



A mouse model to study the alterations in haemostatic and inflammatory parameters induced by *Lonomia achelous* caterpillar haemolymph

M. Barrios^a, P. Taylor^b, A. Rodríguez-Acosta^c, E.E. Sánchez^d, C.L. Arocha-Piñango^a, A. Gil^a, A.M. Salazar^a, Z. Carvajal^a, M.J. Abad^b, B. Guerrero^{a,*}

^aLaboratorio de Fisiopatología-Coagulación Sanguínea, Instituto Venezolano de Investigaciones Científicas, IVIC Apartado 20632, Caracas 1020A, Venezuela

^bLaboratorio de Patología Celular y Molecular, Instituto Venezolano de Investigaciones Científicas (IVIC), Caracas, Venezuela

^cLaboratorio de Inmunología y Ultraestructura, Instituto Anatómico de la Universidad Central de Venezuela, Caracas, Venezuela

^dDepartment of Chemistry and The National Natural Toxins Research Center, Texas A&M University-Kingsville, Kingsville, TX 78363, USA

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ABSTRACT

A mouse model was established to reproduce the haemorrhagic syndrome which occurs in humans after accidental contact with the hairs of the caterpillar *Lonomia achelous* (LA) and measures the haemostatic and inflammatory alterations that occur as a result of this contact. Mice were injected intradermally with different doses (0.4, 0.8 and 1.6 mg/animal) of *L. achelous* haemolymph (LAH). Haematological (haemoglobin, haematocrit, platelet count, differential leukocyte count), haemostatic (fibrinogen, plasminogen, factor XIII [FXIII], fibrinolytic activity) and inflammatory parameters (tumour necrosis factor alpha [TNF- α], nitric oxide [NO]) were measured at different times up to 48 h. C57BL/6 mice responded to LAH injection, in terms of these parameters, in a manner similar to that seen in humans, whereas the BALB/c mice were unresponsive. In C57BL/6 mice injected with LAH, time course measurements showed: a) a reduction in the haemoglobin, haematocrit, fibrinogen, FXIII and plasminogen levels, b) no effect on the platelet count and c) immediate leukocytosis and an increase in the fibrinolytic activity in plasma. An inflammatory response (TNF- α) was observed within 1 h post-injection, followed by a more persistent increase in serum NO. These findings suggest that C57BL/6 mice represent a useful model of the haemorrhagic syndrome observed in humans who have suffered contact with the caterpillar, permitting a deeper understanding of the role of the inflammatory response in the haematological and haemostatic manifestations of this syndrome.

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1. Introduction

In 1977, a haemorrhagic syndrome was described in Venezuela, which resulted from contact with the caterpillar of

the moth *Lonomia achelous* (Arocha-Piñango et al., 1977). Clinically, the affected subjects showed haematomas, ecchymosis, haematuria and digestive, pulmonary, peritoneal and cerebral haemorrhages that could be fatal. In some cases, recently formed wounds reopened and began to bleed. Haematological tests showed mild anaemia with leucocytosis, prolonged prothrombin, partial thromboplastin and thrombin times. They also showed a decrease in fibrinogen, factor V, FXIII, plasminogen and α 2-antiplasmin levels. In addition, increased factor VIII:c, von Willebrand factor, and fibrin degradation products/D-dimers levels were observed. Antithrombin III and platelet levels remained normal, while factor VII, factor II and protein C levels varied (Arocha-Piñango

Abbreviations: LA, *Lonomia achelous*; LAH, *Lonomia achelous* haemolymph; NO, nitric oxide; TNF- α , tumour necrosis factor alpha; FXIII, factor XIII; t-PA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor type 1; u-PA, urokinase plasminogen activator; IL-1, IL-6 e IL-8, Interleukins 1, 2 and 8.

* Corresponding author. Tel.: +58 212 5041180; fax: +58 212 5041086.

E-mail addresses: belsyguerrero@yahoo.es, bguerrerr@ivic.gov.ve (B. Guerrero).

et al., 1992). Several activities similar to, or directed against blood clotting factors have been identified in LA, such as fibrinolytic enzymes which degrade fibrinogen producing abnormal fibrin degradation products, prothrombin activators, one direct and one Xa-like factor, a thermo-stable factor V activator, a thermo-labile factor V inhibitor, a factor XIII proteolytic/urokinase-like activity and a kallikrein-like activity (Arocha-Pinango et al., 2000).

The inflammatory response helps to maintain haemostasis through various mechanisms including endothelial cell activation, platelet activation and aggregation, thrombin generation, and fibrinolytic system activation (Cambien et al., 2003; Cicala and Cirino, 1998; Levi et al., 2002; Joseph et al., 2002). Many types of venom affect both the haemostatic system as well as the inflammatory branch of the immune system, leading to pro-inflammatory cytokine and chemokine release (Petricevich, 2004; White, 2005; Levi and Van Der Poll, 2005). The principle inflammatory mediators involved appear to be nitric oxide (NO), tumour necrosis factor alpha (TNF- α) and interleukins 1 and 6 (IL-1/6). TNF- α , produced mainly by macrophages and lymphocytes, exerts potent effects on non-immune targets such as fibroblasts and endothelial cells as well as other cells of the immune system (Voronov et al., 1999; Kaplan and Silverberg, 1988).

The clinical manifestations observed in the haemorrhagic syndrome induced by *L. achelous* caterpillar may be explained by these activities affecting haemostatic mechanisms directly, but may also involve closely linked pro-inflammatory processes. Establishing a mouse model may permit a further understanding of the interaction between the venom components and the haemostatic and innate immune systems.

2. Materials and methods

2.1. Reagents

Human fibrinogen was purchased from Kabivitrum Ltd. (Stockholm, Sweden), urokinase, tissue plasminogen activator (t-PA) and urokinase from American Diagnostica Inc. (Greenwich CT, USA), chromogenic substrate S-2251 from Chromogenix, Biogenic S.A. (Mölnådal, Sweden). Ethyl ether, trichloroacetic, acetic and hydrochloric acid from Merck (Darmstadt, Germany). Sodium citrate, ethylenediamine-tetraacetic acid (EDTA), ammonium oxalate, calcium chloride, bovine thrombin, monodansylcadaverine (MDC), dithiothreitol (DTT), N-N dimethyl-casein, Trizma base, lipopolysaccharides (LPS from *E.coli* serotype 055:B5) and imidazole and other analytical reagents were obtained from Sigma Chemical Co. (St Louis, USA). Clone 164 WEHI cells were obtained from the American Type Culture Collection (VA, USA).

2.2. Mice

Male C57BL/6 and BALB/c mice, between 10 and 12 wk of age, were obtained from the Instituto Venezolano de Investigaciones Científicas animal facility. Mice were acclimated for at least 1 wk before beginning each experiment and received water and food *ad libitum*.

2.3. Ethical statement

This research complies with the bioethical norms established by the "Principles of laboratory animal care" (NIH, 1985) and the sub-committee of animal models of the International Society of Thrombosis and Haemostasis (Giles, 1987).

2.4. *L. achelous* haemolymph (LAH)

Caterpillar colonies of *L. achelous* were collected from the trunk of a *Tapirira guianensis* tree in a forested zone of Bolivar and Anzoátegui states, Venezuela. Haemolymph was aspirated from the ventral cavity with a Pasteur pipette, and stored at -80°C until use. The haemolymph protein concentration was measured by the method of Lowry et al. (1951).

2.5. Fibrinolytic activity

Fibrinolytic activity was studied by the fibrin plate method as described by Marsh and Arocha-Piñango (1972). Fibrin plates were prepared on 3-cm Petri dishes; 1.5 mL fibrinogen solution (10% plasminogen as contaminant, 0.1% in 5 mM imidazole saline buffer, pH 7.4) was allowed to clot by adding 75 μL of bovine thrombin (10 IU/mL, in 0.025 M CaCl_2). The mixture was incubated at $22-25^{\circ}\text{C}$ for 30 min and then 10 μL of sample was applied to the fibrin film. After 24 h incubation at 37°C , fibrin hydrolysis was measured as the product of the smallest and largest diameter of lysis and expressed in mm^2 .

2.6. Amidolytic activity

The amidolytic activity of LAH was evaluated using a micromethod standardized in our laboratory (Guerrero and Arocha-Pinango, 1992). A mixture of the recommended buffer for each substrate (80 μL), LAH (10 μL at 2 mg/mL) and 10 μL of the specific substrate (final concentrations: 0.60 mM S-2238, 1.20 mM S-2288, 0.80 mM S-2222, 0.80 mM S-2251 or 0.16 mM S-2444) were placed in the wells of 96 well polystyrene plates. After incubation at 37°C for 15 and 30 min, the absorbance at 405 nm was measured. One unit of amidolytic activity was expressed as ΔA 405 nm/min.

2.7. Treatment of mice with LAH

Groups of mice ($n = 5$ per sampling time and per dose) were inoculated intradermally (ID) with 0, 0.4, 0.8 or 1.6 mg of LAH in 100 μL of sterile PBS. An inflammation control group was injected with 10 μg of LPS. Blood was collected at 0.25, 0.5, 1, 3, 6, 12, 24 and 48 h. Different animal groups were assayed at each sampling time.

2.8. Blood samples

Blood was collected from the mice by cardiac puncture under anaesthesia with ether. Between 1 and 1.2 mL of blood were obtained from each animal. For the haemostatic determinations, 0.5 mL was added to 50 μL of 3.8% sodium

citrate as anticoagulant, 0.5 mL was adjusted to 0.1% EDTA for the haematological tests, while serum was obtained from the remnant. All parameters were measured in triplicate.

2.9. Haematological parameters

Haematological measurements included: haemoglobin concentration using a kit from Chemroy Biochemical (USA) based on the cyanomethaemoglobin method (Van Kampen and Zijlstra, 1965); haematocrit by the micro-haematocrit method; leukocyte count with Turk solution and platelet count by the method of Brecher and Cronkite (1950). Cell counts were performed in a Neubauer chamber.

2.10. Haemostatic parameters

Fibrinogen, plasminogen and FXIII were determined according to the methods described by Barrios et al. (2009). a) Fibrinogen was determined by the gravimetric method (Ingram, 1952). b) Factor XIII by a modification of the fluorescence method of Coggan and Board (1984), which is a qualitative method based in the incorporation of the MDC fluorescent amine into casein mediated by the transamidase activity of factor XIIIa. c) Plasminogen by a modification of the method of Mussoni et al. (1979). f) *Endogenous fibrinolytic activity*: This activity was determined by the fibrin plate method (Marsh and Arocha-Piñango, 1972) using the plasma euglobulin fraction, obtained by mixing 0.9 mL of 0.01% (v/v) acetic acid with 0.05 mL citrated plasma in a pre-cooled Eppendorf tube, which was then kept on ice for 30 min followed by centrifugation at 1000 g for 10 min at 4 °C. The supernatant was discarded and the euglobulin precipitate redissolved in 50 µL of 0.05 M Tris buffer, pH 7.4. Ten microlitres of the sample (euglobulin fraction) were applied to the fibrin film and the diameter of lysis measured after 24 h at 37 °C.

2.11. Determination of serum TNF- α and NO concentrations

Bioactive serums TNF- α level were determined using a WEHI cytotoxicity assay, based on that previously described by Espevik and Nissen-Meyer (1986). Briefly, WEHI 164 cells were incubated first in the presence of 1 µg/mL actinomycin D (3 h) then with serial dilutions of the mouse sera for an additional 18 h. Cell viability was measured with the chromogen 3(4,5-dimethylthiazol-2-yl) 2,5 diphenyltetrazolium bromide (MTT), and the concentration calculated from a standard curve obtained with recombinant TNF- α .

Serum NO was measured using the Griess reaction (Sandoval-Chacón et al., 1998), after reduction of nitrates to nitrites with cadmium granules (Cortas and Wakid, 1990).

2.12. Statistical analysis

The haematological and haemostatic data were presented as the mean and standard deviation of 3 experiments. The Student's *t*-test analysis was used to study possible differences between the control and experimental groups. A value of $p < 0.05$ was assumed as statistically significant for all experiments. Values for the haemostatic and inflammatory parameters were compared by linear regression to give a Pearson correlation coefficient, *r* (Excel, Microsoft Office, Microsoft Corporation).

3. Results

3.1. Biochemical characterization of LAH

Table 1 shows the characteristics of the LAH used in these studies, in terms of the urokinase-, plasmin- and factor Xa-like amidolytic activities as well as the fibrinolytic activity on fibrin plates, with the latter activity being the most noticeable.

3.2. LAH toxicity in C57BL/6 and BALB/c mice

In order to establish a mouse model to reproduce the haemorrhagic syndrome observed in humans after contact with *L. achelous* caterpillar venom, we measured haematological and haemostatic parameters in both C57BL/6 and BALB/c mice 96 h after injection with 1.6 mg of LAH. Previous studies have shown that higher doses induced the death of C57BL/6 mice. BALB/c mice showed very little response to LAH in terms of the parameters measured in this study, whereas C57BL/6 mice showed significant falls in leukocyte, haemoglobin, haematocrit, fibrinogen, plasminogen and factor XIII, as well a pronounced fibrinolytic activity in the euglobulin fraction obtained from the plasma of these animals (Table 2). The platelet counts did not change in any significant way. The results indicate that C57BL/6 represent a more useful model for further studies.

3.3. Effect of LAH on haemostatic, haematological and inflammatory parameters in C57BL/6 mice

The effects of LAH at 3 different doses (0.4, 0.8 and 1.6 mg/animal) on these parameters were measured at different times (0.25, 0.5, 1, 3, 6, 12, 24 and 48 h) (Fig. 1). Haemoglobin levels dropped significantly from 12 h

Table 1

Characteristics of *Lonomia achelous* haemolymph.

Protein (mg/mL)	Amidolytic activity ^a (mUA/min/µg)			Fibrinolytic activity ^b (mm ² /µg)
	Urokinase-like (S-2444)	Plasmin-like (S-2251)	Factor Xa-like (S-2222)	
16.8 ± 1.4	31.6 ± 4.4	41.6 ± 6.1	18.4 ± 1.9	289.0 ± 15.6

^a Amidolytic activity determined using chromogenic substrates.

^b Fibrinolytic activity determined as area of lysis on fibrin plates.

Table 2
Haematological and haemostatic parameters in C57BL/6 and BALB/c mice.

Parameter ⁺	C57BL/6		BALB/c	
	Control	LAH	Control	LAH
Haemoglobin (g/L)	13.4 ± 1.1	11.3 ± 0.4*	13.3 ± 1.0	14.6 ± 0.2
Haematocrit (%)	39.0 ± 1.0	32.0 ± 2.0*	39.0 ± 2.0	40.0 ± 1.0
Leucocytes (×10 ³ /μL)	6.9 ± 1.2	7.8 ± 0.9*	6.7 ± 1.5	5.7 ± 1.2
Platelets (×10 ³ /μL)	985 ± 139	993 ± 121	782 ± 164	969 ± 116
Fibrinogen (g/L)	2.6 ± 0.4	1.1 ± 0.3*	2.4 ± 0.3	2.6 ± 0.6
Factor XIII (%)	94 ± 14	67 ± 12*	118 ± 20	100 ± 10
Plasminogen (%)	142 ± 18	45 ± 13*	80 ± 19	96 ± 10
Lysis area (mm ²)	0	618 ± 276*	0	0

Mice were injected ID with 1.6 mg of LAH in 100 μL PBS, or with PBS alone. Blood was collected 48 h post-injection. Mean ± S.D. of 3 experiments, 5 mice/experiment. **p* < 0.05 with respect to the control group.

onwards, most markedly at the highest dose tested (1.6 mg) at 24 h, whereas the haematocrit dropped significantly at 24 h with a slight recovery at 48 h. After an initial sharp rise in the total leukocyte count after 15 min; however it dropped significantly in a biphasic fashion at 1 and 24 h. The percentage neutrophil count rose significantly (*p* < 0.01) between 3 and 12 h, but then decreased significantly at 48 h (*p* < 0.05) at all doses tested. Almost exactly the opposite occurred with the percentage lymphocyte count, with a significant fall (*p* < 0.05) between 3 and 12 h, and an increase to above control level at 48 h. All parameters returned to normal levels within 2–3 days thereafter (results not shown).

Important changes were observed after LAH injection in all four haemostatic parameters measured (Fig. 2). Significant reductions in both plasma fibrinogen and plasminogen were observed at all doses, falling to the lowest levels at

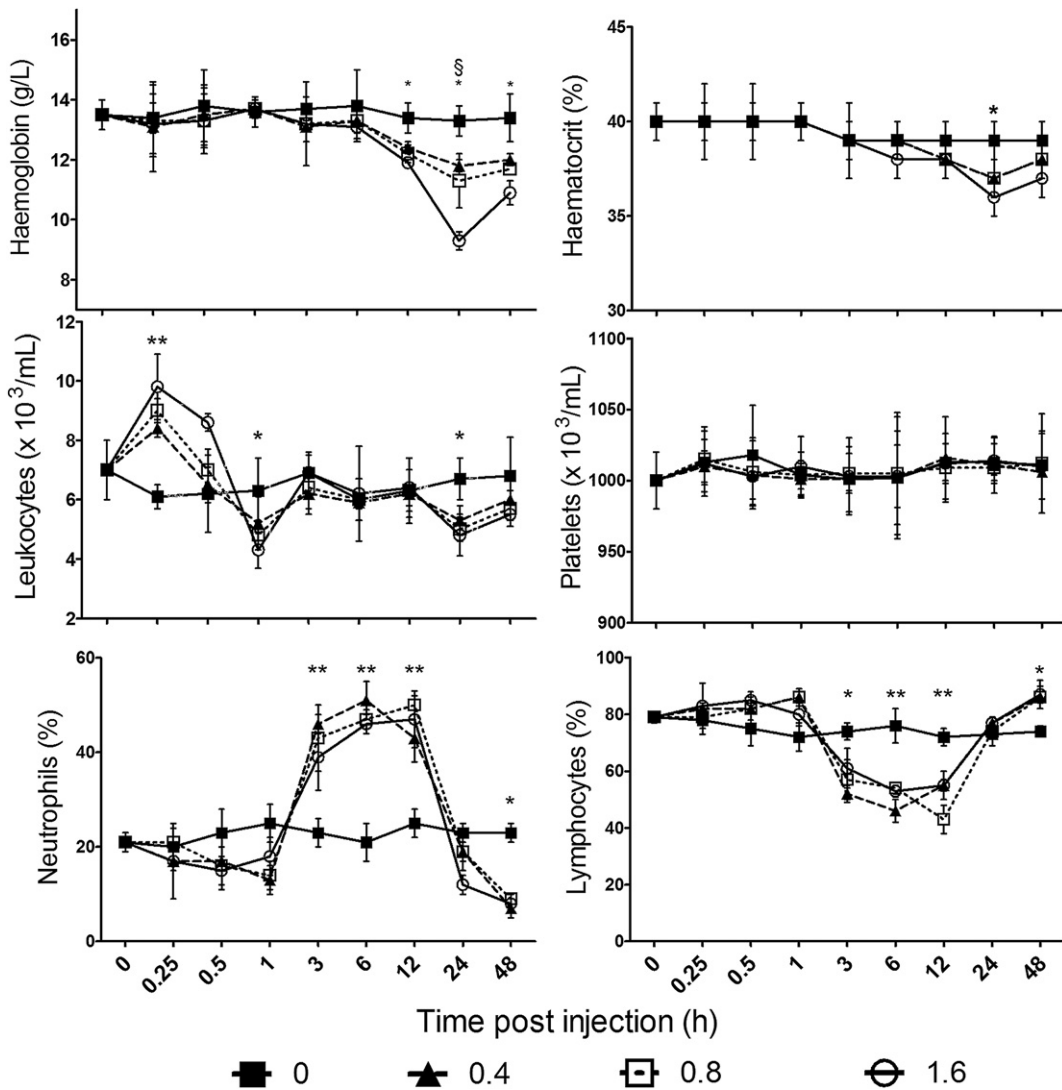


Fig. 1. The effect of different doses of LAH on haematological parameters in C57BL/6 mice. Mice were injected ID with 3 different doses of LAH (mg/animal) or sterile PBS (control), and then bled at different times. Mean ± S.D. of 3 experiments, 5 mice/experiment. **p* < 0.05, all groups with respect to the control group. §*p* < 0.01, 1.6 mg/animal group with respect to the control group.

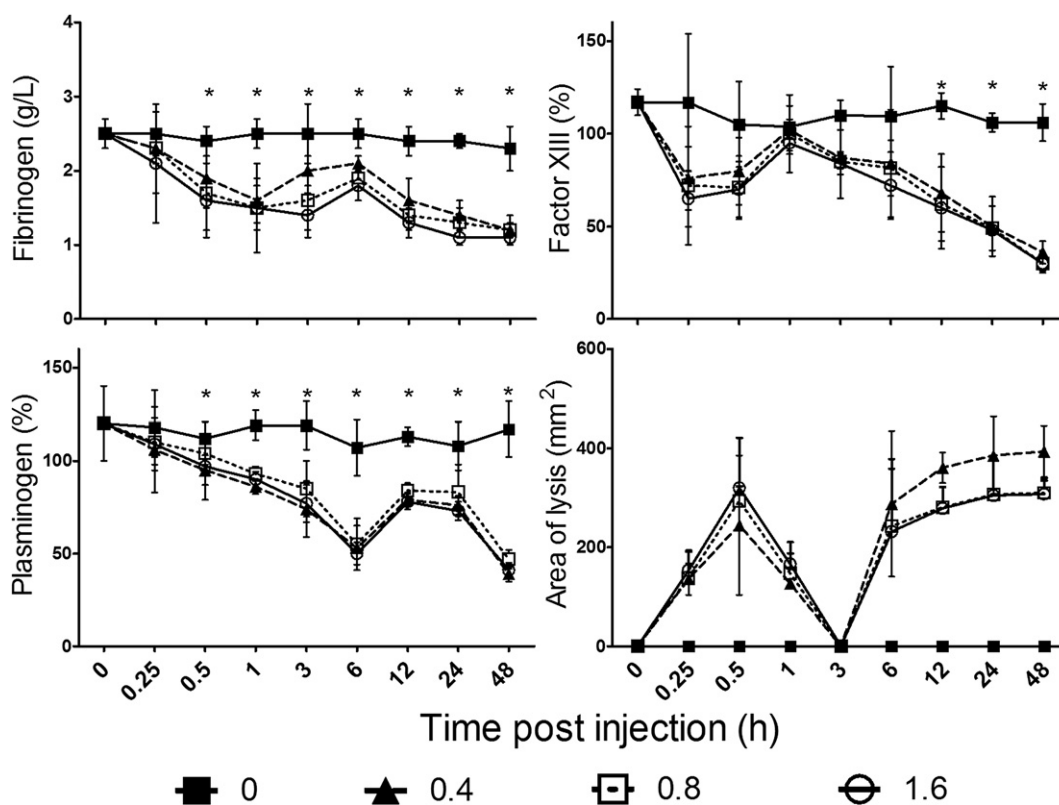


Fig. 2. The effect of different doses of LAH on haemostatic parameters in C57BL/6 mice. Mice were injected ID with 3 different doses of LAH (mg/kg) or sterile PBS (control) and then bled at different times. Mean \pm S.D. of 3 experiments, 5 mice/experiment. * $p < 0.05$, all groups with respect to the control group.

48 h. An immediate sharp drop in factor XIII was observed at 15 min, followed by a temporary recovery at 1 h, then a steady continuous fall up to 48 h. Fibrinolytic activity, absent in the plasma of the control animals, showed a significant increase over time in the LAH-injected animals, which was biphasic due to a sharp drop in this activity to basal levels between 1 and 3 h. The highest activity was observed at 48 h. LAH, even at maximum doses, did not induce overt bleeding in any of the mice over the time of observation, but preliminary histological studies of renal and hepatic tissues did suggest a degree of bleeding at the microscopic level (results not shown).

Fig. 3 shows the inflammatory response to LAH injection. TNF- α increased sharply at 1 h, coincidentally with the control LPS-induced peak, then fell immediately to nearly basal levels. Interestingly, this peak was only observed with the lowest dose of LAH. Nitric oxide levels also rose quickly with all 3 doses of LAH, peaking at 6 h, and then falling to lower, but not basal, levels at 48 h.

3.4. Correlation between haemostatic and inflammatory parameters

In order to determine whether there was any correlation between the haemostatic and inflammatory parameters over time, we performed a linear correlation test on their values in animals receiving the 0.4 mg dose of LAH (Table 3). This dose produced the greatest TNF- α response with

a significant negative correlation between TNF- α and fibrinogen levels (Pearson correlation coefficient $r = -0.8$) and a strong positive correlation between TNF- α and factor XIII ($r = 0.9$). TNF- α levels did not correlate with either plasminogen or fibrin lysis. On the other hand, the serum NO concentration showed a moderate negative correlation with plasminogen, fibrinogen and factor XIII levels ($r = -0.6$, -0.2 and -0.3 , respectively), but not with fibrin lysis.

4. Discussion

C57BL/6 and BALB/c mice, multipurpose strains commonly used in a variety of fields including immunology, were compared in terms of their suitability as a model to investigate the haemostatic and inflammatory alterations that take place as a result of accidental contact with the caterpillar *L. achelous*. The secretions of LA, which cause a severe haemorrhagic syndrome in humans, contain different proteolytic activities against haemostatic factors and adhesion proteins (Arocha-Pinango et al., 2000; Arocha-Pinango and Guerrero, 2001, 2003). In this context, a mouse model would serve to determine the participation of inflammatory processes in the clinical symptoms observed in human patients. Male mice were used in this study due to the well-established role of female sex hormones in immune function (Diodato et al., 2001) as well in haemostatic parameters. Many factors influence

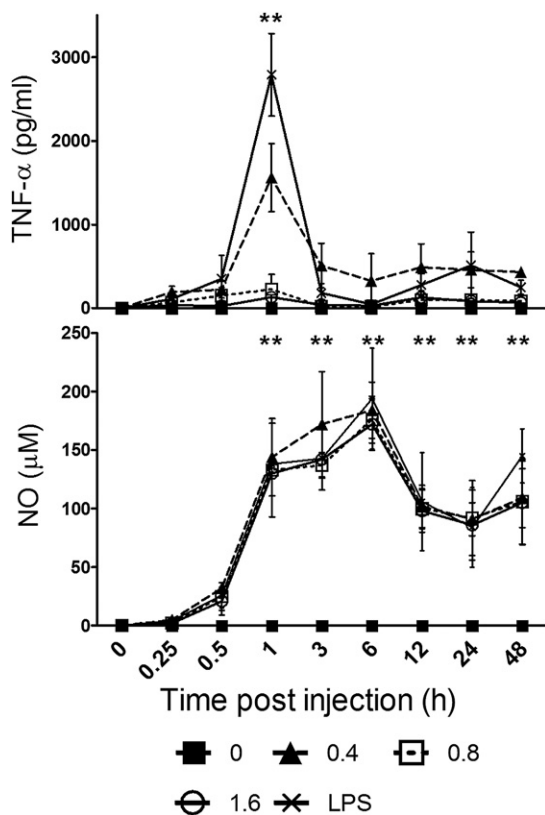


Fig. 3. The effect of different doses of LAH on inflammatory parameters in C57BL/6 mice. Mice were injected ID with 3 different doses of LAH (mg/animal) or sterile PBS (control), and then bled at different times. Mean \pm S.D. of 3 experiments, 5 mice/experiment. $**p < 0.05$, all groups with respect to the control group.

haemostatic changes, and gender plays an important role with the influence due to the steroid sex hormones (Schwartz and Penckofer, 2001). However, further studies will show whether gender differences are important in this particular model.

The initial results, showing that C57BL/6 mice are more susceptible to the venom than BALB/c, concur with those showing that these mice are more sensitive than BALB/c to *Tityus serrulatus* scorpion venom (Petricevich and Pena, 2002; Barros et al., 1998; Tambourgi et al., 1998). We observed a decrease in haematocrit and haemoglobin 3 h

Table 3

Correlation between TNF- α and NO levels and the haemostatic parameters.

	Pearson correlation coefficient (<i>r</i>)	
	TNF- α	NO
Fibrinogen	-0.8 ^a	-0.2 ^b
Factor XIII	0.9 ^a	-0.3 ^b
Plasminogen	0.009	-0.6 ^b
Fibrin lysis	0.3	0.1

Linear correlation test of pooled values in animals receiving the 0.4 mg dose of LAH.

^a Significant correlation between TNF- α and fibrinogen and FXIII levels.

^b Significant correlation between NO and fibrinogen, FXIII and plasminogen levels.

after LAH injection in C57BL/6 mice, a state of anaemia which has also been reported in both animals and humans after contact with other types of venom, including those from snakes and spiders (Marval et al., 1999; da Silva et al., 2003; Arocha-Pinango and Guerrero, 2003; Tavares et al., 2004; Rodríguez-Acosta et al., 1996). Seibert et al. (2003, 2004) reported that *Lonomia obliqua* caterpillar venom had a direct cytolytic effect on human and Wistar rat erythrocytes *in vitro*, as well as intravascular haemolysis, anaemia, and increases in reticulocytes, haptoglobin and haemoglobin in the rats *in vivo*. These findings may be explained by direct and indirect haemolytic effects of the venom, as well as through a loss of blood cells from a leaky vasculature. The erythrocyte swelling and general polychromatophilia which appear in the 24 h blood smears also suggest a direct haemolytic effect of the venom. The leucocytosis observed 30 min after LAH is also common in envenomation, probably due to an inflammatory response which involves the release of white blood cells from bone marrow stores. This is followed by a tendency to lymphopenia (at 1 and 24 h, Fig. 1), which appears to be a result of concomitant neutrophilia, another important indicator of an inflammatory response, and lymphopenia, which may be due to marginalization of this cell population as well as a direct cytotoxic effect of the LAH. Such variations in different white populations are common in inflammation and have been reported in different types of envenomation (da Silva et al., 2003; Emslie-Smith and Harris, 1989; Sano-Martins et al., 1995).

In the present study the haemostatic alterations were evaluated by platelet count, fibrinogen, FXIII and plasminogen levels as well by euglobulin lysis, which indicates alterations in the coagulation as well as the fibrinolytic systems. The platelet count did not vary, a finding also reported in human subjects (Arocha-Pinango and Guerrero, 2001). As in humans (Arocha-Pinango and Guerrero, 2003), the fibrinogen, FXIII and plasminogen levels in C57BL/6 mice all fell after injection of LAH and were still low in some animals followed up to 120 h (results not shown). This contrasts with previous findings from our laboratory in rabbits that recovered in terms of these parameters, between 72 and 96 h after injection (Marval et al., 1999), suggesting that the C57BL/6 mouse is a more sensitive model.

To evaluate the endogenous fibrinolytic activity present in plasma, a very simple test on plasminogen-rich fibrin plates was used. The area of fibrin lyses caused by adding plasma or precipitate of euglobulins indicated the presence of plasminogen activators or a direct plasmin activity. This test has the advantage that physiologic substrates and citrated plasma may be used, it is inexpensive and the reagents are readily accessible (Barrios et al., 2009). At 15 min–1 h post-injection, the plasma euglobulin fractions were active on the fibrin plates probably due to a direct fibrinolytic activity of circulating LAH proteases. LAH contains such plasmin-like activity, as shown in Table 1, and at these times plasminogen levels were almost normal (80–90%). After a period with no fibrinolytic activity in the plasma at 3 h, which may be due to enzymatic inactivation by physiological inhibitors or some other modulatory processes (Laing and Moura-da-Silva, 2005), the activity

rose sharply from 6 h onwards. This phase may be associated with endogenous activation of plasminogen by factors in LAH, as the Pg levels by then had dropped to 40% compared to that of the control, or with the activation of cells such as monocytes, macrophages and endothelial cells that could be secreting endogenous plasminogen activators (Heiden et al., 1996). The biphasic kinetic responses are similar to those seen in human haemorrhagic syndrome following contact with the caterpillars, where there is an initial phase of haemostatic factor consumption, a temporary recovery, followed by another decrease in their levels (Arocha-Pinango et al., 1992).

Mouse models have demonstrated an inflammatory response after envenomation by snakes, spiders, scorpions and insects. Reports show a rise in the serum levels of NO and other pro-inflammatory mediators such as TNF- α , IL-1, IL-6 and IL-8, activation of peritoneal macrophages, splenocytes, platelets and endothelial cells, as well as an increase in the expression of adhesion molecules, such as E-selectin (Tambourgi et al., 1998; Zamuner et al., 2001; D'Suze et al., 2003; Domingos et al., 2003; Petricevich, 2004; Pereira et al., 2005; Chaves et al., 2006).

This is the first report of a similar inflammatory response, in this case of TNF- α and NO, as a result of contact with Lepidoptera of the *Lonomia* genus. Interestingly, the greatest systemic TNF- α response was observed with the lowest dose of LAH (0.4 mg). One possible explanation is that the response was already at maximum with the lower dose, and that secreted TNF- α was degraded by the proteases present in the higher doses of venom. This proteolytic effect on TNF- α has been described after injection of *Bothrops jararaca* venom (Clissa et al., 2001).

The TNF- α and NO responses to an acute inflammatory stimulus show approximately the same time course, with the NO peak being slightly delayed, probably due mainly to the important role of TNF- α in the induction of inducible nitric oxide synthase (iNOS) (Petricevich, 2004). In this model, both mediators began to rise at 30 min post-injection, but NO levels remained high at later times when TNF- α had already dropped to near basal levels. This may be due to a sustained level of activation of immune and endothelial cells that are the principle sources of NO. These cells, as part of this inflammatory response, also synthesize and secrete plasminogen activators which may explain the prolonged activation of the endogenous fibrinolytic system and fall in plasminogen and fibrinogen levels (Clissa et al., 2001). Fibrinogen levels may take weeks to recover after human contact with this caterpillar (Arocha-Pinango et al., 1992).

The systemic changes observed in the haemorrhagic syndrome induced by the haemolymph of the *L. achelous* caterpillar may be due, directly or indirectly, to components of the venom that activate endogenous pro-inflammatory processes, which may then in turn contribute to those changes. To determine whether there is any correlation between the haemostatic and inflammatory parameters, without drawing any conclusion as to causality, we performed a simple test of linear correlation between the pooled data at all times for the dose of 0.4 mg of LAH, which had given the highest TNF- α response. The negative correlation between TNF- α and fibrinogen levels may be due to this cytokine causing the release of

plasminogen activators (t-PA and u-PA) from endothelial cells, with the subsequent formation of plasmin. Also, TNF- α activates neutrophils that release elastase. Both plasmin and elastase degrade fibrinogen, thus possibly contributing to the observed decrease in the plasma concentration (Heiden et al., 1996).

Factor XIII is found in two zymogenic forms, plasmatic and intracellular, in platelets, monocytes and macrophages. The degranulation of platelets, as well as the activation of macrophages induced by TNF- α , may explain the positive correlation between the levels of this cytokine and FXIII (Hettasch and Greenberg, 1998; Adány and Bárdos, 2003).

The negative correlation between serum NO and the fibrinogen, FXIII and plasminogen concentrations may in part be explained by post-translational modification of the fibrinogen (S-nitrosylation) and FXIII (nitration) molecules. Modification of fibrinogen leads to accelerated clot formation, and a reduction in its circulating levels, whereas nitration of FXIII inhibits its enzymatic activity (Catani et al., 1998; Vadseth et al., 2004). In addition, NO inhibits PAI-1 leading to an increase in t-PA and u-PA activation, resulting in activation of plasminogen and a decrease in its circulating levels (Sakamoto et al., 2004).

Animal venoms are a complex mixture of toxins that cause a wide variety of biological effects, including activation of the inflammatory cascade and of the haemostatic system. Examination of the interplay between these two systems following envenomation is important both in terms of our understanding of them under physiological conditions, and the understanding of the pathological consequences of the envenomation. These results identify the C57BL/6 mouse as a promising model for the study of the *L. achelous* haemorrhagic syndrome and the role of the inflammatory response in its progression. Further studies will address the true importance of the inflammatory response in the haematological alterations observed in this work.

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Conflict of interest statement

We have no conflict of interest to declare.

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