentally, the number of patients presenting local skin and systemic symptoms attributed to accidental contact with caterpillar bristles unexpectedly increased here. The envenomation syndrome caused by contact with caterpillars of the genus Lononmia is characterized by initial local skin irritation, edema, hemorrage and pain, usually followed by swelling of regional lymph nodes, disseminated subcutaneous hemorrhages, and bleeding from mucosa and pre-existing healing scars. Marked alterations of blood clotting, including decreased fibrinogen, factor V, factor XIII, plasminogen, and α 2 anti-plasmin, and increases in fibrinolytic activity, fibrinogen degradation products and activated thrombin may occur.1–6 Attempts at reversing these clinical manifestations with anti-fibrinolytic drugs such as aprotinin and e-aminocaproic acid, associated with whole blood, fresh-frozen plasma or cryoprecipitates, failed and indeed instead thrombin may occur.7,8 Antivenoms are effective treatment for patients bitten by venomous animals, a process of manufacturing a specific antinomeric serum by immunizing horses with Lononmia caterpillar bristle extracts (LBE) was developed. Lononmia caterpillar bristle extracts exhibited several protein bands on SDS-PAGE, induced blood coagulation abnormalities and lethality in mice, and stimulated specific antibody production in horses. Sera obtained from immunized horses were rich in anti-LBE specific antibodies distributed among the horse IgG isotypes. These antibodies had the ability to recognize various LBE antigens as well as to neutralize their coagulopathy-inducing activity. The antivenom manufactured by the developed process was composed of purified and sterilized F(ab')2, with ED50 = 38.61 μL, potency = 0.29 mg / ml, and 95% confidence limit of potency 0.20–1.36.

MATERIALS AND METHODS

Animals. Lononmia obliqua caterpillars were obtained from “Empresa Brasileira de Pesquisas Agropecuárias” (EMBRAPA), RS, Brazil and Centro de Vigilância Epidemiológica, RS, PR and SC, Brazil. The larvae were maintained at the Laboratório de Parasitologia, Instituto Butantan, São Paulo, SP, Brazil, under wild-like conditions and were fed tree leaves. Ten adult horses, register numbers 508-1, 508-1, 671-1, 207-2, 556-2, 550-3, 720-3, 796-3, 926-3, and 935-3, weighing 400–450 Kg were maintained at Facenda São Joaquim, Instituto Butantan. BALB/c and outbreed Swiss mice were supplied by Instituto Butantan. All animals used in this work were maintained and treated under strict ethical conditions according to the “International Animal Welfare Recommendations.”11–13

Antigen preparation and analysis. Caterpillars were anesthetized with CO2 and their bristles removed and transferred to vessels pre-cooled at 4°C. A volume of cool phosphate-saline buffer (PBS), pH 7.4, was added to attain a 10% final extract solution. The bristles were homogenized by shaking the solution and the insoluble material removed by centrifugation for 10 min at 600g. The supernatants (LBE) were stored as 1.0 ml aliquots and frozen at −70°C. Before use, LBE samples were centrifuged again and filtered under sterile conditions, protein concentration determined and adjusted to a final concentration of 2 mg/ml.

Tissue distribution. Samples of LBE were diluted in sterile saline to 0.1 mg/ml and 1 μg of extract was labeled with 37 MBq (1 mCi) of [125I] in the presence of 10 μg of Chloramine-T, according to the original Chloramine-T tech-
nique. To remove free $^{125}$I, the labeling mixture was purified by gel filtration on a G-25 column. Three hundred microliters of $^{125}$I-LBE (500,000 counts) were injected i.p. into BALB/c mice (three animals per group). The animals were anesthetized and euthanized at 1, 2, and 24 hr after injection. Samples of blood, urine and fragments of lungs, liver, kidney, spleen, brain, heart and skeletal muscle were excised and relative radioactivities determined using a γ-counter. Results were expressed as the number of counts/mg of tissue or counts/ml of blood or urine.

**Lethality and extract-induced blood coagulation abnormalities.** Swiss mice (18–20 g), a minimum of 4 animals per group, were injected i.p. and i.v. with various amounts of LBE and observed during a 72-hr period. Along this period the number of deaths were recorded. The 50% lethal dose (LD$_{50}$), expressed in micrograms of venom per mouse, was calculated by a Probit test. The ID$_{50}$, incoagulation-inducing dose 50%, was expressed as the minimal dose of LBE that delays the blood clotting time in 50% of mice for at least 15 min. Blood clotting time was determined just before, and at 2, 8, and 24 hr after i.p. injection. Neutralization experiments were performed in two protocols: **a)** Pre-incubation of LBE and antivenom. Three hundred microliter samples of LBE, containing 3 ID$_{50}$, were incubated at 37°C for 30 min with various dilutions of antivenom. Residual activity was then tested by i.p. injection into four mice per group. Antivenom neutralizing ability was expressed as the effective dose 50% (ED$_{50}$), defined as the antivenom/venom ratio at which LBE-induced blood incoagulability was reduced in 50% of injected mice. **b)** Antivenom and venom were injected independently, without pre-incubation. Three hundred microliters of LBE containing 3 ID$_{50}$ were injected i.p. and antivenom was administered 15 min before, at the same time or 30 min after venom injection. Two hours later, blood samples were analyzed for evidence of clotting. The effective dose of antiserum was calculated by Probit analysis.

**Immunization and bleeding.** Anti-LBE polyclonal antibodies were obtained by immunizing adult horses s.c. with LBE (1.5 mg) emulsified in Complete Freund’s Adjuvant (CFA), distributed in 10 distinct dorsal sites. After one month, similar doses of the antigen dissolved in 0.15 M NaCl were injected into the granuloma formed around the sites previously injected. Boosters were repeated twice at one-week intervals. Blood samples were collected by puncture of the jugular vein just before each immunization injection and sera were stored at 20°C. One week after the last immunization, when the antibody serum titer against LBE had attained an appropriate value, the horses were bled, and a volume of blood corresponding to one-twelfth of their body weight was collected in anticoagulant. Plasma and cells were separated by gravity sedimentation, and the cells re-infused into the corresponding horse through the jugular vein. Plasmas were pooled and stored at 4°C.

**F(ab’)$_2$ preparation.** Serum rich in F(ab’)$_2$ was produced as described and prepared according to recommendations of the World Health Organization. Ammonium sulfate was added to the plasma pool obtained from the horses immunized with LBE to 12% saturation; the precipitate containing fibrinogen was discarded. The ammonium sulfate concentration of supernatants was increased to 24%, the mixture allowed to stand overnight, centrifuged and the supernatant discarded. The immunoglobulin-rich precipitate was resuspended in saline, and the pH adjusted to 3.5 with concentrated HCl. The protein solution was made 37°C and pepsin added (1.0 g enzyme/60 g plasma protein). Following an incubation period of 40 min at 37°C, the pH, temperature, and ammonium sulfate concentration were increased to 5.2, 55°C and 28%, respectively. The precipitate containing F(ab’)$_2$, native immunoglobulins, and irrelevant proteins was dissolved in 0.15 M NaCl and dialyzed against 0.15 M NaCl until complete removal of ammonium sulfate. The preparations were analyzed for the presence of F(ab’)$_2$, and specific antibodies against Lonomia antigens, and submitted to quality control procedure to verify absence of bacterial contamination, bacterial endotoxin and toxic substances. The final product was adjusted to contain the desired neutralizing antibody titer in less than 10 mg of protein/ml, and labeled as antilonomous serum.

**Detection of antibodies against Lonomia caterpillar bristle extracts.** Antibodies against Lonomia antigens were assayed by ELISA. Briefly, 96-wells plates were coated overnight at 4°C, with 100 µl of LBE solution containing 10 µg/ml in carbonate buffer, pH 9.6. The wells were blocked for six hours at 37°C with 0.01 M PBS containing 1% gelatin and 0.05% Tween 20. After washing, samples of 100 µl of serial dilutions of horse serum (up to 2 x 10$^{-3}$) were incubated for 120 min at room temperature. The wells were washed again and a 1:4,000 solution of biotin-conjugated sheep anti-horse immunoglobulins was added. The plates were incubated for 35 min at 37°C. After washing, a 1:4,000 solution of streptavidin-conjugate peroxidase was added and the plates were kept at 37°C for 20 min. The reaction was developed by the addition of 0-phenylenediamine dihydrochloride (OPD) and H$_2$O$_2$ and read at 492 nm. The titers of serum or antivenom preparation were expressed as the highest dilution giving an OD twice the background value. Distribution of antibodies against LBE among the horse IgG isotypes was also analyzed by ELISA, as previously standardized. In this case, goat anti-horse IgGa, IgGb, IgGc, and IgG(T) (Bethyl Laboratories I.C.N., Montgomery, AL) were used followed by peroxidase-labeled mouse anti-goat IgG, (Sigma Chemical Co, St Louis, MO).

**Electrophoretic profile of LBE (SDS-PAGE).** LBE samples containing 10 µg of total protein were applied to 15% SDS-polyacrylamide gels and electrophoresis carried out as previously described, with some modifications. The gels were silver stained. Prestained SDS-PAGE Standards (BioRad, Hercules, CA) containing phosphorylase B (112 kDa), bovine serum albumin (84 kDa), ovalbumin (53 kDa), carbonic anhydrase (34 kDa), soybean trypsin inhibitor (28 kDa) and lysozyme (20 kDa) were run in parallel.

**RESULTS**

**Lonomia bristle extracts.** LBE obtained by the described method was limpid but green in color. The protein concentration, as determined by the Lowry method, varied from 0.2 to 6 mg/ml. By SDS-PAGE analysis, LBE exhibited electrophoretic profiles containing approximately 13 well-defined protein bands (Figure 1).

**Biodistribution of LBE components among organs and**
previously weighed. At 1 hr after dioactivity in suitable samples of tissues, blood and urine, labeled-extract components was monitored by quantitating radioactive LBE corresponding to 10^9 /H9262 relations. The proteins were silver stained.20 Prestained SDS-PAGE dodecyl polyacrylamide gel electrophoresis, under reducing conditions, separated by precipitation with ammonium sulfate. The presence of antibodies against Lonomia caterpillar antigens was determined for samples of LBE, the blood clotting time was normal 24 hr after envenomation.

Lethality. The LD_{50} of Lonomia bristle extract following i.v. injection was 0.19 mg LBE/18–20 g mouse. When the mice were injected i.p. with crescent LBE protein concentration no death was observed with up to 1 mg LBE/18–20 g Swiss mouse. Some animals that received 1.5 to 2.5 mg LBE/18–20 g mouse died in a period of 24 to 72 hr, but the relative doses for inducing the same effect varied between assays, with variable corresponding LD_{50}.

Antibody production. Figure 4 shows that horses immunized with Lonomia bristle extract produced high titer antibodies. The antibody titers increased sharply after 37 days, attaining maximum levels after two additional boosters. (Figure 4). Six of 10 horses responded vigorously with antibody titers greater than 256,000. Two to three years after initial immunization, these animals have retained detectable titers of specific immunoglobulins, which increased following LBE booster. Horse IgG, IgGc, and IgG(T) were of similar titers of antibodies against LBE antigens, while IgGc was lowest (Figure 5).

Serum-neutralization: To demonstrate that antilonomic serum preparations could be used as effective therapeutics, it was essential to show that they neutralized LBE-induced blood incoagulability. Using the ID_{50} values previously determined for Lonomia bristle extract, we examined the relative efficiency of the horse antivenom in neutralizing clotting alterations. Standard assays were used: antivenom and venom were either pre-incubated or independently injected into mice. The antilonomic horse serum containing native IgG molecules, batch 9408129, was capable of inhibiting the blood incoagulability when previously incubated with the extract (3 ID_{50}) for 30 min at 37°C before i.p. and i.v. injection into mice (Table 1). In experiments in which the antivenom was injected 30 min before or at the same time as the extract containing 3 ID_{50}, the blood-clotting times of injected mice were similar to the mice that received saline. This dose of antivenom was previously known to be effective in inhibiting the putative coagulating factors present in extract. When, however, the antivenom was injected 15 min after the extract, the protection capacity was reduced by 50% as compared with control mice (Table 2). This antivenom gave an ED_{50} = 38.61 μl and a potency of 0.29 mg/ml, CL 95% = 0.20–1.36, when assayed with 3 ID_{50}.

Refining and standardization of antilonomic serum. Plasmas from horses immunized with LBE were digested with pepsin and the correspondent F(ab’)2 preparations were separated by precipitation with ammonium sulfate. The presence of antibodies against Lonomia caterpillar antigens was tested by ELISA, Western blot and immunodiffusion methods. The antibody titers were usually between 32,000–250,000; two of them reached 2 × 10^4. These preparations also contain specific antibodies capable of neutralizing blood clotting alterations and lethality induced by LBE. Neutralization was highly effective when the antivenom was incubated with caterpillar extract prior to injection. The antilonenic activity remained in these organs and or remained in kidney tissue. After 2 hr, a lower, but significant extension of radioactivity has found principally in the kidneys, blood and urine (Figure 2). Radioactivity was also detected, at lower levels, in lungs, liver, spleen, heart, skeletal muscle and brain. After 2 hr, a lower, but significant level of radioactivity remained in these organs and fluids, but was almost completely excreted after 24 hr. At 1 and 2 hr after envenomation, most radioactivity was already eliminated by urine or remained in kidney tissue.

Hemostatic disturbances. Figure 3 shows that injection of mice with LBE delayed the blood clotting time, in a dose-related manner, as compared with control animals that received saline. Maximal activity was found at 7.5 μg/18–20 g mouse, i.p. injected. With these, or higher doses, the blood became incoagulable during the entire 3 hr period of observation. Smaller doses delayed the blood clotting time to a lesser degree. When the animals were injected i.p. with up to 30 μg of LBE, the blood clotting time was normal 24 hr after injection. The dose of 60 μg (i.p.) induced a slight prolongation of the blood clotting time as detected 24 hr after envenomation.
omic sera prepared to be used in immunotherapy for victims of lonomism were standardized according to the WHO recommendations, including the quality control tests (bacterial and fungal sterility, abnormal toxicity (safety), pyrogen test, potency and physio-chemical controls: appearance, pH measure, total protein content, total nitrogen content). One ml of these antivenoms was capable of neutralizing the toxic activities present in 3 ID₅₀ of caterpillar bristle extract.

DISCUSSION

Local inflammation at the contact sites denoted by hyperemia, edema and swelling of regional lymph nodes are the usual symptoms observed in accidents with caterpillars (erucism). Patients envenomed by contact with caterpillars of the genus *Lonomia* may additionally develop more severe systemic clinical manifestations and the incidence of death
FIGURE 4. Horses antibody titers against Lononmia-caterpillar bristle extract (LBE). Horses were primed with LBE (1.5 mg) in complete Freund’s adjuvant and boosted 4 weeks after with the same antigen in 0.15 M NaCl. Blood samples were collected just before the first immunization dose and 30, 37 and 42 days after. In A, the sera were diluted as indicated and antibodies against Lononmia bristle antigens assayed by ELISA using LBE as the antigen source. In B, the horses sera antibody titers, after the third hyperimmunization process. Groups of horses that have had been immunized since the first year (508-1; 589-1), since the second year (671-2; 207-2; 556-2) and horses never-before immunized were used (550-3; 720-3; 796-3; 926-3; 935-3).

is high. Subcutaneous hemorrhages and systemic reactions associated with severe coagulopathy are the major pathogenic alterations observed in lonomism. Lononmia bristles contain proteins and perhaps other substances that induce coagulopathy, complement activation and phospholipase A2 activity (Rocha-Campos AC and others, unpublished data). These impressive reactions observed in patients after skin contact with the caterpillars were not, however, entirely reproduced in experimental animals. Coagulopathy, reflected in terms of prolonged time of unclotted blood, is a measurable parameter observed in rats and mice. Toxicity expressed by lethality was observed in mice. Therefore, these parameters, in association with the immunochemical methods, were used to follow antibody production in immunized horses and to evaluate their final efficiency in neutralizing Lononmia-caterpillar bristle biological activities.

Lononmia-caterpillar bristle extracts, when analyzed by SDS-PAGE, under reducing conditions, exhibited at least 13 well-defined protein bands (Figure 1). The fact that these proteins represent at least some of the toxic factors present in the extracts is predictable. Some components including a procoagulant factor and a phospholipase A2 have been partially purified, although the unequivocal purified proteins corresponding to each biological activity seen and the targets of specific monoclonal antibodies have not yet been determined. This extract, used in the simulation of the enven-
neutralization, is capable of inducing coagulopathy in mice. The effect is dose-dependent and was observed consistently after two hours, when small doses of LBE were used (1 ID<sub>50</sub>). As both the complement activating components and blood inducing coagulopathy, were retained in dialyzed LBE which have been used as starting material to be applied on chromatography columns, these components appear to be large rather than small molecules.

Two hours after i.p. injection into mice, Lonomia-caterpillar components pass into the blood circulation and attain high levels in the kidneys and in urine, being almost completely cleared during the following 24 hr (Figure 2). Although smaller amounts could be detected in some organs such as the heart, lungs, spleen, brain and skeletal muscles, there is no evidence to indicate that particular toxic components adhere to or are taken up by pre-existing cells in these organs or structure. On the contrary, the strongest evidence so far accumulated suggests that the targets for the toxic components are circulating plasma proteins such as blood clotting factors<sup>3</sup>–<sup>5</sup> and complement system components.

The Lonomia-caterpillar antigens are strong immunogens for horses. Following conventional immunization involving priming with the antigens in complete Freund’s adjuvant, some modifications were scheduled, mainly in the protein concentration, number of sites and intervals of immunizations. Around 80% of the immunized animals responded with a high titered antibody response as evaluated by ELISA. In contrast to horses immunized with Bothrops and Crotalus venoms in which the antivenom antibodies are concentrated mostly in the IgG(T) isotype, followed by IgGa,<sup>18</sup> the antibodies produced by horses immunized with Lonomia-caterpillar antigens, except for IgGc, are almost similarly distributed among the IgGa, IgGb and IgG(T) isotypes (Figure 5). The small amounts of extract employed during the whole process of immunization (total of 6 mg/450 kg horses/year), distributed by multiple sites of dorsal inoculation, contrast with others schemes of sera production, which use two or three times more antigen. It was also observed that some horses immunized with LBE two or three years previously, maintained high levels of specific antibodies, even after 12 months without receiving LBE-antigens, and upon booster responded with an even higher antibody titer. These data taken together reinforce the suggestion that they are strong immunogens.

Quantitation of the antivenom-neutralizing capacity for use as immunotherapy continues to be a difficult task. First, the animal models did not reproduce entirely the envenomation syndrome they produce in humans. We have suggested that animals expressing hemostatic alterations after LBE-envenomation are adequate models for evaluating the antilonomonic sera efficacy.

Second, our data have obvious methodological uncertainties, primarily that the caterpillar extracts contain toxins yet not fully investigated. Our work aimed to obtain an antivenom to treat lonomism taking the following concerns into account: a) selection of plasma with high antibody titers and with presumably high affinity; b) development of antivenoms capable of neutralizing the biological activities clearly observed in victims, and, c) conformation of the methods with the World Health Organization recommendations for preparation and use of antivenoms in immunotherapy.<sup>15</sup> The first concern was accounted for by using plasmas exhibiting high antibody titers collected from horses after long immunization periods. The second was addressed by using carefully standardized in vivo and in vitro methods for determination of the antivenom neutralizing potency.

### Table 1

Neutralization of Lonomia-caterpillar bristle extract (LBE)-induced blood incoagulability. LBE containing 3 ID<sub>50</sub>* (minimal dose that extends the clotting time >900 sec in at least 50% of the group) were incubated at 37°C for 30 min with antivenoms, and injected i.p. and i.v. into mice (four mice per group). After 120 min samples of blood were collected and the clotting times recorded.

<table>
<thead>
<tr>
<th>Injection (route)</th>
<th>Dose of antivenom (ml)</th>
<th>Clotting time average (sec)</th>
<th>Protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.p.</td>
<td>&gt;900</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>90</td>
<td>100</td>
<td></td>
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<tr>
<td>200</td>
<td>120</td>
<td>100</td>
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<tr>
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<td>90</td>
<td>900</td>
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<td>0</td>
<td>&gt;900</td>
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<tr>
<td>i.v.</td>
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<tr>
<td>0</td>
<td>&gt;900</td>
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</tbody>
</table>

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*1 ID<sub>50</sub>i.v. = 0.4 μg; † ID<sub>50</sub>i.p. = 10 μg
Groups of mice injected with saline (control mice) were always included.

### Table 2

Neutralization of Lonomia-caterpillar bristle extract (LBE)-induced blood incoagulability using protocols in which antivenom was administered before, together, or after envenomation. Control mice received only LBE. Antivenin (60 μl) was administered i.p. in mice 30 min before, simultaneously (0), and 15 min after i.p. injection of LBE (3 ID<sub>50</sub>). Blood samples were collected 120 min later and the clotting times determined.

<table>
<thead>
<tr>
<th>Time of antivenin injection (min)*</th>
<th>Clotting time average (sec)*</th>
<th>Protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-30</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>100</td>
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<td>80</td>
</tr>
<tr>
<td>&gt;900</td>
<td>0</td>
<td>0</td>
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* Each point represents the mean of 3 determinations in groups of 4 mice each.
plasmas were used to prepare antivenoms composed of purified F(ab′)_2. The antivenom sera produced for use in treating victims of lonomism usually contain ED₅₀ and potency around 40 µl and 0.29 mg/ml, respectively.

Antivenoms continue to be the only specific treatment for envenomation syndromes, particularly in the treatment of snake bite victims where a number of the venom components involved in the pathophysiological mechanisms are well known. Antitoxic serum is an antivenom in which the manufacturing process involves a venom source containing poorly studied toxic components and thus requires careful clinical trials and laboratory-based studies to determine its neutralizing properties, targets of action, and safety. The preliminary use of these antivenom serum (Fan HW and others, unpublished data) has restored the blood clotting alterations to normal levels in victims of lonomism more rapidly than in victims who could not be treated with the antivenoms (due to unpredictable circumstances, such as their distance from health facilities). Most importantly, after the introduction of this antivenom in the treatment of this type of envenoming, no more deaths were recorded.

In memoriam: This article is dedicated to the memory of Dr. Eva Maria Antonia Kelen, a careful scientist, for her recognized contributions to the body of knowledge regarding the action of animal venoms on the blood clotting system. Her efforts and enthusiasm were decisive factors for the introduction of Lonomia obliqua toxin studies in the Instituto Butantan, São Paulo, Brazil, including the development of the antivenom serum described herein.

Acknowledgments: We gratefully thank Edson Hideki Sakaguti and the staff of the Fazenda São Joaquim for technical assistance and Ms. Nicola Conrans for English review of the manuscript.

Financial support: This study was supported by FAPESP, Procs. 95/05501-8, 96/5895-9, 92/1564-7, and 97/13259-8; CNPq, Procs. (delaetor No.) 521941/1995-4, Fundação Butantan and Fundação Nacional da Saúde, Ministério da Saúde do Brasil.

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