




## ACUTE-PHASE REACTIONS, INCLUDING CYTOKINES, IN PATIENTS BITTEN BY *BOTHROPS* AND *CROTALUS* SNAKES IN BRAZIL.

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**ABSTRACT.** Thirty-one patients bitten by venomous snakes in Botucatu area (State of São Paulo - Brazil), sixteen by *Bothrops* spp. and fifteen by *Crotalus durissus terrificus*, were studied. The group comprised twenty-nine males and two females, ranging from fourteen to sixty-three years of age (mean  $33 \pm 15$ ). Leukocytosis with neutrophilia and lymphopenia, increase of mucoproteins and C-reactive protein, decrease of total serum protein and albumin, were observed on the first day after the accident. In addition, increased serum levels of the cytokines IL-6 and IL-8, but not of IL-1b and TNF- $\alpha$ , were observed. The alterations were generally more intense in patients bitten by *Crotalus durissus terrificus* than by *Bothrops* spp. It is concluded that these snakebite envenomations closely resemble an acute trauma, inducing a typical acute-phase response.

**KEY WORDS:** snake venom, acute-phase reactions, cytokines, C-reactive protein, inflammation.

### INTRODUCTION

Ophidic accidents are a serious health problem for the tropical countries<sup>(3,6,33,36)</sup>. In Brasil every year about 20.000 ophidic accidents occur, 2.000 of them only in State of São Paulo<sup>(3,6)</sup>. Among the accidents occurring in the Botucatu area, interior of São Paulo State, about 80% are caused by three species of *Bothrops* (*B. jararaca*, *B. alternatus* and *B. neuwiedi*), and 20% by *Crotalus durissus terrificus*<sup>(7)</sup>.

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Crotalid snake venoms are known to induce a complex series of inflammatory events concomitantly to a considerable tissue damage<sup>(9,29)</sup>. However, our knowledge of the characteristics of the inflammatory responses, and their relationship with damage to tissues, is still very limited, especially at the clinical level. This study attempts to define some of the early inflammatory changes caused by crotalid snake venoms in humans, with the aim of contributing to a better understanding of the pathophysiology of envenomation, and therefore, a basis for improved future treatment strategies.

## PATIENTS AND METHODS

Thirty-one patients bitten by venomous snakes; 16 by *Bothrops* spp. and 15 by *Crotalus durissus terrificus* were studied. The patients were admitted to the Tropical Diseases Service, School of Medicine of Botucatu. Twenty-nine patients were males and two females, ranging from 14 to 63 years of age (mean  $33 \pm 15$ ). From the 31 patients, 19 of them brought the snake and 12 did not. From the 19 snakes, 10 belonged to the *Bothrops* genus and 9 were *Crotalus durissus terrificus*. The 12 patients who did not bring the snake were diagnosed by their clinical symptoms.

After clinical evaluation, all patients were submitted to specific antivenom therapy, according to the severity of each case by intravenous route. Patients from the *Bothrops* group received from 100 to 300 mg of antivenom and those of the *Crotalus* group received from 200 to 300 mg.

All 31 patients were tested for hematocrit, hemoglobin, white blood cells count, erythrocyte sedimentation rate, clotting time, total protein, albumin, globulins and mucoproteins, at the first and the 30th days after the day of envenomation<sup>(27)</sup>.

Cytokine levels were tested in 16 patients, 8 from the *Bothrops* group and 8 from *Crotalus* group. Blood samples were collected during the first five days after the accident, and the serum levels of interleukin-1b (IL-1b), IL-8 and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), were determined using monoclonal antibody-based ELISA systems (Biotrak, Amersham International, United Kingdom) according to the manufacturers instructions. Serum IL-6 was quantified using the B9 hybridoma proliferation bioassay, as previously described<sup>(23)</sup>. The guides for the cytokine levels were the following:

IL-1b: detection limit: 4 pg/ml; serum levels above this value considered elevated;

IL-6: detection limit: 3 pg/ml; serum levels above 5 pg/ml considered elevated;

IL-8: detection limit: 10 pg/ml; serum levels above this value considered elevated;

TNF- $\alpha$ : detection limit: 15 pg/ml; serum levels above 25 pg/ml considered elevated.

All sera were stored frozen at  $-20^{\circ}\text{C}$  until assayed.

Serum C-reactive protein levels were determined in 4 patients, 2 from the *Bothrops* group and 2 from the *Crotalus* group, immediately, on the first three days and on the seventh day following the accident.

Statistical analyses were performed by the Students t-test, by the Mann Whitney test and by the Wilcoxon test comparing the results of the first and the 30th day. The cytokines were analyzed by the Mann Whitney test comparing the results between *Bothrops* and *Crotalus* group<sup>(32,37)</sup>.

## RESULTS

[Table 1](#) and [Table 2](#) summarize the hematological and blood and serum analyses. It was observed leukocytosis, with neutrophilia, lymphopenia, anaeosinophilia, increase of bands, mucoproteins and clotting time and decrease of total proteins, erythrocyte sedimentation rate and albumin, on the first day after the accident.

**TABLE 1** - Hematological findings in patients bitten by *Bothrops* spp. and *Crotalus durissus terrificus*, measured on the first and 30th days after the treatment. Results are reported as means  $\pm$  SD. Bands, basophils and eosinophils are reported as medians.

HEMATOLOGICAL FINDINGS	PATIENTS				STATISTICAL ANALYSES
	<i>Bothrops</i> (n = 16)		<i>Crotalus</i> (n = 15)		
	1st day	30 st day	1st day	30 th day	
Hematocrit (%)	42.8 $\pm$ *3.7	44.3 $\pm$ *3.9	43.4 $\pm$ 3.8	43.7 $\pm$ 5.0	*=0.05 < p < 0.10
Hemoglobin (g%)	13.6 $\pm$ *1.1	14.1 $\pm$ *1.2	13.7 $\pm$ 1.6	13.8 $\pm$ 1.6	* = p < 0.05
White blood cells (n./mm <sup>3</sup> )	8063.0 $\pm$ *, #2503.0	6261.0 $\pm$ *2137.0	15087.0 $\pm$ #, **5921.0	7847.0 $\pm$ **3255.0	* = p < 0.05 ** = p < 0.01 # = p < 0.001
Neutrophils (n./mm <sup>3</sup> )	4920.0 $\pm$ *, #2499.0	3223.0 $\pm$ *1767.0	10549.0 $\pm$ #, **3681.0	3977.0 $\pm$ **2494.0	* = p < 0.05 **, # = p < 0.001
Lymphocytes (n./mm <sup>3</sup> )	1936.0 $\pm$ *1026.0	2332.0 $\pm$ *740.0	1769.0 $\pm$ #800.0	2781.0 $\pm$ #1028.0	*=0.05 < p < 0.10 # = p < 0.01
Monocytes (n./mm <sup>3</sup> )	336.0 $\pm$ *207.0	299.0 $\pm$ 165.0	541.0 $\pm$ *, #342.0	355.0 $\pm$ #227.0	* = p < 0.05 #=0.05 < p < 0.10
Basophils (n./mm <sup>3</sup> )	0.0	21.0	0.0	0.0	n.s.
Eosinophils (n./mm <sup>3</sup> )	319.5 *, #	209.0 *, **	0.0 #, ##	472.0**, ##	*=0.5 < p < 0.10 ## = p < 0.001, #, ** = p < 0.05
Bands (n./mm <sup>3</sup> )	100.5 *	25.0 *	315.0 #	0.0 #	*=0.05 < p < 0.10 # = p < 0.01

n.s. = not significant

**TABLE 2** - Blood and serum analyses (erythrocyte sedimentation rate, clotting time, urea, total proteins, albumin, globulin and mucoproteins) in patients bitten by *Bothrops* spp. and *Crotalus durissus terrificus*, measured on the first and 30th days after the treatment. Results are reported as means  $\pm$  SD. Clotting time and urea are reported as medians.

BLOOD AND SERUM ANALYSES	PATIENTS				STATISTICAL ANALYSES
	<i>Bothrops</i> (n = 16)		<i>Crotalus</i> (n = 15)		
	1st day	30 th day	1st day	30th day	

Erythrocyte sedimentation rate (mm/hour)	14.69 ±* 13.32	16.87 ± 11.30	6.27 ±*, # 5.98	17.53 ± # 14.84	* = p < 0.05 # = p < 0.01
Clotting time (minutes)	8.00 *	n.p.	20.00 *	n.p.	* = p < 0.02
Urea (mg%)	25.50	21.00	29.00 *	23.00 *	* = p < 0.05
Total protein (g%)	6.19 ±* 0.56	7.03 ±* 0.50	6.60 ± # 0.69	7.03 ± # 0.65	* = p < 0.001 # = p < 0.02
Albumin (g%)	4.06 ±* 0.69	4.44 ±* 0.37	3.92 ± # 0.36	4.33 ± # 0.51	* = p < 0.05 # = p < 0.02
Globulins (g%)	2.13 ±*, # 0.81	2.64 ±* 0.40	2.69 ± # 0.46	2.69 ± 0.67	* = p < 0.01 # = p < 0.05
Mucoproteins (mg %)	11.04 ± 3.89	9.96 ± 4.23	11.95 ±* 4.56	9.68 ±* 3.11	*=0.05< p <0.10

n.p. = not performed

**Table 3** shows that the cytokine serum levels were increased within the first five days after the accident. The IL-1b were increased only in four patients from *Crotalus* group. The IL-6 levels ranged in *Bothrops* group from 0.0 to 116 pg/ml (median=11) and in *Crotalus* group from 6 to 223 pg/ml (median=101). The IL-8 levels ranged in *Bothrops* group from 0.0 to 2088 pg/ml (median=0.0) and in *Crotalus* group from 0.0 to 21968 pg/ml (median=1281). IL-6 serum levels were higher (p=0.041) and IL-8 serum levels presented high tendency to be increased in patients from *Crotalus* group (0.052 < p < 0.065). TNFα were similar in both groups studied.

**TABLE 3** - Increased serum cytokine levels in patients bitten by *Bothrops* spp. and *Crotalus durissus terrificus*, measured within the first five days after the accident.

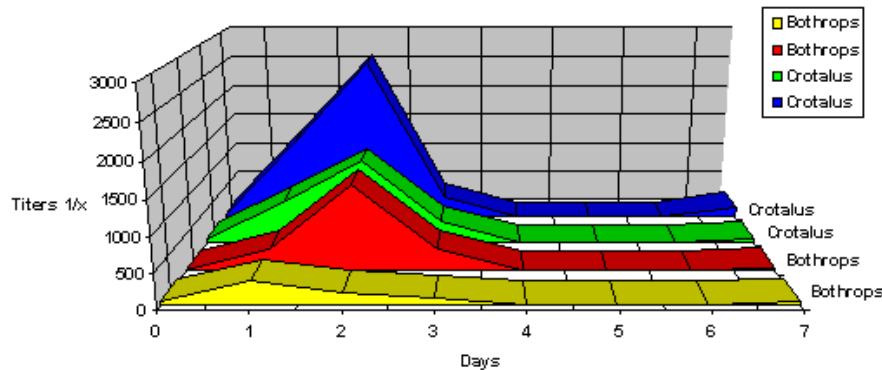
PATIENTS	CYTOKINE LEVELS (pg/ml)			
	IL-1b	IL-6	IL-8	TNFα
<i>Bothrops</i> (n = 8)	-	116	-	-
	-	38	-	-
	-	21	-	-
	-	7	-	-
	-	-	-	-

	-	-	-	-
	-	13	-	-
	-	9	2088	-
<b>Medians</b>	<b>0.0(a)</b>	<b>11(c)</b>	<b>0.0(e)</b>	<b>0.0(g)</b>
<i>Crotalus</i> (n = 8)	-	100	-	-
	-	102	-	-
	45	111	2144	-
	14	6	1526	-
	-	8	-	-
	60	223	21868	-
	66	89	1702	-
	-	141	1307	-
<b>Medians</b>	<b>7(b)</b>	<b>101(d)</b>	<b>1281(f)</b>	<b>0.0(h)</b>

axb, p=0.110; cxd, p=0.041; exf, 0.052<p<0.065; gxh, not significant

**Figure 1** shows the time course of C-reactive protein which peaked between 1 and 2 days after the accident.

**FIGURE 1:** Increased C-reactive protein levels in 4 patients, 2 of them bitten by *Bothrops* spp. and 2 bitten by *Crotalus durissus terrificus*.



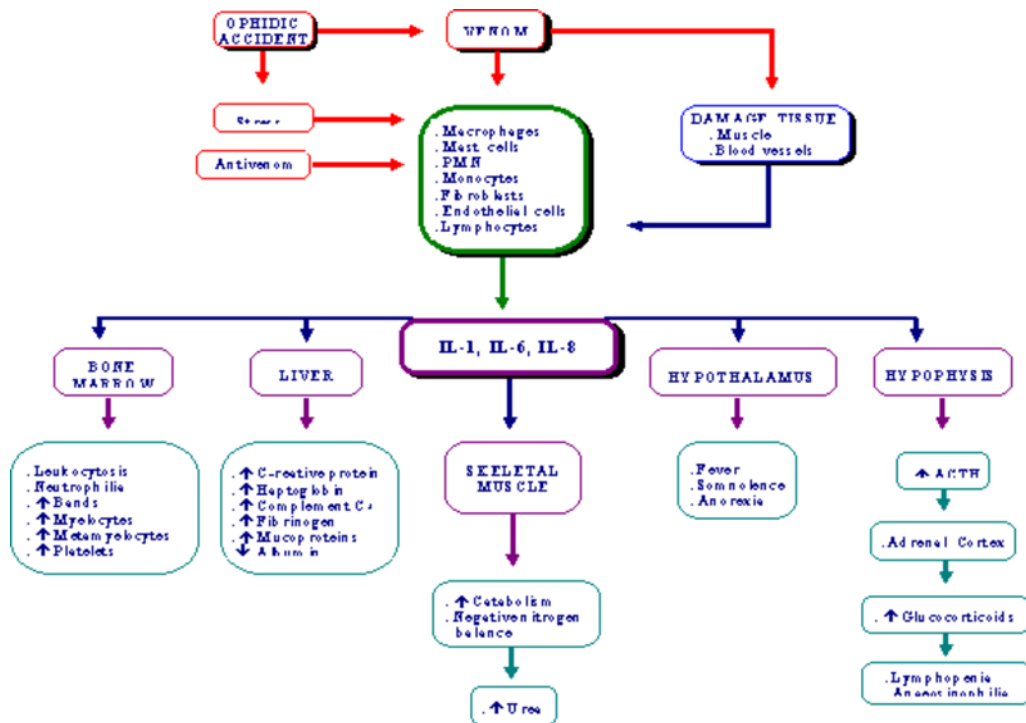
## DISCUSSION

The patients bitten by venomous snake presented an acute-phase reaction, characterized by a moderate leukocytosis, with neutrophilia, lymphopenia, anaeosinophilia, increase of mucoproteins, clotting time and C-reactive protein and decrease of total proteins, erythrocyte sedimentation rate and albumin, on the first day after the accident. In addition, pro-inflammatory cytokine levels were increased within the first five days, especially IL-6 and

IL-8. These alterations were in general more intense in patients bitten by *Crotalus* than by *Bothrops* snakes.

These observations suggest that ophidic accident shares many characteristics of an acute trauma, as shown in **Figure 2**. It is possible that the ophidic venom components interact with the target cells (macrophages, mast cells, platelets, polymorphonuclears, fibroblasts, endothelial cells and/or lymphocytes) inducing and releasing inflammatory mediators, among them cytokines, such as IL-6 and IL-8. Interleukin-6, which appeared increased in almost all of the patients studied, is known to induce a wide variety of biological effects. Upon the hypothalamus giving rise to fever, anorexia and somnolence<sup>(12,20,28,35)</sup>. It also acts on the hypophysis leading to secretion of adrenocorticotrophic hormone which interacts with the adrenal cortex releasing glucocorticoids which in turn cause lymphopenia<sup>(10,24)</sup>.

**FIGURE 2** - The acute phase response of the organism caused by the ophidic accident



In the skeletal muscle the cytokines promote a negative nitrogen balance increasing urea<sup>(12,30)</sup>. Interleukin-6 acts on the bone marrow inducing and releasing the young white blood cells, such as bands and myelocytes<sup>(12,28)</sup> and promoting the production of platelets<sup>(19)</sup>. It also acts on the liver increasing the production of C-reactive protein, complement factor C3, fibrinogen and mucoproteins<sup>(11,13,15)</sup>. On the other hand, IL-1b down-regulates the albumin production<sup>(26,31)</sup>.

Although it is easier to diagnose the acute phase reaction when the IL-6 is increased, it is very difficult to do so in ophidic accident by routine tests because the erythrocyte sedimentation rate decreases and clotting time increases. This is due to the fact that the fibrinogen is consumed<sup>(2,22,34)</sup>. In addition, C3 is consumed by antivenom treatment<sup>(8,16,25)</sup>.

These clinical observations agree with the experimental studies conducted by Lomonte *et al.*<sup>(23)</sup>, who injected venom from *Bothrops asper* into mice. These authors observed an increase of IL-6 after the envenomation which peaked between 3-6 hours and returned to normal values at 12 hours. Although serum TNF-a and IL-1b were not detectable, preliminary observations indicate a local production of these cytokines, evidenced by immunohistochemical techniques (Lomonte B, Tarkowski A & Hanson LÅ, unpublished results).

Emslie-Smith & Harris<sup>(14)</sup>, observed lymphopenia in rats inoculated with notechis II-5, a potent myotoxin isolated from the venom of the Australian tiger snake (*Notechis scutatus*). On the other hand, from published data, these authors identified lymphopenia in only two cases of snakebite in man<sup>(17,18)</sup> and suggest that lymphopenia might be an important early indicator of serious envenomation. We agree with these authors because, frequently in severe envenomation caused by *Crotalus* snakes, lymphopenia and anaesinophilia are frequently observed<sup>(6)</sup>. These alterations may be caused by excessive release of glucocorticoids from adrenal cortex in intensive stressful situation.

The increase of IL-8 observed in patients bitten by *Crotalus durissus terrificus* may have an important action in the respiratory tract as observed in some severe accidents. Recently, Amaral *et al.*<sup>(1)</sup> observed acute respiratory insufficiency in patients bitten by *Crotalus* snakes and attributed these alterations to the venom action on the peripheral nervous system. On the other hand, Hoch *et al.*<sup>(21)</sup> observed increased concentrations of IL-6 and IL-8 in patients with severe accidental trauma. These authors suggested that IL-8 contributes to the development of the adult respiratory distress syndrome. It is possible that in severe envenomation caused by *Crotalus* snakes, the increased levels of the IL-8 may have some effect in the lungs, perhaps contributing to the respiratory insufficiency observed in these patients.

In addition, it is possible that the antivenom has some effect on the immune system. Bielory *et al.*<sup>(8)</sup> observed discreet increase of C-reactive protein and erythrocyte sedimentation rate in patients treated with equine anti-thymocyte globulin. These alterations were observed from the seventh day after the treatment.

At present it is unknown if the induction and release of pro-inflammatory cytokines is due to a direct action of venom components on cells of the immune system, or if it is an indirect response to the local tissue damage caused by some venom components, such as myotoxins and hemorrhagic toxins. *In vitro*, it was observed that *Bothrops asper* venom lacked direct stimulating activity on cultured murine splenic cells, in terms of cytokine secretion or proliferation. Also, the ability of a purified venom component, myotoxin II, to induce a strong IL-6 response, is suggestive of an indirect mechanism inducing an acute-phase response, probably initiated by the local tissue damage<sup>(23)</sup>.

In conclusion, we suggest<sup>(4,5)</sup> that the ophidic venom, especially *Crotalus* venom, by releasing cytokines and other inflammatory mediators, produces a clinical and biochemical situation like an acute trauma.

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