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Local inflammation, lethality and cytokine release in mice injected with *Bothrops atrox* venom

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We have provided evidence that: (a) lethality of mice to crude *Bothrops* venom varies according the isogenic strain (A/J > C57Bl/6 > A/Sn > BALB/c > C3H/HePas > DBA/2 > C3H/He); (b)BALB/c mice (LD_{50}=100.0 μg) were injected i.p. with 50 μg of venom produced IL-6, IL-10, INF-γ, TNF-α and NO in the serum. *In vitro* the cells from the mice injected and challenged with the venom only released IL-10 while peritoneal macrophages released IL-10, INF-γ and less amounts of IL-6; (c) establishment of local inflammation and necrosis induced by the venom, coincides with the peaks of TNF-α, IFN-γ and NO and the damage was neutralized when the venom was incubated with a monoclonal antibody against a 60 kDa haemorrhagic factor. These results suggest that susceptibility to *Bothrops atrox* venom is genetically dependent but MHC independent; that IL-6, IL-10, TNF-α, IFN-γ and NO can be involved in the mediation of tissue damage; and that the major venom component inducers of the lesions are haemorrhagins.

Key words: *Bothrops atrox*, Cytokines, Animal venoms, Mediators of inflammation

Introduction

Bites by snakes belonging to the genus *Bothrops* sp. can result in an immediate local lesion. This progresses from an acute inflammatory reaction to an impressive tissue destruction.[1] Systemic effects of the venom at various grades of severity include blood incoagulability and acute kidney failure.[2,3] Although some venom components are endowed with the ability to reproduce in experimental animals some local or systemic lesions such as haemorrhage (haemorrhagins) and blood incoagulability (thrombin-like factors) respectively, observations in human victims have been isolated and characterized, the intimate events elapsing from the venom penetration to the establishment of the lesions are not yet known. Among these are included the venom components and the host mediators involved in the recruitment of leukocytes from the circulation to the site of the tissue injury, the elicitation of specific leukocyte populations, activation or damage of the endothelial cells lining the post capillary venules at the site at which extravasation of leukocytes and/or erythrocytes occur.[4]

In order to address these issues, basic information obtained in a representative experimental animal model using adequate venom samples as inflammatory inducer are needed. Previous reports have shown that susceptibility to *Crotalus durissus terrificus* venom varies according to the strain of mice used, A/J and DBA/2 have been shown to be the most susceptible and BALB/c and C57Bl/6 the most resistant strains.[5]

It is well known that lethality and toxicity of snake venoms can vary according to the age, sex, nutritional state and geographic regions where the animal were captured.[6]

Thus, aiming to minimize the experimental bias BALB/c mice, a strain of mice moderately susceptible to the *B. atrox* venom action and a mixture of venom obtained from several adult snakes from the same geographic region were used throughout all experiments.

The present study shows: that the susceptibility to *B. atrox* venom varies according to the strain of mice and that cytokines are released *in vivo* during envenoming and *in vitro* upon stimulation with the venom. The inflammatory effects induced by this venom are also described at microspical level. As haemorrhage is one of most conspicuous tissue lesions produced by *Bothrops* sp. venom, the blocking action of a monoclonal antibody against a haemorrhagic factor present in *B. atrox* venom was tested.

Materials and methods

Chemicals, reagents and buffers

RPMI 1640 medium, actynomycin D, ortho-phenyldiamine (OPD), nicotinamide-adenine dinucleotide

phosphate (NADPH), flavine adenine dinucleotide (FAD), sodium nitrate reductase, polyoxyethilene sorbitan (Tween 20), Concanavalin A (Con A) and LPS serotype O111:B4 were purchased from Sigma (St Louis, MO), fetal calf serum (FCS) was purchased from Fazenda Pig (Rio de Janeiro), murine anti-IL-6 (clones: MP5–20F3 and MP5–32.C.11), rIL-6, anti-IL-10 (clones JESS-2A5 and SXC-1), rIL-10, anti-IFN-γ (clones XMG 1.2, AN18), rIFN-γ were purchased from PharMingen (Toreyana, San Diego) and rTNF-α was from Boehringer Mannheim (Indianapolis).

Venoms

Bothrops atrox, venom, was provided by the Laboratório de Herpetologia, Instituto Butantan, São Paulo, Brazil. The venom was collected, pooled, lyophilized and stored at –20°C. Samples were prepared in 0.15 M phosphate buffered saline (PBS) pH 7.2 at use.

Animals

Male mice of the strains C57Bl/6 (H-2^b), BALB/c (H-2^d), C3H/He (H-2^k), C3H/HePa (H-2^k), DBA/2 (H-2^d), A/J (H-2^a), A/Sn (H-2^a) weighing 18–22 g and aged 5–6 weeks, were obtained from the Biotério de Camundongos Isogênicos, Universidade de São Paulo, São Paulo, Brazil and used throughout the experiments. The animals were maintained and used under strict ethical conditions according to the animal welfare international recommendations (Commitee Members, International Society on Toxinology, 1991).

Antibodies

A monoclonal antibody against a haemorrhagic factor recognizing a 60 kDa component was prepared and immunochemically analysed by the authors (manuscript in preparation).

Lethality and LD_{50} calculation

The lethal toxicity of *B. atrox* venom was assessed in isogenic mouse strains by intradermal injection of different venom concentrations in 0.1 ml of 0.85% NaCl solution. Five animals were used for each venom dose. The LD_{50} was determined by the Spearman-Karber method[7] and calculated according to probit analysis.[8]

Venom treatment

Groups of four mice per group were injected i.p. with 500 µl of saline containing 50 µg of venom.

Sera and cell suspensions

Blood was collected from the retro-orbital plexus at different times after venom injection. Cells were obtained either from spleens or from the peritoneal cavity of mice previously injected with 50 µg of venom.

Cell culture supernatants

Spleen cells were obtained from venom or saline injected mice 3 h before harvesting. Cells were cultured in supplemented RPMI-1640 medium at 10^5 cells/well in 5% CO_2 at 37°C. Cells received *in vitro* either 250 ng of venom or 1 µg of LPS or 1 µg of Con A.

Adherent peritoneal cells were collected from the same donors of spleen cells. Cells were cultured in RPMI-1640 medium supplemented with 2-ME, L-glutamine, and 5% FCS, and left to adhere onto 24 microwell plates for 24 h at a initial concentration of 10^6 cells/well in 5% CO_2 at 37°C before *in vitro* addition of 250 ng of venom or 1 µg of LPS. Supernatants were collected at 48 h of culture.

IL-6, IFN-γ and IL-10 concentrations in serum or spleen or adherent peritoneal cell cultures

The presence of cytokines IL-6, IL-10 and IFN-γ in mouse sera was determined by indirect ELISA following the procedures described elsewhere.[9] Briefly, ELISA plates were coated with 100 µl (1 µg/ml) of monoclonal antibodies anti-IL-6, anti IFN-γ or anti-IL-10 in 0.1 M sodium carbonate coating buffer, pH 8.2, and incubated for 6 h at room temperature. The wells were then washed with 0.1% PBS/Tween 20 and blocked with 100 µl of FCS 10% PBS for 2 h at room temperature. After washing, duplicate mouse serum samples of 50 µl were added to each well. Recombinant murine cytokines were used to generate standard curves. After 18 h of incubation at 4°C the wells were washed and incubated with 100 µl (2 µg/ml) of the biotinylated monoclonal antibodies anti-IL-6, anti-IFN-γ or anti-IL-10 respectively as second antibodies for 45 min at room temperature. After a final wash, the reaction was developed by the addition of OPD to each well. Optical densities were measured at 405 nm in a microplate reader. IL-6, IFN-γ and IL-10 levels are expressed as ng/ml. The detection limits of these assays were 0.78 ng/ml for IL-6 and IL-10 and 7.8 ng/ml for IFN-γ.

TNF-α concentration in serum or spleen or adherent peritoneal cell cultures

The presence of TNF-α in mice sera was evaluated by the method of Ruff and Gifford.[10] Briefly, L929 cells maintained in RPMI 1640 medium supplemented with 5% FCS were plated at 3×10^5 cells/well on a 96-multiwell tissue culture plate and incubated at 37°C for 18 h in a 5% CO_2 atmosphere. Duplicate samples of 100 µl of mouse sera collected from the saline or venom injected animals were serially diluted in RPMI 1640 medium containing 1.0 µg/ml of actinomycin D

and added to the L-929 cell cultures. After 18 h of incubation, the supernatants were removed and the remaining live cells assessed by fixing and staining with 1% crystal violet. Absorbance [A] was measured in each well at 620 nm in a microplate reader. Cytotoxicity was calculated by the formula:

$$\text{Cytotoxicity} = \frac{\text{A control} - \text{A sample}}{\text{A control}} \times 100$$

Titres were calculated as the reciprocal of the dilution of the sample in which 50% of the cells in the monolayers were lysed. TNF-α activity is expressed as ng/ml, estimated from the ratio of a 50% cytotoxic dose of the test to that of the standard mouse recombinant TNF-α The detection limit of this assay was 3.429 pg/ml.

Nitrite concentration in serum, spleen or adherent peritoneal cell cultures

The nitrite levels in mice serum as an indication of NO production were determined as described previously.[11] Briefly, 40 µl of each mouse serum sample were incubated in a 96- well, flat-bottom plate with 40 µl of the reduction solution (NADPH 1.25 mg/ml; FAD 10.4 mg/ml; KH_2 PO_4 0.125 M) containing 0.5 U NO_2^- reductase for 2 h at 37°C; after incubation, 80 µl of Griess reagent (0.1% naphtylenediamine hydrocloride, 1% sulphonylamide, 3% H_3PO_4) were added to each well. The optical densities were measured at 540 nm in a micro plate reader. NO_2^- concentrations were determined using a standard curve of $NaNO_3$ ranging from 1.25 to 270 mM and expressed as nmol/10^5 cells. Nitrite levels in the cell culture supernatants were determined by mixing equal volume of Griess reagent and and processed as above.

Results

Determination of LD_{50}

Groups of five BALB/c, C3H/He, C3H/Pas, C57Bl/6, DBA/2, A/J and A/Sn mice were injected i.p. with different doses of *B. atrox* crude venom and the death survival ratio determined after 48 h. The LD_{50} value was calculated by probit analysis at 95% confidence. As shown in Table 1 the LD_{50} were:137.0 µg, 136.0 µg and 129.0 µg for DBA/2, C3H/HePas and C3H/He respectively and 100.0 µg, 95.2 µg, 90.8 µg and 89.4 µg for BALB/c, A/Sn, C57Bl/6 and A/J strains respectively.

Local inflammatory reaction induced by crude *Bothrops atrox* venom

Subcutaneous injection of 50 µg of crude *B. atrox* venom induces an inflammatory reaction characterized macroscopically by an early appearance of edema and erythema followed later by intense haemorrhage culminating in local tissue necrosis. Fragments of tissues collected from the injected sites after 24 and 48 h, showed the presence of a leukocyte infiltrate rich in polymorphonuclear cells with some macrophages, lymphocytes and erythrocytes immersed in a hyaline exudate (Fig. 1A). As indicated by the arrows the skeletal muscle cells surrounded by the infiltrating leukocytes are disrupted. In contrast, fragments of tissues collected from the corresponding skin sites injected with the same doses of crude venom pre-incubated with a monoclonal antibody against a 60 kDa purified haemorrhagic component did not show patent inflammatory signals except for the presence of a discrete exudate between the skeletal muscle cells (Fig. 1B).

Table 1. Susceptibility to *Bothrops atrox* venom of different strains of isogenic mice

Strain*	Haplotype	DL 50**
BALB/c	a	100.0 (80–120)
C3H/He	k	129.0 (91–169)
C3H/HePas	k	136.0 (136–179)
C57Bl/6	b	90.8 (81–105)
DBA/2	d	137.0 (116–189)
A/J	a	89.4 (82–97)
A/Sn	a	95.2 (86–112)

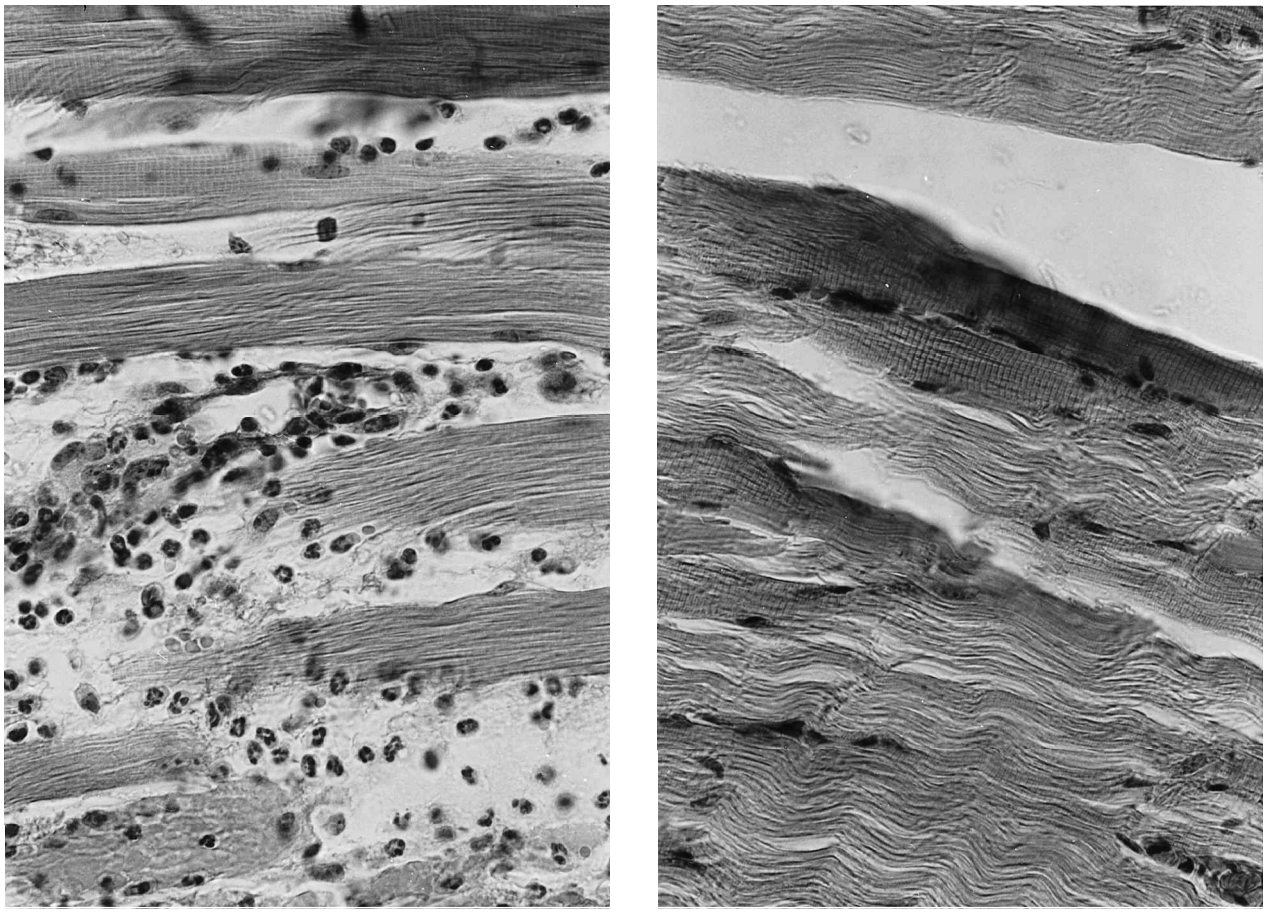
*Mouse 20 ± 2 g.
**95% confidence limit.
Mice were i.p. injected with different venom concentrations of *B. atrox* venom. DL 50% was estimated by probit analysis.

In vivo inflammatory cytokines and nitrite release upon venom injection

Groups of BALB/c mice were injected i.p. with 50 µg of *B. atrox* venom and samples of blood were collected after 2, 4, 12, 18 and 24 h. Cytokines and NO_2^- were individually titrated in the sera. IL-6, IL-10 and IFN-γ attained maximal release after 4 h (Fig. 2A,B,C). following venom injection, while a second peak for IFN-γ was at 12 h TNF-α started to appear after 4 h attaining a peak at 6 h declining thereafter (Fig. 2D). NO_2^- starts to appear at 2 h attaining a peak at 4 h and declining at 6 h (Fig. 2E).

Ex vivo inflammatory cytokines and nitrite release upon venom injection

The cytokines which were detected in serum are mostly synthesized by macrophages. In order to better understand the mechanism of the cell activation by venom spleen cells or adherent peritoneal cells obtained from the same mice pre-stimulated *in vivo* with *B. atrox* venom were analysed in cultures.



(A) (B)

FIG. 1. Photomicrography of subcutaneous tissues obtained from: (A) BALB/c mice s.c. injected with 50 μg of ***B. atrox*** venom, tissue samples were collected 24 h after fixed in 10% paraformaldehyde and HE stained. (B) BALB/c mice s.c. injected with 50 μg of ***B. atrox*** venom previously incubated with 50 μl of monoclonal antibody (59.1) against 60 kDa haemorrhagic factor. Arrows indicate PMN, macrophages, lymphocytes and a disrupted capillary. Magnification 300 ×.

Splenocytes

Groups of BALB/c mice were injected ip with venom 3 h before cell harvesting. Venom, LPS or Con A were added to splenocyte cultures and IL-6, IL-10, IFN-γ, TNF-α and NO_2^- were measured in these supernatants after 48 h. Figure 3A shows that in contrast with LPS and Con A the addition of venom to cells obtained either from mice previously injected with saline or venom does not induce IL-6 release. Release of IL-10, however, was induced by venom, LPS and Con A either in animals injected with venom or with saline (Fig. 3B). Release of IFN-γ was only obtained by Con A. (Fig. 3C). TNF-α and NO_2^- were not detected in these supernatants (data not shown).

Resident adherent cells

Figure 4 shows that resident peritoneal cells either obtained from animals injected with venom or saline produce significant amounts of IL-6, IL-10 and IFN-γ after venom or LPS challenge. TNF-α and NO_2^- were not detected in these supernatants (data not shown).

Discussion

These data have shown that subcutaneous injection of *B. atrox* crude venom in mice induced a set of gross inflammatory signals: plasma exudation, leukocyte migration, vascular wall damage resulting in haemorrhage and skeletal muscle cell disruption. Some or all of these effects caused by *B. atrox* venom have been already described either in human victims of snake bite or in experimentally injected animals.[12,13] The aim of this work has been to provide an experimental model to study *in vivo* and *in vitro* the production and release of mediators (pro-inflammatory substances and cytokines) known to be involved in leukocyte migration, vascular wall damage and in cell activity or death[14] and to analyse the lesions induced by *B. atrox* crude venom. Such an experimental model should involve animals genetically homoge-

neous and moderately susceptible to the venom toxic effects, as well as venom samples containing all the presumptive toxic components. This was achieved by pre-determining the LD_{50} of the venom in several isogenic strains of mice. Among the analysed strains A/Sn, A/J and C57Bl/6 were significantly more susceptible to the venom lethal effects than C3H/He, C3H/HePas and DBA/2 resistant strains. BALB/c mice were found to be a moderately susceptible strain. The genetic influence on an experimental animal susceptibility to the lethal venom action is not novel, since genetic background also influences susceptibility of mice to the lethal activity present in *C. d. terrificus* venom.[5] Distribution of susceptibility or resistance to the lethal activities of the venoms among different mouse strains varies according to the species of

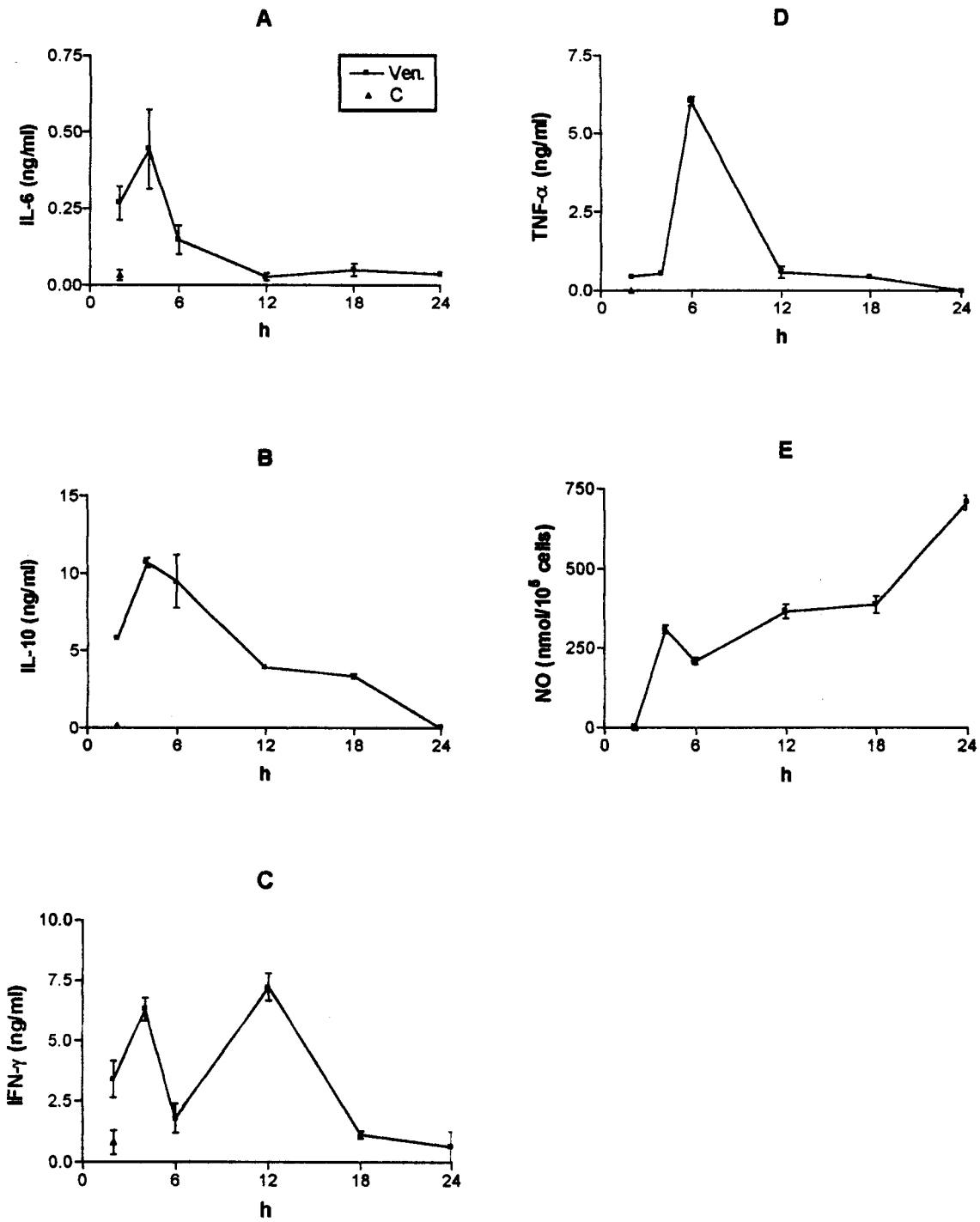


FIG. 2. Cytokine release in serum of BALB/c mice ip injected with 50 μg of *Bothrops atrox* venom. Blood was collected at 2, 4, 6, 8 12, 18 and 24 h and control blood was collected from saline injected mice. The presence of (A) IL-6, (B) IL-10, (C) INF-γ was determined by ELISA; (D) TNF-α was assayed by its cytotoxic effect on L929 cells; (E) NO was determined by a modification of the Griess method. Each point represents the mean ± SD of duplicate samples.

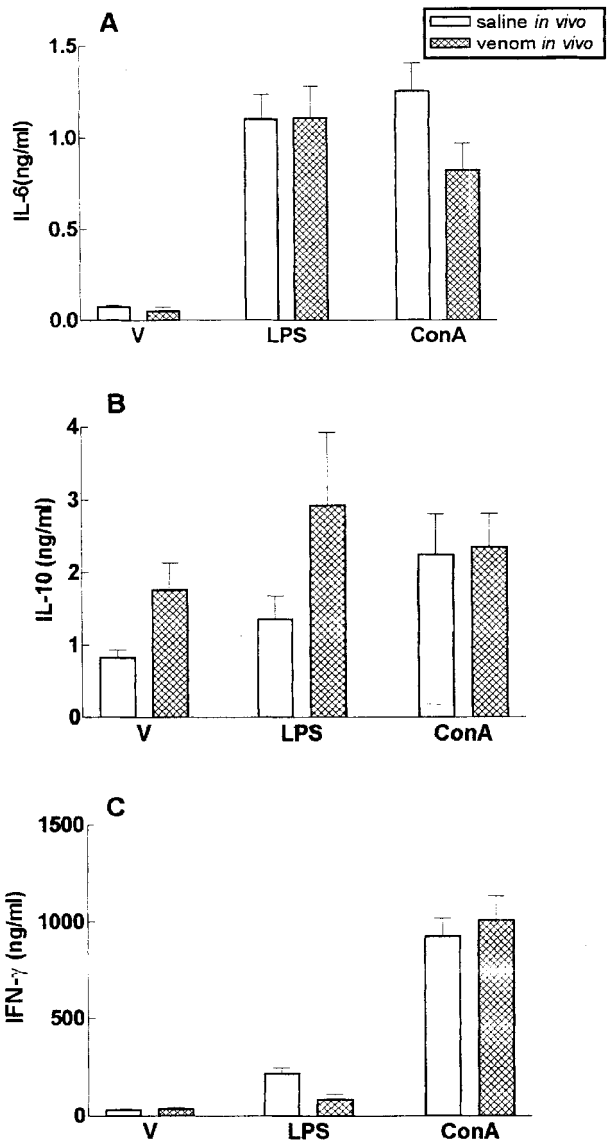


FIG. 3. *Ex vivo* cytokine release in supernatants of spleen cells collected from BALB/c mice i.p. injected with 50 μg of *B. atrox* venom. Spleen cells were harvested 3 h after saline or venom injection, maintained *in vitro* and challenged either with 250 ng of venom, 1 μg of LPS or with 1 μg of Con A and incubated for 48 h (A) IL-6, (B) IL-10, (C) INF-γ, were assayed by ELISA. Each point represents the mean ± SD of duplicate samples of four animals.

venom. A/J and DBA/2 mice are the most susceptible to the *C. d. terrificus* and are the most resistant to the *B. atrox* venom. These observations are compatible with the fact that while in *C. d. terrificus* venom the lethality is confined to neurotoxins such as crotamine and crotoxin, in *Bothrops* sp. the lethality appears to be dependent on the inflammatory factors such as thrombin-like, haemorrhagins, phospholipases and proteases enzymes.

Plasma exudation and oedema at the skin sites of venom injection can be at least partially explained by local release of the well-known mediators of the early events of the acute inflammatory events such as bradykinin[15] and anaphylatoxins.[16] In fact *Bothrops* venom contains proteolytic enzymes capable of promoting *in vitro* cleavage of plasma kallikreinogen and/or the complement components C3 and C5, releasing bradykinin[15] and C3a and C5a,[17] respectively. The local release of these vasoactive peptides although presumptive has not yet been experimentally proven. Although C3a and C5a are potent chemoattractants for PMN leukocytes and monocytes,[18] accumulation of macrophage-like cells, lymphocyte scattering among the inflamed tissues, the cell wall damage and skeletal muscle cells disruption cannot be explained by the local formation of those mediators. Published results obtained in other inflam-

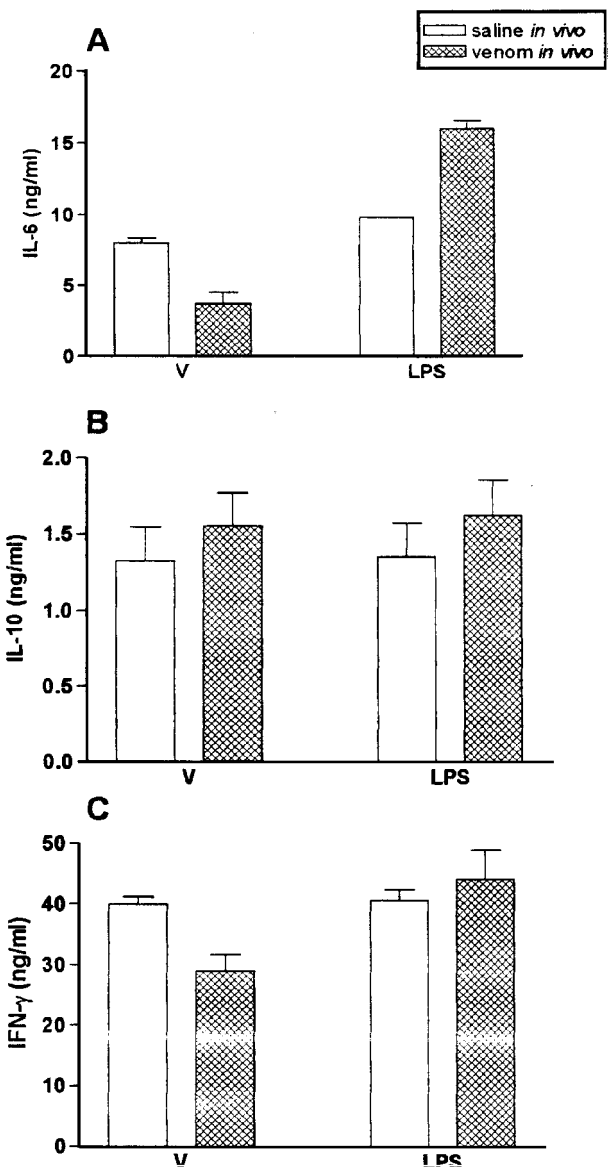


FIG. 4. *Ex vivo* cytokine release in supernatants of spleen cells collected from of BALB/c mice ip injected with 50 μg of *B. atrox* venom. Adherent peritoneal cells were harvested 3 h after saline or venom injection, maintained *in vitro* and challenged either with 250 ng of venom or 1 μg of LPS and incubated for 48 h. (A) IL-6, (B) IL-10, (C) INF-γ, were assayed by ELISA. Each point represents the mean ± SD of duplicate samples of four animals.

matory models have indicated that pro-inflammatory substances and some cytokines are deeply involved in the activation of the endothelial cells and leukocytes leading them to express on their cell surface adhesion molecules as E-selectin and P-selectin and the corresponding specific ligands PSGL-1 and Sialyl Lewis and others.[19] Besides, some cytokines such as IL-8 are chemoattractants for basophils and T lymphocytes[20–22] and potent activators of these cells.[23]

The cytokine profiles observed *in vivo* upon *B. atrox* venom injection shows that production of IL-6, IL-10 and IFN-γ attained maximal values at 4 h following venom injection. While the first two cytokines decay along the following hours becoming almost undectable, IFN-γ serum levels attain a second higher peak 12 h later. TNF-α in contrast, appears at a later time in serum reaching high amounts 18 h after the venom injection (Fig. 2). NO_2^- serum levels, on the other hand, starts to appear even before 4 h. following venom injection increasing slowly but consistently reaching maximal values after 24 h. These results agree with previous reports showing that IL-6 is released in Swiss mice injected with *B. asper* venom[13] and human victims of snake bite.[12] Although the first authors were not able to detect TNF-α in mice injected with *B. asper* venom, others presented indirect evidence indicating that this cytokine can be produced along envenomation by snake venom.[24] These authors provided evidence that metallo-proteases from *B. jararaca* and *Echis pyramidium leakey* venoms can cleave pro-TNF-α into its mature form *in vitro*. Besides, they have shown that TNF-α antibody significantly reduced the resultant necrotic lesions in mice injected with *E. p. leakey* venom. IL-10 is capable of mediating suppression on activated macrophages and monocytes leading to striking down-regulation of numerous inflammatory monokines as TNF-α, IL-1,IL-6, IL-8, GM-CSF and G-CSF.[25,26] IL-10 is also capable of effectively protecting mice from endotoxin-induced shock, a lethal inflammatory reaction mediated by monokines as TNF-α and IL-10.[27] A similar suppressor effect of IL-10 was also found on NO production by activated macrophages.[28] Our data shows that the IL-10 peak at 4 h after venom injection coincides with TNF-α and NO_2^- low serum levels are compatible with these observations.

Some coincidences or discrepancies were also observed when cytokine production was analysed *in vitro* using either spleen cells or adherent peritoneal cells obtained from BALB/c mice injected and challenged *ex vivo* with venom: (a) IL-6 was not produced by spleen cells; (b) IL-10 was produced both by spleen or peritoneal cells; (c) IFN-γ was produced by adherent peritoneal cells but not by spleen cells.As the *in vivo* venom stimulated cells responded *in vitro* to the classical cytokine inducers, these discrepancies cannot be imputed to general deregulation of the machinery used to produce cytokines. Production of IL-10 by spleen cells and peritoneal adherent cells agrees with the well-established observations showing that this cytokine is produced by a large array of cells in which T and B lymphocytes and macrophages are included.[29] Depending on the relapsing time, the production of the various cytokines will be influenced by the ones that are first released into the surrounding millieu, interfering therefore with maturation or secretion of the others or with the expression of their correspondent receptors.

The discrepancies detected in the cytokine profiles produced *in vivo* and *ex vivo* can be explained by the interplay involving the factors governing the production of the concerned proteins, the lack of some essential factors for the synthesis or expression of a particular cytokine or by the low sensitivity of the assay methods of detecting smaller amounts of cytokines. As a monoclonal antibody specific for a 60 kDa *B. atrox* haemorrhagic factor was able to block leukocyte infiltration and haemorrhagic activities of the venom. Such findings suggest that the haemorrhagic components could be the conspicuous inflammatory inducers present in the venom. The monoclonal certainly recognizes and blocks the action of the haemorrhagic factor presumably by combining with a functionally important epitope.

On the basis of our data, we can hypothesize that *B. atrox* venom can trigger various host inflammatory mechanisms by using the same or some components acting in concert: (a) the early plasma exudation can be mediated by local release of bradykinin and histamin; (b) polymorphonuclear cells adherence and migration may be triggered by C3a, C5a and or IL-8; (c) the endothelial cells belonging to the blood vessels crossing the tissue sites injected with the venom can be activated and express P-selectin and E-selectin under the paracrinal action of histamine, thrombin-like factor and TNF-α; (d) basophils and lymphocyte accumulation can be mediated by IL-8; and (e) skeletal muscle cell disruption can be induced either directly by enzymes as proteases, phospholipase A_2, haemorrhagins or even other cytolitic components present in the venom, or indirectly by mediators released from invading leukocytes.

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