Role of crotoxin, a phospholipase A<sub>2</sub> isolated from Crotalus durissus terrificus snake venom, on inflammatory and immune reactions

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Background: Crotoxin (CTX) is a potent neurotoxin from Crotalus durissus terrificus snake venom (CdtV) composed of two subunits: one without catalytic activity (crotapotin), and a basic phospholipase A<sub>2</sub>. Recent data have demonstrated that CdtV or CTX inhibit some immune and inflammatory reactions. Aim: The aim of this paper was to investigate the mechanisms involved in these impaired responses.

Materials and methods: Male Swiss mice were bled before and at different intervals of time after subcutaneous injection of CTX or bovine serum albumin (BSA) (control animals). The effect of treatments on circulating leukocyte mobilisation and on serum levels of interleukin (IL)-6, IL-10, interferon (IFN)-γ and corticosterone were investigated. Spleen cells from treated animals were also stimulated in vitro with concanavalin A to evaluate the profile of IL-4, IL-6, IL-10 or IFN-γ secretion. Cytokine levels were determined by immunoenzymatic assay and corticosterone levels by radioimmunoassay. To investigate the participation of endogenous corticosteroid on the effects evoked by CTX, animals were treated with metyrapone, an inhibitor of glucocorticoid synthesis, previous to CTX treatment.

Results: Marked alterations on peripheral leukocyte distribution, characterised by a drop in the number of lymphocytes and monocytes and an increase in the number of neutrophils, were observed after CTX injection. No such alteration was observed in BSA-treated animals. Increased levels of IL-6, IL-10 and corticosterone were also detected in CTX-injected animals. IFN-γ levels were not modified after treatments. In contrast, spleen cells obtained from CTX-treated animals and stimulated with concanavalin A secreted less IL-10 and IL-4 in comparison with cells obtained from control animals. Metyrapone pre-treatment was effective only to reverse the neutrophilia observed after CTX administration.

Conclusions: Our results suggest that CTX may contribute to the deficient inflammatory and immune responses induced by crude CdtV. CTX induces endogenous mechanisms that are responsible, at least in part, for these impaired responses.

Key words: Crotoxin, Phospholipase A<sub>2</sub>, Inflammation reaction, Immune reaction, Endogenous glucocorticoids, Crotalus durissus terrificus snake venom

Introduction

Crotoxin (CTX), a β-neurotoxin from the venom of South American rattlesnake Crotalus durissus terrificus, is responsible for neuromuscular transmission blocking, myotoxic effects and lethality induced in vivo by the venom.<sup>1,2</sup> CTX is a heterogeneous protein, composed of a weakly basic phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and high enzymatic basic activity (CB), and an acidic polypeptide devoid of toxic and enzymatic activities, called crotapotin (CA).<sup>3</sup>

Although CA is enzymatically and pharmacologically inactive,<sup>2</sup> it does enhance the toxicity of PLA<sub>2</sub>.<sup>4,5</sup> On interaction with membranes or phospholipid vesicles, the complex dissociates: CB binds to the membrane, and CA is released from the complex.<sup>6,7</sup>

In spite of its high PLA<sub>2</sub> content, crude Crotalus durissus terrificus snake venom (CdtV) induces a short-duration oedema,<sup>8,9</sup> a scarce cell infiltration in subcutaneous tissues, and inhibits some activities displayed by inflammatory macrophages.<sup>5</sup> In addition, it has been demonstrated that CTX or CdtV
reduces the humoral immune response to classical protein antigens without inhibition of the contact hypersensitivity reaction elicited by sensitising hapten.

Inflammatory and immune responses are thought to be regulated by cellular and vascular events that involve a number of secreted substances. Endogenous glucocorticoids (GCS), whose secretion is elevated during the early phase of the acute inflammatory reaction, regulate the subsequent development of the process by suppressing both vascular and cellular events. In this context, endogenous GCS interfere with vasodilatation and vascular permeability, and secretion of chemical mediators. GCS also acts on the acquired specific immune response by inhibiting T-cell activation and/or modulating synthesis of Th0, Th1 and Th2 cytokines.

The aim of the present study was to investigate the role of CTX on impaired inflammatory and immune response elicited by crude venom. In this context, the effects of CTX on circulating leukocyte distribution and on the profile of pro- and anti-inflammatory cytokine secretion were evaluated. In addition, the effect of CTX on endogenous GCS secretion and its consequent interference on effects observed were also investigated. The results obtained show that in vivo injection of CTX evokes marked alterations on distribution of circulating leukocytes, and on cytokine and corticosterone secretion. CTX treatment inhibited the secretion of interleukin (IL)-4 and IL-10 by spleen cells stimulated with concanavalin A (ConA). The increased levels of the endogenous corticosteroid after CTX injection interfere only with the increased number of peripheral neutrophils. Taken together, the data presented in this study suggest that CTX is able to evoke endogenous mechanisms, such as the reduction of mononuclear cells on circulation and secretion of anti-inflammatory cytokines and glucocorticoids, that may contribute to the lack of local inflammatory reaction during envenomation by CdtV and to the immunosuppression evoked by this venom.

Materials and methods

Animals

Male BALB/c mice weighing 18–20 g were obtained from Animal Facilities from Institute Butantan. Animals were allowed a standard diet and water ad libitum.

Venom and purified toxin

Lyophilised crude CdtV was a pool obtained from several specimens of C. durissus terrificus snakes and supplied by the Laboratory of Herpetology, Institute Butantan. CTX was isolated from crude venom in a single-step purification process by anion-exchange chromatography using a Mono-Q HR 5/5 column in a FPLC system (Pharmacia, Uppsala, Sweden). Fractions (1 ml/min) were eluted by a linear gradient of NaCl (0–1 M in 50 mM Tris–HCl; pH 7.0). Before pooling, fractions containing CTX were checked for homogeneity by non-reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (12.5%), and PLA2 activity on a synthetic chromogenic substrate was assessed.

Determination of blood leukocyte counts

Blood was collected from the orbital plexus of mice before and at varying intervals of time after subcutaneous injections of 5 μg of CTX or equivalent concentration of bovine serum albumin (BSA) (control animals). Total leukocyte number was determined in a Neubauer haemocytometer and differential counts in blood smears.

Determination of immunoglobulin G1 anti-human serum albumin antibody production

Mice were immunised with human serum albumin (HSA) plus adjuvant (Al(OH3); 100 μg, subcutaneously) 6 h after subcutaneous injection of 5 μg of CTX. Control animals received the same amount of HSA and CTX. The serum immunoglobulin (Ig)G1 anti-HSA response was evaluated by enzyme-linked immunosorbent assay (ELISA) 1 week after the challenge with HSA. Briefly, microtitre plates, previously coated with HSA (2 μg/ml), were incubated for 1 h with different serum dilutions. After washing, plates were incubated with peroxidase-labelled goat anti-mouse IgG1 antibodies. The reaction was developed by the addition of substrate solution (0.4 mg/ml of o-phenylenediamine plus H2O2 in 0.15 M citrate buffer; pH 5.0). Absorbances at 492 nm were recorded in a microplate reader and results were expressed as the values of absorbance given at the reciprocal of serum dilution.

Determination of cytokine levels in serum or in supernatant from spleen cell cultures obtained from CTX-treated animals

Mice were subcutaneously injected with 5 μg of CTX or BSA and blood and spleen cells were collected after different intervals of time. Spleen cell suspensions (5 × 106 cells/well), obtained from mice 6 h after CTX or BSA treatment, were stimulated in vitro with ConA (5 μg/ml) for 24 or 48 h at 37°C. IL-4, IL-6, IL-10 and interferon (IFN)-γ levels were determined in blood and culture supernatant by a specific two-site sandwich
ELISA using the following monoclonal antibodies: for IFN-γ, XMG 1.2 and biotinylated NA 18; for IL-4, BVD-1D11 and biotinylated BVD6-24G2; for IL-6, MP520F3 and biotinylated MP532C1L; and for IL-10, C252–2A5 and biotinylated SXC-1. Binding of biotinylated antibodies was detected using streptavidin-biotinylated horseradish peroxidase (Amersham Int., Uppsala, Sweden) and ABTS (Sigma Chemical Co., St. Louis, MO, USA) solution in 0.1M citrate buffer + H₂O₂. Samples were quantified by comparison with standard curves of recombinant mouse cytokines. Standard curves were as follows: IL-4, 2.2–540 pg/ml; IL-6, 2.93–2000 pg/ml; IL-10, 30–540 pg/ml; IFN-γ, 20–1620 pg/ml. All the antibodies were purified by protein G-Sepharose chromatography from hybridoma cell culture supernatants, and biotin-labelled as needed. Hybridomas and recombinant standard cytokines were a gift from Dr R.L. Coffman (DNAX Research Institute, Palo Alto, CA, USA).

**FIG. 1.** Effect of crotoxin (CTX) on circulating leukocyte mobilisation. Blood was collected from orbital plexus of mice before or at different intervals of time after subcutaneous injection of 5 μg of CTX (●) or equivalent concentration of bovine serum albumin (BSA) (●) dissolved in 100 μl of sterile saline. Total leukocyte number was determined in a Neubauer haemocytometer, and lymphocyte, monocyte or neutrophil numbers were quantified in blood smears. Values represent the mean ± SEM of six animals in each group. *p < 0.01 in comparison with values obtained in BSA-treated animals.

**Determination of corticosterone levels**

The effect of CTX on GCS serum levels was investigated by determining the corticosterone serum concentration by radioimmunoassay as described by Abraham.32 Animals were treated subcutaneously with 5 μg of CTX or BSA and blood was collected from the abdominal aorta at varying intervals of time.

**Metyrapone treatment**

Metyrapone (Ciba-Geigy, Sào Paulo, Brazil), dissolved in phosphate-buffered saline solution (PBS) (pH 7.2, containing 10 mM sodium phosphate, 150 mM NaCl), was administered to block synthesis of glucocorticoids, without causing a typical deficiency of mineralocorticoids.33 Two daily doses of 30 mg/kg each were given intraperitoneally at 12 h intervals for...
Control animals received the same amount of PBS. The CTX treatment was initiated 2 h after the last dose and experiments were carried out according to the procedures already described regarding leukocyte mobilisation, cytokine and glucocorticoid secretions, and IgG1 production.

Analysis of data

Means and SEM of all data are presented and compared by Student’s t-test or analysis of variance. When appropriate, the data were analysed by the Newman–Keuls test.

Results

Effects of CTX on circulating leukocyte mobilisation

The subcutaneous injection of CTX evoked significant alterations on the pattern of circulating leukocytes, without inducing changes in the total number of white cells (Fig. 1). In spite of marked alterations on the number of polymorphonuclear and mononuclear cells, the values of total leukocytes were not modified. Two hours after CTX treatment, an intense drop in the number of lymphocytes and an increase of neutrophils were observed. These alterations evolved until 6–8 h, when the maximal responses occurred. Additionally, CTX evoked a drastic drop in the number of monocytes 6 h after injection. Values were normalised 24 h after CTX administration (Fig. 1). No significant alterations were observed in animals treated with BSA (Fig. 1).

Levels of serum inflammatory mediators and endogenous glucocorticoids after in vivo CTX treatment

Figure 2 depicts cytokine and glucocorticoid concentrations in the serum of mice after CTX administration (5 μg, subcutaneously). Ninety minutes after CTX injection, a discrete increment of IL-10 was detected in the serum, which returned to basal values 180 min after administration. IL-6 serum levels also increased at 180 min after injection and remained high up to 540 min. In addition, endogenous corticosterone was released into the circulation 180 min after CTX treatment and the peak in serum levels of this steroid was detected between 6 and 9 h. In contrast, IFN-γ levels were not modified after CTX injection (data not shown). Sera obtained from control animals did not present significant alterations on the profile of increments of the endogenous substances studied (data not shown).

Effect of CTX treatment on cytokine production by spleen cells

Spleen cells isolated from mice were investigated regarding their ability to secrete cytokines after in vitro ConA stimulation. Results presented in Figure 3 show that in vivo CTX treatment inhibited the production of IL-4 and IL-10 by spleen cells, when they were stimulated for 24 or 48 h. No interference of IL-6 or IFN-γ production after ConA stimulation was detected.
Interference of in vivo metyrapone treatment on effects induced by CTX

As described, in vivo CTX treatment induces a significant increase in the corticosterone secretion. To investigate the participation of this endogenous GCS on the effects evoked by CTX, animals were treated with metyrapone, an inhibitor of corticosteroid synthesis, previous to CTX administration. The effectiveness of metyrapone treatment was confirmed by a

FIG. 3. Effect of in vivo crotoxin (CTX) treatment on cytokine secretion by spleen cells stimulated with concanavalin A (ConA). Spleen cells were collected from animals 6 h after bovine serum albumin (BSA) (■) or CTX (□) treatment. A sample of 5 × 10⁶ cells/well were stimulated with ConA (0.5 ml of 10 μg/ml) during 24 or 48 h at 37°C in an air chamber (5% CO₂). Cytokine contents were determined by enzyme-linked immunosorbent assay in cell supernatants. Values represent the mean of duplicate cultures ± SD. * p < 0.05 and ** p < 0.001 in comparison with values obtained in BSA-treated animals.

FIG. 4. Interference of metyrapone treatment on cytokine secretion induced by crotoxin (CTX). Animals were pre-treated with metyrapone (30 mg/kg, intraperitoneally; every 12 h, for 3 days) or equivalent volume of phosphate-buffered saline (PBS). Two hours after the last injections, CTX (5 μg/100 μl, subcutaneously) was administered. Spleen cells were collected 6 h after toxin injection, and 5 × 10⁶ cells/well were stimulated with concanavalin A (0.5 ml of 10 μg/ml) during 24 or 48 h at 37°C in an air chamber (5% CO₂). Cytokine levels in cell culture supernatants were determined by enzyme-linked immunosorbent assay. Results represent the mean of duplicate cell cultures ± SD from six animals treated with PBS and CTX (□) or with metyrapone and CTX (■).
marked reduction on corticosterone levels before CTX injection (data not shown). Results showed, first, that metyrapone administration did not affect the altered cytokine production by spleen cells stimulated with ConA. Cells obtained from metyrapone- and CTX-treated animals produced equivalent amounts of IL-4 and IL-10 to those collected from control animals, treated with PBS and CTX (Fig. 4). Second, the results showed that metyrapone treatment did not interfere with the described inhibition of IgG1 anti-HSA antibody production by CTX treatment, performed 6 h before HSA immunisation (Fig. 5). Finally, on the contrary, metyrapone pre-treatment abolished the increase in circulating neutrophil counts induced by CTX, but it did not interfere with the distribution of other leukocytes. Numbers of lymphocytes and monocytes in circulating blood of metyrapone- and CTX-treated animals were similar to those observed in blood of animals treated with PBS and CTX (Fig. 6).

Discussion

Class II phospholipase A$_2$ (PLA$_2$) is responsible for arachidonic acid mobilisation from cell membranes and is believed to play a key role in inflammation. CTX, the most abundant component of CdtV, is a complex formed by a non-toxic and non-enzymatic subunit A and a venom class II PLA$_2$, which has properties of lethal $\beta$-neurotoxins and exhibits high enzymatic activity. CTX exists in several isoforms in the same venom. It is more toxic and shows less enzymatic activity than its dissociated PLA$_2$ subunit.

It has been demonstrated that crude CdtV induces impaired local inflammatory reaction characterised by negligible oedema and by the absence of leucocyte infiltration. Also, antibody production is impaired in horses after CdtV administration. The determination of the total number of white blood cells present in the circulation provides an important representation of the status of activation of the immune system and of the profile of distribution of these cells in the organism. Marked alterations in the number of circulating leucocytes are associated with inflammatory reactions, which reflect mobilisation of these cells from their marginal and/or bone marrow pools. Our results demonstrated that CTX injection induces marked neutrophilia and drastic decrease in the number of lymphocytes and monocytes at 6–8 h after treatment. The functional significance of these effects and the mechanisms involved has not yet been elucidated. The same pattern of response has been shown during acute stress conditions. The anatomical sites where lymphocytes and monocytes migrate from the blood after CTX treatment are not known. No changes in the lymphocyte numbers were detected in lymph nodes located near to the CTX injection area or in the spleen (data not shown). It is possible that this mononuclear cell behaviour after CTX administration may contribute to the impaired immune and inflammatory responses evoked by crude venom already described. There are evidences that these changes can significantly alter the capacity of the immune system to mount an antigen-specific-cell mediated immune response in vivo. This hypothesis will be further investigated. The mobilisation of neutrophils into circulation is of interest, since local injection of CTX does not evoke migration of neutrophils to tissues (data not shown). Thus, it is possible that neutrophilia may be secondary to endogenous mechanisms elicited by CTX.

The inflammatory and immune responses are thought to be regulated by a series of cellular and molecular events that involve a number of cytokines.
Data here presented show that in vivo CTX treatment induces increase in IL-10 and IL-6 levels without altering IFN-γ levels in serum. On the contrary, CTX inhibits the production of IL-4 and IL-10 by splenic cells after ConA stimulation, with no alteration of IFN-γ and IL-6 production.

IL-10 was described originally as a cytokine produced by murine Th2 clones, which inhibits the synthesis of several cytokines by Th1 clones and decreases the proliferation and/or IL-2 production by T cells. These effects are achieved by impairing the expression of some co-stimulatory molecules and by altering some functions of professional antigen-presenting cells such as macrophages. IL-10 also acts selectively on the macrophages by inhibiting the expression of co-stimulatory cell surface B7 molecules. It has been shown that resident or inflammatory macrophages have impaired spreading and phagocytic functions after CdtV treatment. As macrophages play an important role in the innate and T-cell-dependent immune responses, it is proposed that macrophages, whose phenotype or activities could be depressed through the early IL-10 production, might be implicated in the depressed responses induced by CTX.

In agreement with our results, increased plasma levels of IL-6 were detected in patients bitten by C. durissus terrificus. IL-6 has been found to play a central role in defence mechanisms, including the acute phase reactions, haematopoiesis and immune response. Concentrations of IL-6 in plasma rise markedly in response to tissue injury and correlates with plasma corticosteroid hormone increments. IL-6 stimulates the hypothalamic–hypophisis–adrenal axis...
inducing corticosteroid secretion.\textsuperscript{19} Elevation of glucocorticoid levels in the circulation is related to altered distribution of peripheral leukocyte subpopulations. Alterations observed during stress conditions, such as infection,\textsuperscript{50} are significantly reduced in adrenalectomised animals.\textsuperscript{30,40} Through interaction with specific receptors, secreted glucocorticoids mediate the decrease in number of lymphocytes and monocytes during stress.\textsuperscript{30} The lymphocyte diminution in blood is due to retention of cells in the lymphatic circulation.\textsuperscript{28} Indeed, neutrophilia induced by secreted glucocorticoids is dependent on mobilisation of polymorphonuclear cells into the circulation from the marginated and blood marrow pools.\textsuperscript{51}

The elevated IL-6 levels in serum of CTX-treated animals, and the observations that elevated serum glucocorticoids levels promote alterations on white blood cells\textsuperscript{32} similar to those evoked by CTX, suggested a possible role for CTX on corticosteroid hormone secretion. Data in the present study demonstrate that CTX induces glucocorticoid secretion, reflected by elevation of corticosterone serum levels, and corroborate the observations of Chisari \textit{et al.}\textsuperscript{55} that CdtV or CTX treatments evoke ACTH and corticosterone secretion \textit{in vivo} and \textit{in vitro}.

Corticosteroids modulate several events related to inflammatory and immune reactions. In this context, vascular and cellular events of inflammatory reaction, cytokine secretion and antibody production are influenced by elevated levels of corticosteroids.\textsuperscript{11–27} For these reasons, it is plausible that elevated levels of corticosteroids after CTX injection could explain, at least in part, the absence of inflammatory effects during human or experimental envenomation and the impaired IgG that CdtV or CTX treatments evoke ACTH and corticosterone secretion.

In summary, our results suggest that, despite its high PLA\textsubscript{2} activity, CTX induces endogenous mechanisms, evidenced by alterations on circulating leukocyte distribution and cytokine and glucocorticoid secretion. These mechanisms may contribute to the impaired inflammatory reaction and to the immunosuppression elicited by CdtV. Endogenous corticosteroids are not responsible for such impaired immune response. The role of the drastic drop in circulating mononuclear cells and the participation of anti-inflammatory cytokines on the impaired responses elicited \textit{in vivo} by CTX need to be further investigated.

\textbf{References}

1. Bon C, Bouchier C, Choumet V, Faure G, Jiang MS, Lambertz MP, Radvangy F, Salou B. Crotoxin, half-century of investigations on a phospholipase A\textsubscript{2} neurotoxin. \textit{Acta Physiol Pharmacol Latinoam} 1989; 39: 439–448.

2. Gopalakrishnakone P, Hawgood BJ. Morphological changes induced by crotoxin in murine neuron and neuromuscular junction. \textit{Toxicon} 1984; 22: 791–804.

3. Bon C, Choumet V, Faure G, Jiang MS, Lambertz MP, Radvangy F, Salou B. Biochemical analysis of the mechanism of action of crotoxin, a phospholipase A\textsubscript{2} neurotoxin from snake venom. In: Dolly O.J. ed. \textit{Neurotoxins in Neurochemistry}. Chichester: Ellis Horwood, 1998:52–63.

4. Bon C. Synergism of the two subunits of crotoxin. \textit{Toxicon} 1982; 20: 105–109.

5. Choumet V, Bouchier C, Delot E, Faure G, Salou B. Development of a polyclonal antibody against crotoxin. \textit{Toxicon} 1996; 39: 197–202.

6. Bon C, Jeng TW. Crotoxin: a possible mechanism of action. \textit{Adv Cytobiomembran} 1979; 3: 231–235.

7. Radvangy F, Bon C. Investigations on the mechanism of action of crotoxin. \textit{J Physiol (Paris)} 1984; 78: 18–23.

8. Santoro ML, Sousa e Silva MCC, Gonçalves, LRC, Mariano, M. The venom of South American rattlesnake inhibits macrophage functions and is endowed with anti-inflammatory properties. \textit{Acta Physiol Pharmacol Latinoam} 1990; 29: 189–195.

9. Santoro ML, Sousa e Silva MCC, Gon alves LRC, Almeida/Santos SM, Cardoso DE, Laporta-Ferreira IL, Suiquim, Peres C, Sano-Martins IS. Comparison of the biological activities in venoms from three subspecies of the South American rattlesnake (\textit{Crotalus durissus terrificus, C. durissus cascavella} and C. durissus collilineatus). \textit{Compar Biochem Physiol} 1999; 122: 61–73.

10. Cardoso B, Mota I. Effects of \textit{Crotalus} venom on the humoral and cellular immune responses. \textit{Toxicon} 1997; 35: 607–612.

11. Sternberg V, Bouley MG, Katz BM, Lee KJ, Parmar SS. Negative endocrine control system for inflammation in rats. \textit{Agents Actions} 1990; 29: 189–195.
12. García Leme J, Farsky SP. Hormonal control of inflammatory responses. Med Inflamm 1993; 2: 181–198.
13. Chrousos GP. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. N Engl J Med 1995; 332: 1351–1362.
14. García Leme J, Wilhelm DL. The effects of adrenalectomy and corticosteroid one on vascular permeability responses in the skin of the rat. Br J Exp Pathol 1975; 56: 402–407.
15. García Leme JG, Schapoval EE. Stimulation of the hypothalamic-pituitary-adrenal axis by compounds formed in inflamed tissue. Br J Pharmacol 1975; 53: 75–83.
16. Moraes FR, García Leme J. Endogenous corticosteroids and insulin in acute inflammation. Microvasc Res 1982; 23: 281–293.
17. Oyanagui Y. Physiological regulation of vascular permeability by endogenous glucocorticoids and active oxygen. Inflammation 1983; 7: 81–89.
18. Boschetto P, Musajo FG, Tognetto L, Bocasci M, Mapp CE, Barnes PJ, Fabbrini LM. Increase in vascular permeability produced in rat airways by PAF. potentiation by adrenalectomy. Br J Pharmacol 1992; 105: 588–592.
19. Flower RJ, Parente L, Persico P, Salmon JA. A comparison of the acute inflammatory response in adrenalectomised and sham-operated rats. Br J Pharmacol 1986; 87: 57–62.
20. Moraes FR, Bechara GH, Moraes JR. Effect of alloxan diabetes and adrenalectomy on carrageenin-induced pleurisy in the rat. Braz J Med Biol Res 1987; 20: 47–53.
21. Farsky SH, Sannomiya P, García-Leme J. Secreted glucocorticoids regulate leukocyte-endothelial interactions in inflammation. A direct vital microscopic study. J Leukoc Biol 1995; 57: 379–386.
22. Tjandra K, Kubis P, Rioux K, Swain MG. Endogenous glucocorticoids inhibit neutrophil recruitment to inflammatory sites in cholestatic rats. Am J Physiol 1996; 270: G821–G825.
23. Leach M, Hutchinson P, Holdsworth SR, Morand EF. Endogenous glucocorticoids modulate neutrophil migration and sialo-Pselectin but not neutrophil phagocytic or oxidative function in experimental arthritis. Clin Exp Immunol 1998; 112: 385–388.
24. Perretti M, Becherucci C, Scapigliati G, Parente L. The effect of adrenalectomy on interleukin-1 release in vitro and in vivo. Br J Pharmacol 1989; 98: 1137–1142.
25. Fantuzzi G, Di Santo E, Sacco S, Benigni F, Ghezzi P. Role of the hypothalamus–pituitary–adrenal axis in the regulation of TNF production in mice. Effect of stress and inhibition of endogenous glucocorticoids. J Immunol 1998; 159: 3552–3555.
26. Swain MG, Appleyard C, Wallace J, Wong H, Le T. Endogenous glucocorticoids released during acute toxic liver injury enhance hepatic IL-10 synthesis and release. J Immunol 1995; 155: 2051–2057.
27. Peers SH, Duncan GS, Flower, RJ. Development of specific antibody and immune-mediated inflammation. In: Bon C, ed. Envenomings and Their Treatments. Lyon: Fund Marcel Mérieux, 1996: 155–159.
28. Milad MA, Ludwig EA, Anne S, Middleton E, Jusko WJ. Pharmacodynamic model for joint exogenous and endogenous corticosteroid suppression of lymphocyte trafficking. J Pharmacokinet Biopharm 1994; 22: 469–480.
29. Huemer C, Brunner R, Hammes E, Muller H, Meyer Zum Buschenfelde KH, Lohse AW. Circadian variations in antigen-specific proliferation of human T lymphocytes and correlation to cortisol production. Psychoneuroendocrinology 1995; 20: 335–342.
30. Dhabhar FS, Miller AH, McEwen BS, Spencer RL. Stress-induced changes in blood leukocyte distribution. Role of adrenal steroid hormones. J Immunol 1996; 157: 1658–1664.
31. Faure G, Clouet M, Bouchet C, Caminard L, Guillaume JL, Monegger B, Vialhoigne M, Ben C. The origin of the diversity of crototoxin isoforms in the venom of Crotalus durissus terrificus. Eur J Biochem 1994; 223: 161–164.
32. Abraham GE. Radioimmunoassay of steroids in biological materials. Acta Endocrinol 1971; 75 (suppl 183): 5–12.
33. Temple ET, Liddle GW. Inhibitors of adrenal steroids biosynthesis. Anna Rev Pharmacol Toxicol 1970; 10: 199–215.
34. Deguchi M, Itohe Y, Matsukawa S, Yamazuchi A, Nakagawa G, Usefulness of methazolamide treatment to suppress cancer metastasis by facilitation of lymphatic fluid. Surgery 1998; 123: 440–449.
35. Pruzanski W, Scou K, Smith G, Raikovic I, Stefanaki E, Vadas P. Enzymatic activity and immunoreactivity of extracellular phospholipase A2 in inflammatory synovial fluids. Inflammation 1992; 16: 451–457.
36. Valentín E, Ghomashchi, F, Gelli MH, Lanzulski M, Lambeau G. On the diversity of secreted phospholipasesA2. Cloning, tissue distribution, and function and expression of two novel mouse group II enzymes. J Biol Chem 1999; 274: 31195–31202.
37. Stocker KR. Medical Use of Snake Venom Proteins. Boston, MA: CRC Press, 1990.
38. Raw I, Higashi HG, Kelen EMA. Antivenom production and organization of the public health system for the treatment of envenomization in Brazil. In: Bon C, ed. Envenomings and Their Treatments. Lyon: Fund Marcel Mérieux, 1996: 155–159.
39. Dhabhar FS, Miller AH, Stein M, McEwen BS, Spencer RL. Diurnal and stress-induced changes in distribution of peripheral blood leucocyte subpopulations. Brain Behav Immun 1994; 8: 66–70.
40. Dhabhar FS, Miller AH, McEwen BS, Spencer RL. Effects of stress on immune cell distribution. Dynamics and hormonal mechanisms. J Immunol 1995; 154: 5511–5527.
41. Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. J Immunol 1989; 142: 2081–2095.
42. Fiorentino DF, Zlotnik A, Vieira P, Mosmann TR, Howard M, Moore KW, O’Garra A. II-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. J Immunol 1991; 146: 3444–3451.
43. Deng L, Shevach EM. II-10 inhibits mitogen-induced cell proliferation by selectively inhibiting macrophage costimulator function. J Immunol 1992; 148: 3133–3139.
44. Deng L, Linsley PS, Huang LY, Germin RN, Shevach EM. II-10 inhibits macrophage costimulator activity by selectively inhibiting the up-regulation of B7 expression. J Immunol 1993; 151: 1224–1234.
45. Scharzt RS. A cell culture model for Th lymphocyte clonal anergy. Science 1998; 24: 1549–1556.
46. Harding FA, McArthur JG, Gross JA, Raulet DH, Allison JP. CD28-mediated signaling costimulatory murine T cells and prevents induction of anergy in TcR clones. Nature 1992; 356: 607–609.
47. Barraiveira B, Lomonte B, Tarkowski A, Hansen LA, Meira DA. Acute-phase reactions, including cytokines, in patients bitten by Bothrops and Crotalus snakes in Brazil. J Vet Anim Toxicol 1995; 1: 1–10.
48. Hirano T, Akira S, Taga T, Kishimoto T. Biological and clinical aspects of interleukin-6. Immunol Today 1990: 11: 444–449.
49. Tilders JJH, DeRijk RH, Van Dam NM, Vincent-NAIM, Schoutus K, Piersons JHA. Activation of the hypothalamic-pituitary-adrenal axis by bacterial endotoxins: routes and intermediate signals. Psychoneuroendocrinology 1994; 19: 209–222.
50. Hermann G, Beck FM, Sheridan JE. Stress-induced glucocorticoid response modulates mononuclear cell trafficking during an experimental influenza viral infection. J Neuroimmunol 1995; 56: 179–186.
51. Nakagawa M, Terasshima T, D’yachkova Y, Bondy GP, Hogg JC, Van Eeden SF. Glucocorticoid-induced granulocytosis: contribution of marrow release and demargination of intravascular granulocytes. Circulation 1998: 24: 2307–2313.
52. Suwa T, Hogg JC, English D, Van Eeden SF. Interleukin–6 induces demargination of intravascular neutrophils and shortens their transit in marrow. Am J Physiol Heart Circ Physiol 2000; 279: H2954–H2960.
53. Chisari A, Spinedi E, Voiron MJ, Giovannattista A, Gaillard RC. A phospholipase A2-related snake venom (from Crotalus durissus terrificus) stimulates neutrophilic and immune functions: determination of different sites of action. Endocrinology 1998: 139: 617–625.
54. Mosmann TR, Coffman RL. Heterogeneity of cytokine secretion patterns and functions of helper T cells. Adv Immunol 1989; 46: 111–147.
55. Zhang X, Brunner T, Carter L, Dutton RW, Rogers P, Bradley L, Sato T, Reed JC, Green D, Swain SL. Unequal death in Th1 helper cell Th1 and Th2 effectors: Th1, but not Th2, effectors undergo rapid Fas-Fas-ligand-mediated apoptosis. J Exp Med 1997; 185: 1837–1849.