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Role of crotoxin, a phospholipase A_2 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 phospolipase A_2. 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 pretreatment 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_2, 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.[1,2] CTX is a heterogeneous protein, composed of a weakly toxic basic phospholipase A_2 (PLA_2) and high enzymatic basic activity (CB), and an acidic polypeptide devoid of toxic and enzymatic activities, called crotapotin (CA).[3] Although CA is enzymatically and pharmacologically inactive,[2] it does enhance the toxicity of PLA_2.[4,5] On interaction with membranes or phospholipid vesicles, the complex dissociates: CB binds to the membrane, and CA is released from the complex.[6,7]

In spite of its high PLA_2 content, crude *Crotalus durissus terrificus* snake venom (CdtV) induces a short-duration oedema,[8,9] a scarce cell infiltration in subcutaneous tissues, and inhibits some activities displayed by inflammatory macrophages.[8] In addition, it has been demonstrated that CTX or CdtV

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reduces the humoral immune response to classical protein antigens without inhibition of the contact hypersensitivity reaction elicited by sensitising haptens.[10]

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.[11-13] In this context, endogenous GCS interfere with vasodilatation and vascular permeability,[14-18] leukocyte mobilisation and functions,[19-23] and secretion of chemical mediators.[24-26] GCS also acts on the acquired specific immune response by inhibiting T-cell activation and/or modulating synthesis of Th0, Th1 and Th2 cytokines.[27-30]

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[31] 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 PLA_2 activity on a synthetic chromogeneic 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 haemocitometer 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 ($Al(OH_3)$; 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.[10] 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 H_2O_2 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 (5×10^6 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

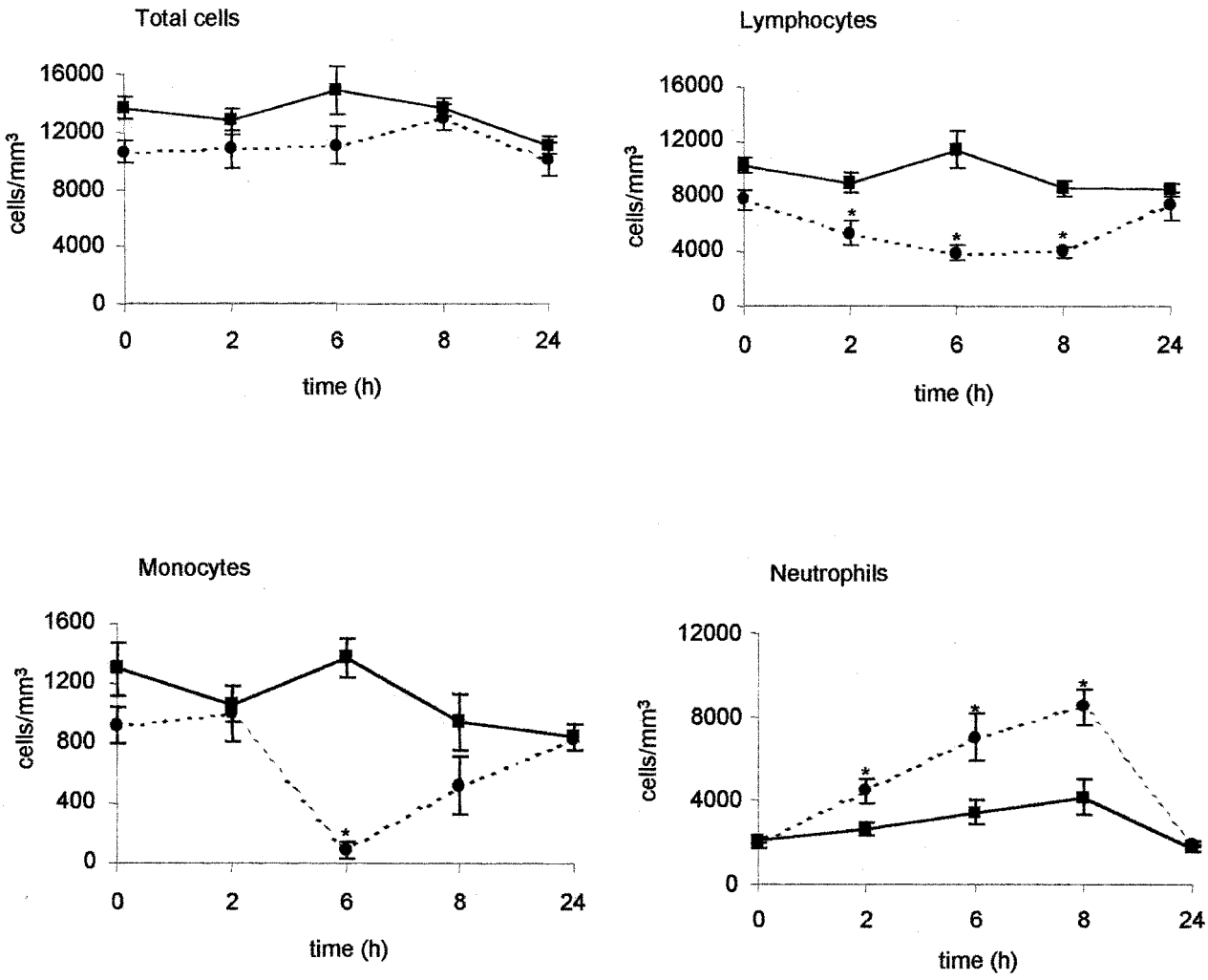

FIG. 1. Effect of crotoxin (CTX) on circulating leukocyte mobilisation. Blood was collected from orbital plexus of mice before or at different intervals of time after subcutaneous injection of 5 μg of CTX (●) or equivalent concentration of bovine serum albumin (BSA) (■) dissolved in 100 μl of sterile saline. Total leukocyte number was determined in a Neubauer haemocitometer, 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.

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 streptoavidin-biotinylated horseradish peroxidase (Amersham Int., Uppsala, Sweden) and ABTS (Sigma Chemical Co., St. Louis, MO, USA) solution in 0.1 M citrate buffer + H_2O_2. 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).

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, São 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

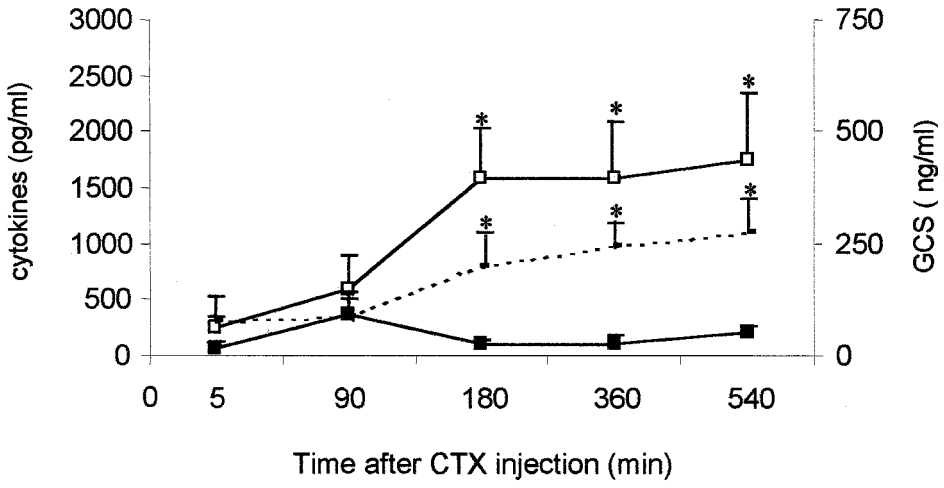

FIG. 2. Effects of crotoxin (CTX) on *in vivo* cytokine and corticosterone secretion. Animals were treated with CTX (5 μg/100 μl, subcutaneously) or equivalent volume of bovine serum albumin (BSA), and blood was collected from orbital plexus at varying intervals of time after injections. Cytokine and corticosterone serum levels were determined by enzyme-linked immunosorbent assay and radioimmunoassay, respectively. Values represent the mean ± SD of 12 animals. (■) IL-10 concentrations, (□) IL-6 concentrations and (– –) corticosterone levels. * $p < 0.05$ in comparison with initial values and with values obtained in BSA-treated animals. GCS, Glucocorticoids.

3 days.[34] 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 IgG_1 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.

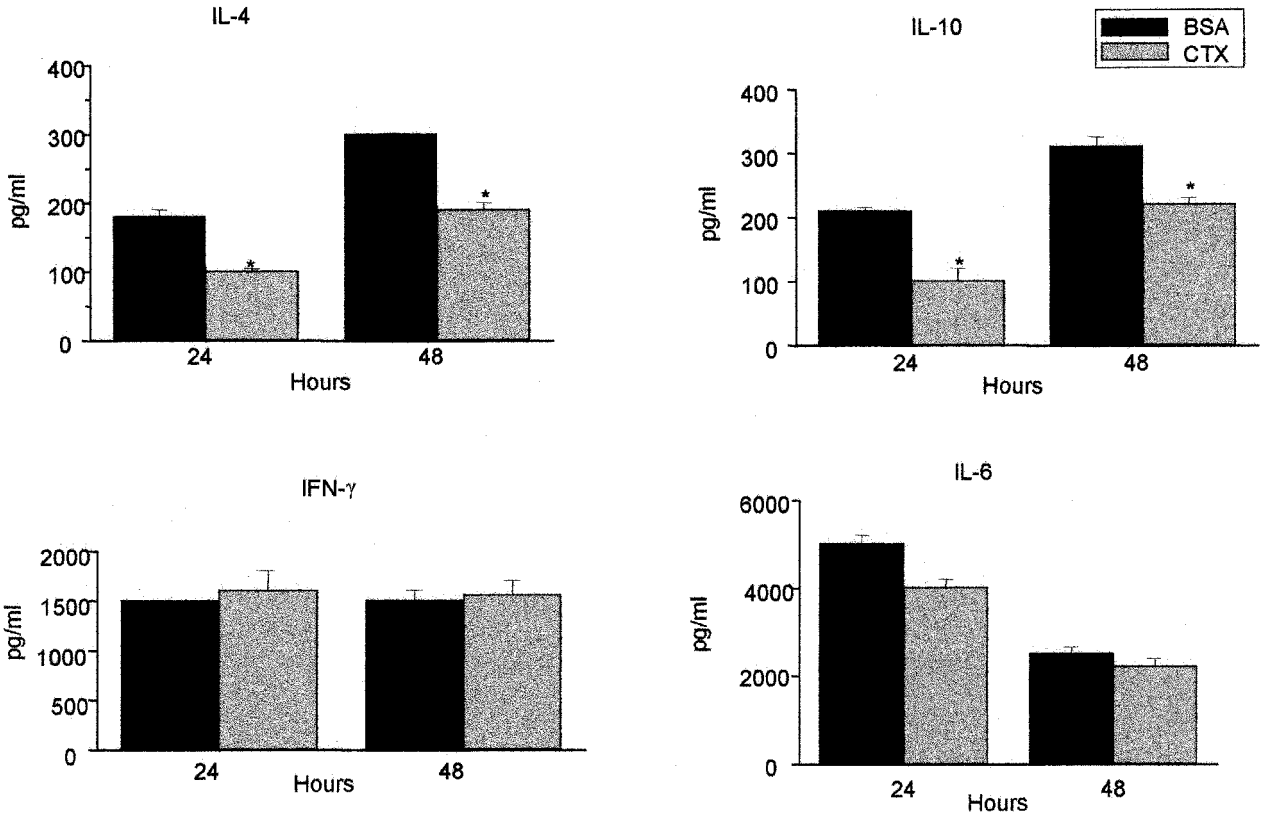

FIG. 3. Effect of *in vivo* crotoxin (CTX) treatment on cytokine secretion by spleen cells stimulated with concanavalin A (ConA). Spleen cells were collected from animals 6 h after bovine serum albumin (BSA) (■) or CTX (□) treatment. A sample of 5×10^6 cells/well were stimulated with ConA (0.5 ml of 10 μg/ml) during 24 or 48 h at 37°C in an air chamber (5% CO_2). 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.

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

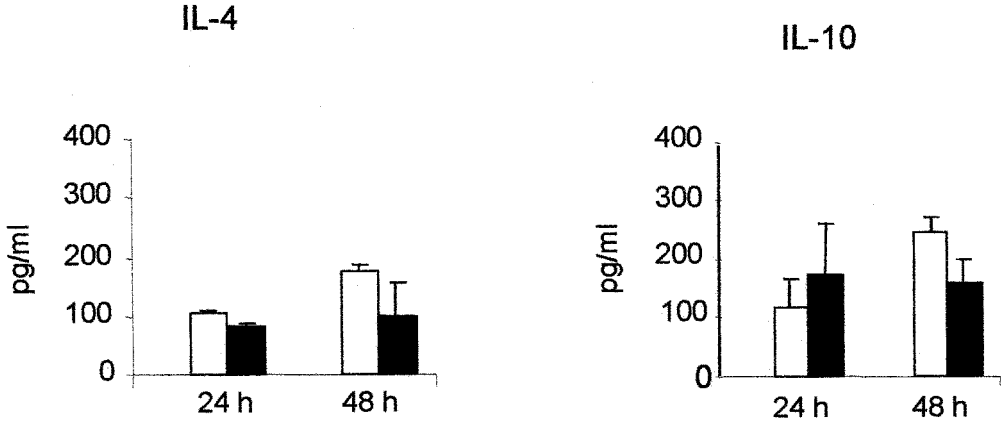

FIG. 4. Interference of metyrapone treatment on cytokine secretion induced by crotoxin (CTX). Animals were pre-treated with metyrapone (30 mg/kg, intraperitoneally; every 12 h, for 3 days) or equivalent volume of phosphate-buffered saline (PBS). Two hours after the last injections, CTX (5 μg/100 μl, subcutaneously) was administered. Spleen cells were collected 6 h after toxin injection, and 5×10^6 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% CO_2). 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 (■).

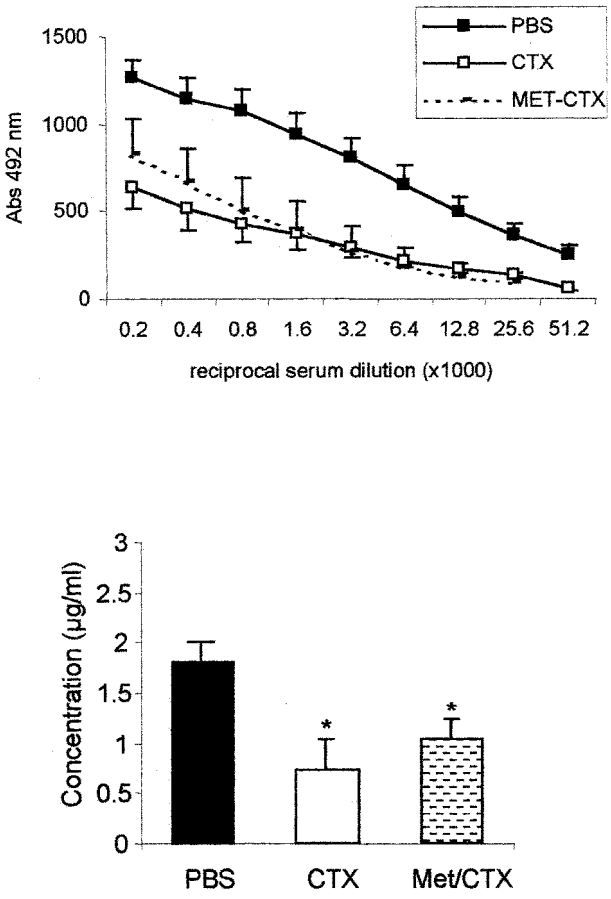

FIG. 5. Effect of metyrapone (MET) treatment on the inhibition of anti-human serum albumin (anti-HSA) HSA immunoglobulin (Ig)G antibody production induced by crotoxin (CTX). Animals were pre-treated with metyrapone (30 mg/kg, intraperitoneally; every 12 h, for 3 days). CTX (5 µg/100 µl) was administered 2 h after the last dose of metyrapone and the mice were immunised with HSA 6 h after CTX. Serum IgG_1 content was evaluated by enzyme-linked immunosorbent assay. Results represent the mean absorbance ± SD of serum obtained from six animals in each group. * $p < 0.05$ in comparison with values obtained in control animals, treated with phosphate-buffered saline (PBS).

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,[10] 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.[35,36] 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 β-neurotoxins and exhibits high enzymatic activity.[2,37] CTX exists in several isoforms in the same venom. It is more toxic and shows less enzymatic activity than its dissociated PLA_2 subunit.[5]

It has been demonstrated that crude CdtV induces impaired local inflammatory reaction characterised by negligible oedema and by the absence of leukocyte infiltration.[8] Also, antibody production is impaired in horses after CdtV administration.[38]

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 leukocytes 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.[30,39,40] 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*.[30] 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.

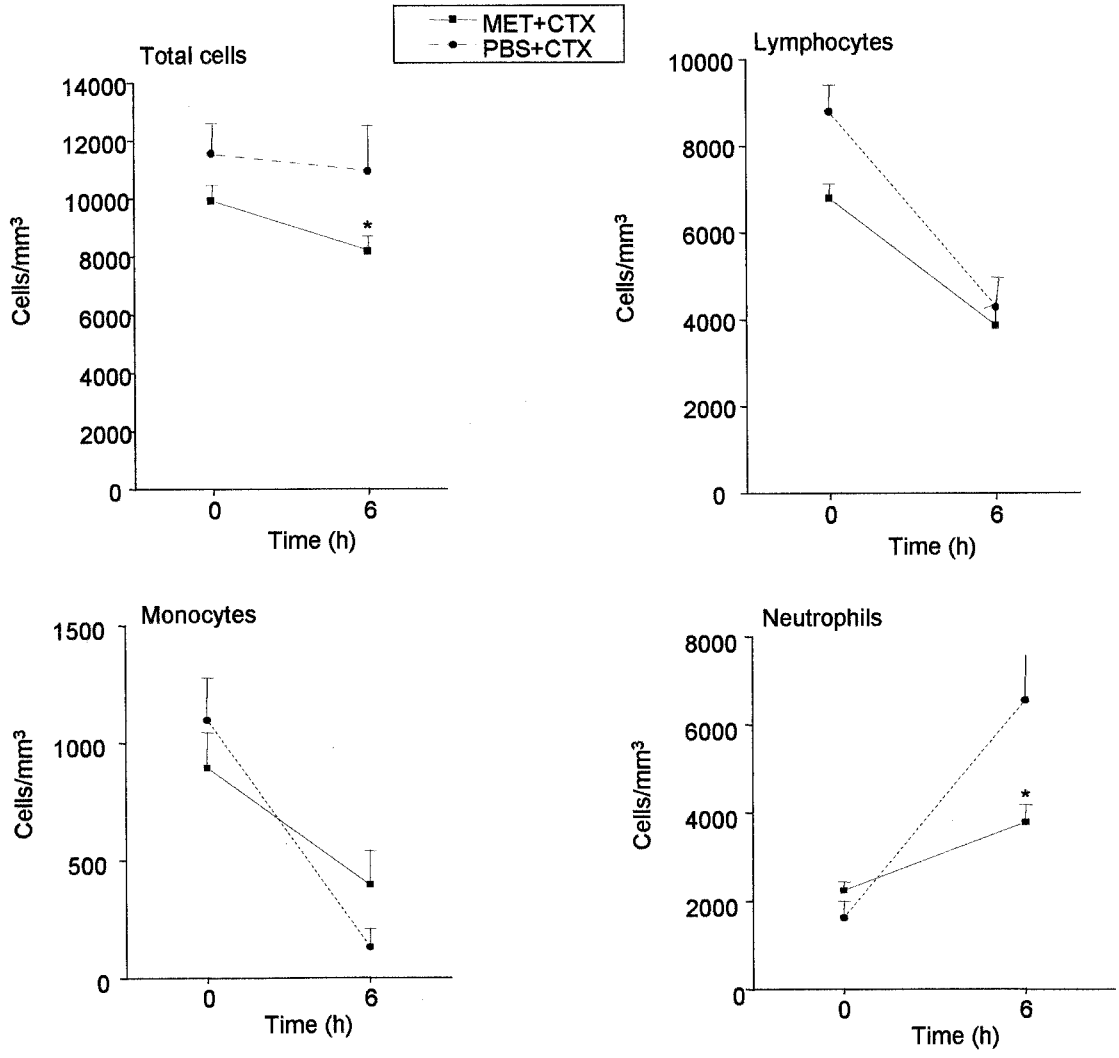

FIG. 6. Effect of metyrapone (MET) pre-treatment on leukocyte mobilisation alterations evoked by crotoxin (CTX). Animals were pre-treated with metyrapone (30 mg/kg, intraperitoneally; every 12 h, for 3 days) or equivalent volume of phosphate-buffered saline (PBS) and, 2 h after the last administration, received CTX (5 μg/100 μl; subcutaneously). Before and 6 h after CTX injection, blood was collected from the orbital plexus, and total leukocyte, lymphocyte, monocyte and neutrophil numbers were determined. Values represent the mean ± SEM of six animals in each group. * $p < 0.05$ in comparison with values obtained in control animals.

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 spleen 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[41,42] 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.[43,44] IL-10 also acts selectively on the macrophages by inhibiting the expression of co-stimulatory cell surface B.7 molecules.[45,46] It has been shown that resident or inflammatory macrophages have impaired spreading and phagocytic functions after CdtV treatment.[8] 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*.[47] IL-6 has been found to play a central role in defence mechanisms, including the acute phase reactions, haematopoiesis and immune response.[48] 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 corticoisteroid secretion.[49]

Elevation of glucocorticoid levels in the circulation is related to altered distribution of peripheral leukocyte subpopulations. Alterations observed during stress conditions, such as infection,[50] are significantly reduced in adrenalectomised animals.[30,40] Through interaction with specific receptors, secreted glucocorticoids mediate the decrease in number of lymphocytes and monocytes during stress.[30] The lymphocyte diminution in blood is due to retention of cells in the lymphatic circulation.[28] Indeed, neutrophilia induced by secreted glucocorticoids is dependent on mobilisation of polymorphonuclear cells into the circulation from the marginated and blood marrow pools.[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[52] 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 *et al.*[53] that CdtV or CTX treatments evoke ACTH and corticosterone secretion *in vivo* and *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.[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_1 production described. To investigate this hypothesis in our experimental procedures, animals were pre-treated with metyrapone, a drug that blocks glucocorticosteroid production but does not interfere with mineralocorticoid activity.[32,33] Our results demonstrated that pharmacological pre-treatment was able to reverse only the neutrophilia evoked by CTX. All other effects were not modified, including the inhibition of IgG antibody production. These results indicate that the increase in corticosteroids was responsible for the neutrophilia, corroborating the hypothesis that the increased numbers of polymorphonuclear cells are not a direct effect of CTX, but instead depend on the induction of an endogenous mechanism. Moreover, the elevated production of endogenous GCS is not related to the reduced immune response pattern induced by CTX. It is important to emphasise that, although CTX induced a significant elevation in serum GCS levels, such an increment is still lower than those reached when exogenous GCS are used in anti-inflammatory or immunosuppressive therapies.

Since the suppression of HSA-specific IgG1 antibodies induced by CTX was not related to the elevated production of endogenous GCS, it could be due to a reduced synthesis of IL-4 in CTX-treated mice. It is well known that differentiation of a Th2-type response and IgG1 isotype switching are dependent on IL-4.[54] Indeed, spleen T cells obtained 6 h after CTX injection secreted much less IL-4 and IL-10, but normal levels of IFN-γ, on *in vitro* stimulation with ConA. This profile of cytokine synthesis was also not changed by the administration of the glucocorticoid inhibitor. These findings might explain, therefore, why Th2-mediated but not Th1-mediated responses where affected in venom-treated mice, as previously demonstrated by Cardoso and Mota.[10] The inhibition of IgG1 antibody production, but not of contact hypersensivity,[10] also discards the possibility that CTX might induce apoptosis later in the immune response, since it is known that Th1 effector cells are much more susceptible than Th2 effectors to this death mechanism.[55]

In summary, our results suggest that, despite its high PLA_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 *in vivo* by CTX need to be further investigated.

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