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# Involvement of formyl peptide receptors in the stimulatory effect of crotoxin on macrophages co-cultivated with tumour cells

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ABSTRACT

Crotoxin (CTX) is the main neurotoxic component of *Crotalus durissus terrificus* snake venom. It inhibits tumour growth and modulates the function of macrophages, which are essential cells in the tumour microenvironment. The present study investigated the effect of CTX on the secretory activity of monocultured macrophages and macrophages co-cultivated with LLC-WRC 256 cells. The effect of the macrophage secretory activities on tumour cell proliferation was also evaluated. Macrophages pre-treated with CTX (0.3 μg/mL) for 2 h were co-cultivated with LLC-WRC 256 cells, and the secretory activity of the macrophages was determined after 12, 24 and 48 h. The co-cultivation of CTX-treated macrophages with the tumour cells caused a 20% reduction in tumour cell proliferation. The production of both $H_2O_2$ and NO was increased by 41% and 29% after 24 or 48 h of co-cultivation, respectively, compared to the values for the co-cultures of macrophages of control. The level of secreted IL-1β increased by 3.7- and 3.2-fold after 12 h and 24 h of co-cultivation, respectively. Moreover, an increased level of $LXA_4$ (25%) was observed after 24 h of co-cultivation, and a 2.3- and 2.1-fold increased level of 15-epi-$LXA_4$ was observed after 24 h and 48 h, respectively. Boc-2, a selective antagonist of formyl peptide receptors, blocked both the stimulatory effect of CTX on the macrophage secretory activity and the inhibitory effect of these cells on tumour cell proliferation. Taken together, these results indicate that CTX enhanced the secretory activity of macrophages, which may contribute to the antitumour activity of these cells, and that activation of formyl peptide receptors appears to play a major role in this effect.



## 1. Introduction

Macrophages play a critical role in a host's defense against cancer. Several studies have demonstrated that when monocytes/macrophages are activated under *in vitro*

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or *in vivo* conditions, they are able to lyse tumour cells. Macrophages exist in two distinct polarisation states, as classically activated macrophages (M1 macrophages) and alternatively activated macrophages (M2 macrophages) (Mantovani et al., 1992; Gordon, 2003). In the initial stage of tumour progression, M1 macrophages release compounds that are cytotoxic to cancer cells, such as reactive nitrogen/oxygen intermediates, tumour necrosis factor α (TNF-α), IL-1β and IL-6 (Roitt and Delves, 1992). The reactive oxygen species (ROS) that are formed during the respiratory burst of the mononuclear phagocytes have been implicated in the mechanism of killing tumour cells. In addition, ROS act as signalling molecules to induce the production of IL-1β and the expression of inducible nitric oxide (iNOS) (Song et al., 2002). Nitric oxide (NO) has been shown to be toxic to tumour cells via mitochondrial damage, inhibition of DNA synthesis and disruption of the flux of substrates through the tricarboxylic acid cycle (Hibbs et al., 1988; Lancaster and Hibbs, 1990; Pellat et al., 1990). The production of IL-6 and TNF-α, which have a regulatory effect on tumour growth, has been implicated as one of the cytostatic/cytocidal factors in the direct anti-tumour activity of the activated macrophages (Hamilton and Adams, 1987; Lewis and McGee, 1992; Paulnock, 1992; Arinaga et al., 1992).

During tumour progression, the secretory activities of these macrophages may become altered, resulting in their being unable to lyse tumour cells (Mosmann et al., 1986; Mantovani et al., 2004, 2005; Sica et al., 2008; for review). Additionally, the lipoxygenase pathway is inhibited in macrophages upon their contact with tumour cells (Calorini et al., 2005). The inhibitory effect of tumour cells on the lipoxygenase activity of macrophages might be important for tumour progression because the lipoxygenase products, such as the lipoxins (LXs) and their analogues, are lipid mediators with anti-angiogenic and anti-tumour activities (Fierro et al., 2002; Hao et al., 2011). LXs are eicosanoids produced from arachidonic acid via the 5-lipoxygenase (5-LO) and 15-lipoxygenase (15-LO) pathways (Serhan et al., 1984) that are involved in a range of physiological and pathophysiological conditions (Serhan et al., 1995). $LXA_4$ and $LXB_4$ are the main LXs produced in mammals. The acetylating of cyclooxygenase-2 (COX-2) by aspirin (Serhan et al., 1995), or in the absence of aspirin, via S-nitrosylation of COX-2 (Birnbaum et al., 2006), or P450-derived 15R-HETE that is substrate for leucocyte 5-LO (Clària et al., 1996), lead to the transcellular biosynthesis of 15-epi-lipoxins (ATL). Released ATL, in particular the 15-epi-$LXA_4$ form, has more potent and longer acting effects than does the native 15S-containing LX form because it is less rapidly inactivated (Serhan et al., 1995; Serhan, 2005; for review). The native LXs and their natural analogue 15-epi-$LXA_4$ modulate inflammation-related signals and may play a role in regulating the genesis and development of tumours (Serhan, 2005; Li et al., 2008) and exert their effects via binding to G-protein-coupled $LXA_4$ receptor (ALXR, also termed FRL1) (Fiore et al., 1994; Ye and Boulay, 1997; Rabiet et al., 2007).

CTX displays an antitumour effect, reducing tumour growth both *in vivo* and *in vitro* (Newman et al., 1993; Donato et al., 1996; Cura et al., 2002; Sampaio et al., 2010 for review). Crotoxin (CTX), the main toxic component of the venom of the South American rattlesnake *Crotalus durissus terrificus*, is a heterodimeric complex consisting of the basic and toxic phospholipase $A_2$ and an acidic, non-toxic, nonenzymatic component named crotapotin (Slotta and Frankel-Conrat, 1938; Bon et al., 1988). In addition to its *in vivo* antitumour activity, CTX, administered intramuscularly daily, inhibited the growth of Lewis lung carcinoma and MX-1 human mammary carcinomas (Newman et al., 1993; Donato et al., 1996; Cura et al., 2002). Five days of treatment with CTX significantly inhibited the growth of tumours in rat paws (Brigatte, 2005). The inhibitory effect of the toxin on tumour growth is abolished by pretreatment with Boc-2, a selective antagonist of the formyl peptide receptor (Faiad et al., 2008).

The immunomodulatory effect of *C. durissus terrificus* venom (CdtV) is retained by its major toxin, CTX, and by the isolated subunits of CTX (CA and CB) (Sampaio et al., 2010 for review). In addition, peritoneal macrophages incubated with CTX released higher $LXA_4$ levels than did non-treated cells (Sampaio et al., 2006b). A single dose of subcutaneously administered CTX promoted an increase of $H_2O_2$ release, NO production and IL-1β and TNF-α secretion by the peritoneal macrophages obtained from Walker 256 tumour-bearing rats fourteen days later. Concomitantly, a reduction in tumour size was observed (Costa et al., 2010).

Despite the intriguing results described above, the effect of CTX on the secretory activity of peritoneal macrophages in a tumour microenvironment has not been determined. The present study investigated the following issues: 1) the effect of CTX on the secretory activity of macrophages co-cultured with LLC-WRC 256 cells, 2) the effect of CTX on tumour cell proliferation and 3) the possible involvement of formyl peptide receptors in the actions of the toxin.

## 2. Materials and methods

### 2.1. Animals

Male Wistar rats weighing 160–180 g were used in this study. All the procedures were performed in accordance with the guidelines for animal experimentation, and the Ethical Committee for the Use of Animals of the Butantan Institute approved the protocol (CEUAIB, protocol number 631/09).

### 2.2. Crotoxin

Lyophilised venom of *C. durissus terrificus* was obtained from the Laboratory of Herpetology, Butantan Institute, São Paulo, Brazil, and stored at −20 °C. Crude venom solution was subjected to anion-exchange chromatography as previously described by Rangel-Santos et al. (2004), using a Mono-Q HR 5/5 column in an FPLC system (Pharmacia, Uppsala, Sweden). The fractions (1 ml/min) were eluted using a linear gradient of NaCl (0–1 mol/L in 50 mmol/L Tris–HCl, pH 7.0). Three peaks (p1, p2 and p3) were obtained: p2 corresponded to the pure CTX fraction (about 60% of the crude venom); peaks 1 and 3 included the other CdtV toxins. Prior to pooling, the fractions containing CTX were tested for homogeneity by non-reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis