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Cytokine profile of Ehrlich ascites tumor treated with *Bothrops jararaca* venom

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We previously demonstrated that *Bothrops jararaca* venom (*Bj*V) has an antitumor effect on Ehrlich ascites tumor (EAT) cells and induces an increase of polymorphonuclear leukocytes in early stages of tumor growth. It has been reported that this venom presents an important inflammatory effect when inoculated in animal models and in human snakebites, and that cytokine levels have been detected in these cases. To evaluate whether the cytokines can be involved with the suppression of the tumoral growth, we evaluate the cytokine profile in the peritoneal cavity of mice inoculated with EAT cells and treated with *Bj*V. Swiss mice were inoculated with EAT cells by the intraperitoneal route and treated with *Bj*V venom (0.4 mg/kg, intraperitoneally), on the 1st, 4th, 7th, 10th, and 13th day. Mice were evaluated for cytokine levels on the 2nd, 5th, 8th, 11th and 14th day. Analysis was performed using an enzyme-linked immunosorbent assay for interleukin (IL)-1α, IL-2, IL-4, IL-6, IL-10, IL-13, and tumor necrosis factor-α (TNF-α) levels in the peritoneal washing supernatant. Results were analyzed statistically by the Kruskal–Wallis and Dunn's tests at the 5% level of significance. We observed that EAT implantation induces IL–6 production on the 11th and 14th days of tumor growth, IL-10 on the 11th day and TNF-α on the 14th day. The treatment with *Bj*V suppresses production of these cytokines. In addition, IL–13 was produced by animals that were inoculated only with venom on the 11th and 14th days, and by the group inoculated with EAT cells and treated with venom on the 2nd and 14th days. Furthermore, we suggest that the IL–6 detected in the present study is produced by the EAT cells and the suppression of its production could be associated with the antitumor effect of *Bj*V.

Key words: Cytokines, Ehrlich ascites tumor, *Bothrops jararaca*, Snake venom

Introduction

We previously demonstrated that *Bothrops jararaca* venom (*Bj*V) has an antitumor effect on Ehrlich ascites tumor (EAT) cells.[1] Other experimental studies have been performed using snake venoms for treating animal tumors, and we observed that there are still many controversies on this subject and that the mechanism of tumor growth inhibition was not clear.[2]

Inflammation induced by snake venoms, with inflammatory cell involvement and cytokine liberation, was suggested as an important factor in tumor growth inhibition.[2] Elevated levels of some cytokines in human accidents with venomous animals[3] and experimental studies[4,5] have been demonstrated.

Literature on neoplasias and cytokines is quite extensive, and its approach in the *in vivo* models is very complex. Several reports have demonstrated that snake venoms may induce increased serum levels of interferon alpha (INFα), tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, IL-8, and IL-10.[3–5]

It has also been demonstrated that certain snake venoms show a proteolytic effect on cytokines. In this sense, it is already known that *Crotalus atrox* venom degrades interferon and IL-2.[6] In addition to the proteolytic effect, the literature also describes an inhibitory effect in cytokine production by certain snake venoms.[7,8]

There are other reports that demonstrate an association between cytokines and tumor growth inhibition. The main cytokines involved in this response are TNF-α,[9,10] IL-1α,[11,12] IL-2,[13] IL-4,[14,15] IL-6,[16,17] IL-10[18,19] and IL-13.[20,21] These cytokines can act as inhibitory substances or growth factors for tumor cells.

In our previous report on this subject, we demonstrated that the *in vivo* treatment of mice bearing EAT

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cells with *Bj*V-induced tumor growth inhibition (70×10^6 EAT cells/ml versus 0.7×10^6 EAT cells/ml, on the 14th day), increased animal survival time (15.5% versus 64.3%), and induced an increase of polymorphonuclear leukocytes in early stages of tumor growth (2nd and 5th days), but the total number of mononuclear leukocytes was not affected.[1] We suggest that the antitumor effect of *Bj*V[1,2,22] is due to the direct action of some substance of the venom, mainly proteases, but could also be by an indirect action, through stimulation of the inflammatory reaction, with involvement of cells and cytokines. To evaluate whether the cytokines can be involved with the suppression of the tumoral growth, we evaluate the cytokine profile in the peritoneal cavity of mice inoculated with EAT cells and treated with *Bj*V.

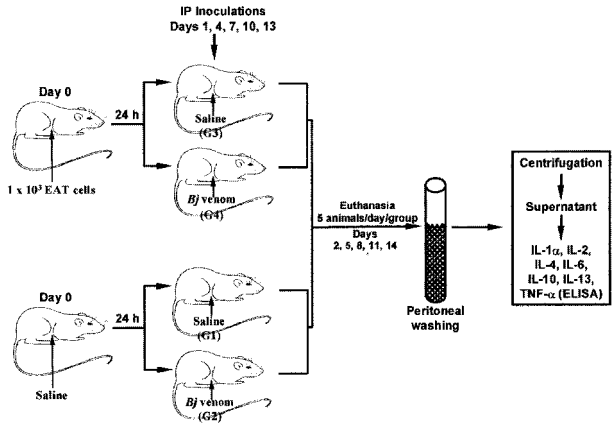

FIG. 1. Experimental design for evaluation of the cytokine profile of mice inoculated intraperitoneally (IP) with EAT cells and treated with *Bj*V (0.4 mg/kg). ELISA, Enzyme-linked immunosorbent assay.

Materials and methods

Animals

Swiss strain male mice, 4–6 weeks old, from our own animal facilities were used throughout the experiment.

Venoms

*Bj*V was obtained from snakes maintained in captivity at The Center for the Study of Venoms and Venomous Animals of São Paulo State University, Brazil. Newly extracted venom was centrifuged for 10 min at 1500 rpm, filtered using a GSWP00250 Millipore filter and then lyophilized. The lethal dose 50 (LD_{50}) for this venom was previously determined as 2.4 mg/kg of animal weight. The venom was stored at 4(C during the experiment.

Ehrlich ascites tumor

The tumor was maintained in Swiss mice in the ascitic form. Tumor cells were collected by aspiration with a Pasteur pipette, centrifuged for 10 min at 200 *g*, and washed twice with phosphate-buffered saline (pH 7.2). In all experimental protocols, mice were injected intraperitoneally with 1×10^3 tumor cells. Cell viability was evaluated by trypan blue exclusion test, and only cell suspensions that presented more than 95% viability were used.

Experimental design (Fig. 1)

Four groups of 25 animals were formed: G1, saline control group; G2, venom control group (0.4 mg/kg); G3, EAT control group; and G4, EAT + 0.4 mg/kg of venom. Animals of groups G3 and G4 were inoculated with 1×10^3 tumor cells/ml into the peritoneal cavity. Treatment began 24 h after tumor cell inoculation and consisted of five intraperitoneal injections at 72-h intervals between each. The saline (G1) and tumor (G3) control groups were injected with saline solution, and the venom control group (G2) and the venom + EAT group (G4) were inoculated with *Bj*V (0.4 mg/kg). On the 2nd, 5th, 8th, 11th, and 14th days after inoculation, five animals from each group were sacrificed in a sulfur ether chamber. After abdominal region asepsis, each mouse was inoculated with 3 ml of saline. After homogenization, the solution containing peritoneal cells was removed and centrifuged, and the supernatant was stored at –20°C. These procedures were repeated twice and data were grouped for statistical analysis.

Immunoenzymatic assay

The cytokine profile was obtained through immunoenzymatic assay (enzyme-linked immunosorbent assay) using supernatant aliquots of the peritoneal washing. The following cytokines were analyzed: TNF-α, IL-1α, IL-2, IL-4, IL-6, IL-10 and IL-13. The protocols, antibodies and reagents employed were as per the manufacturer's recommendations (R&D Systems Inc., Minneapolis, USA).

Statistical analysis

The data were analyzed by the Kruskal–Wallis nonparametric test for independent samples. The differences between the groups were appraised by the Dunn's method. The significance level was 5%.

Results

There were no significant differences observed in IL-1α and IL-2 production to any group throughout the study. However, IL-1α was slightly increased on the 2nd and 5th days for the group inoculated with EAT cells and treated with venom, and IL-2 on the 8th day for the group inoculated only with *Bj*V (Table 1). IL-4

Table 1. Cytokine levels in the experimental groups inoculated with EAT cells and/or 0.4 mg/kg of *Bj*V after 2, 5, 8, 11, and 14 days of tumor growth

Time	Day	Cytokine levels (pg/ml) in the groups			
		Saline	0.4 mg/kg BjV	EAT	EAT + 0.4 mg/kg BjV
IL-1	2	3.6 (6.5)*	2.3 (2.1)	2.8 (2.4)	9.0 (11.8)
	5	3.7 (5.7)	4.5 (8.5)	3.3 (6.1)	8.6 (15.1)
	8	0.2 (8.5)	5.7 (5.0)	2.3 (3.4)	3.3 (4.9)
	11	0.8 (3.6)	1.8 (1.2)	1.6 (5.5)	4.2 (3.4)
	14	0.0 (0.2)	1.3 (1.6)	1.3 (2.6)	0.8 (1.6)
IL-2	2	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (1.1)
	5	0.0 (0.4)	0.0 (3.9)	0.0 (5.4)	1.5 (5.4)
	8	3.2 (3.9)	15.5 (11.1)	0.4 (8.9)	3.6 (3.2)
	11	0.0 (18.0)	0.0 (0.0)	0.0 (0.0)	0.0 (1.1)
	14	0.0 (0.0)	0.0 (5.4)	0.0 (3.2)	0.0 (5.4)

* Each value represent the median (semi-interquartile range) for the cytokine level.

levels were not detected in all groups (data not shown).

Results for IL-6 production are shown in Fig. 2. The group inoculated with EAT cells and treated with saline (G3) presented significantly higher IL-6 levels on the 11th and 14th days in relation to the saline control group (G1). On the contrary, in the group inoculated with EAT cells and treated with *Bj*V (G4), this exponential increase of IL-6 was not observed. IL-6 levels was also observed in the group treated just with venom (G2) in relation to the saline control group (G1) on the 5th day.

The IL-10 analysis showed a significant increase of its level on the 11th day in the group inoculated with EAT and treated with saline (G3) in relation to the saline control group (G1) (Fig. 3).

For IL-13, analysis showed no significant increase in the group inoculated with EAT and treated with saline (G3). On the contrary, significant increases of this cytokine were seen in the EAT and *Bj*V group (G4) on the 2nd and 14th days in relation to the control group (G1). On the 14th day, IL-13 increase in the venom control group (G2) was also significant compared with the EAT and saline group (G3) (Fig. 4).

TNF-α production analysis showed that the group inoculate with EAT cells and treated with saline (G3) presented significantly higher levels on the 14th day than the two non-inoculated groups treated (G2) or not treated (G1) with *Bj*V (Fig. 5).

Discussion

The present study was performed because we were interested in determining whether some cytokine was involved in the process of inhibition of the growth tumoral after treatment with *Bj*V, as previously demonstrated.[1]

The analysis of cytokines in the peritoneal cavity showed that, in the EAT group, TNF-α, IL-6, IL-10 were produced in later stages of tumor growth (11th and/

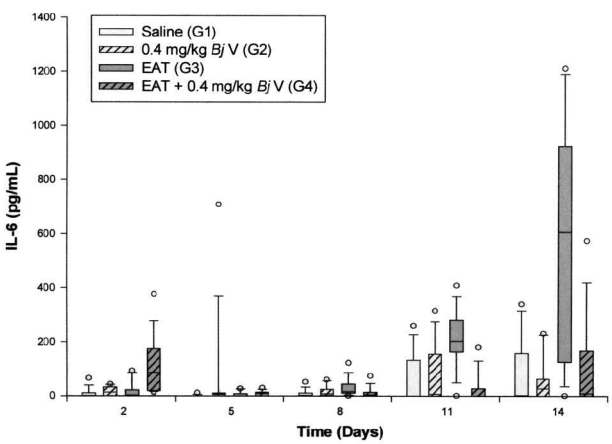

FIG. 2. Box plot of the IL-6 level kinetics in EAT cells treated with *BjV*. Each box represents 25–75% values with the median as an internal line, the error bar represents 10% and 90%, and the circle represents the outliers. Statistics: 2nd day, G4 > (G1 = G2 = G3); 5th day, G2 > G1; 11th day, G3 > (G1 = G4); 14th day, G3 > (G1 = G2 = G4); $p < 0.05$.

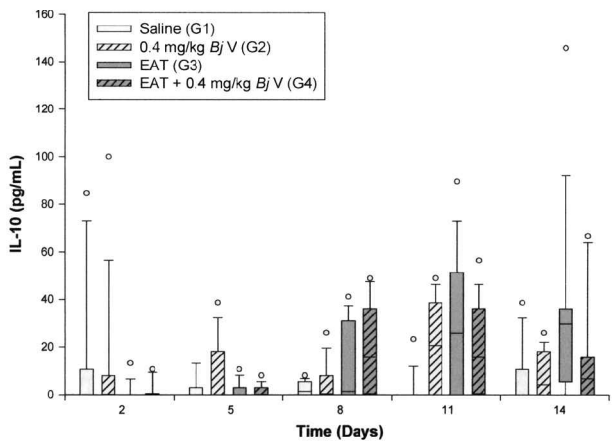

FIG. 3. Box plot of the IL-10 level kinetics in EAT cells treated with *BjV*. Each box represents 25–75% values with the median as an internal line, the error bar represents 10% and 90%, and the circle represents the outliers. Statistics: 11th day, G3 > G1; $p < 0.05$.

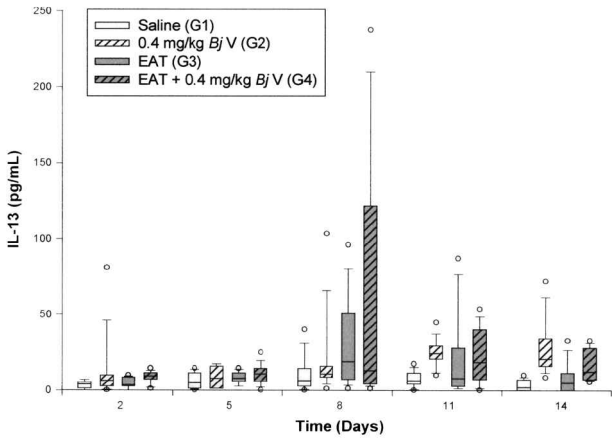

FIG. 4. Box plot of the IL-13 level kinetics in EAT cells treated with *BjV*. Each box represents 25–75% values with the median as an internal line, the error bar represents 10% and 90%, and the circle represents the outliers. Statistics: 2nd day, G4 > G1; 11th day, G2 > G1; 14th day, G2 > (G1 = G3), G4 > G1; $p < 0.05$.

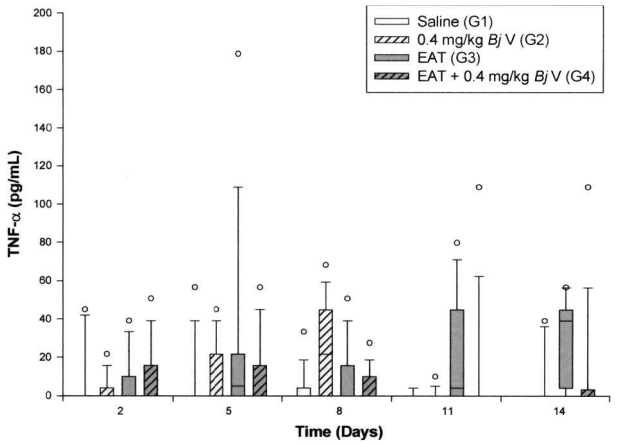

FIG. 5. Box plot of the TNF-α level kinetics in EAT cells treated with *BjV*. Each box represents 25–75% values with the median as an internal line, the error bar represents 10% and 90%, and the circle represents the outliers. Statistics: 14th day, G3 > (G1 = G2); $p < 0.05$.

or 14th day). IL-13 levels was detected mainly in the groups treated with *Bj*V, independently of the presence of EAT cells. IL-1 and IL-2 levels were very low when compared with those obtained for TNF-α, IL-6, IL-10 and IL-13. However, the most important cytokine detected in our protocols was IL-6. After treatment of EAT cells with *Bj*V, the production of TNF-α, IL-6, and IL-10 was suppressed.

There were no significant increases between EAT groups for IL-1α, IL-2, IL-4, and IL-13 throughout the experiment. However, we observed a slight tendency for increased IL-1α levels in the beginning stages of the experiment, in IL-2 levels on day 8 of tumor growth, and in IL-13 during the final stages of the experiment. Although these slight increases are not significant, these cytokines may also be involved in tumor growth inhibition.

Although we did not observe increased IL-4 levels, the structure of L-amino acid oxidase, a very common enzyme in snake venoms, shows a high degree of homology with murine IL-4[23] and therefore it is possible that this enzyme present in *Bj*V[24,25] exerts an antitumor effect on the EAT cells similar to that obtained with IL-4 treatment.[14,15] There are reports in the literature that show the direct toxic effect of L-amino acid oxidase *Ophiophagus hannah* on stomach cancer cells, murine melanoma, fibrosarcoma, and colorectal cancer.[26]

In addition to these studied cytokines, it is important to remember that, in the *in vivo* model, other cytokines may be playing a role in the effect observed after treatment. It is possible that the antitumor effect on EAT treated with *Bj*V is associated with the action of other cytokines possessing antitumor properties, such as IL-12.[27] However, it is also possible that the EAT produce other cytokines promoting its growth; for instance, transforming growth factor-β.[28]

In the present study, comparing cytokine levels in the animals treated only with venom, we observed a significant increase in IL-6 on the 5th day, and IL-13 on the 11th and 14th days in relation to the saline control group. Also, although not statistically significant, we detected an increase in TNF-α and IL-2 on the 8th day and in IL-4 and IL-10 from day 11. These data suggest that treatment with *Bj*V induces a T_{H2} behavior in the cytokine profile during the end stages of the study period, as reported for the *Crotalus durissus terrificus* venom.[7]

After analyzing the literature, we can infer that there is a close relationship between snake venoms, cytokines, and tumor cells. However, the literature on this subject is controversial and shows that treatment of tumors with snake venoms may or may not induce an anti-neoplastic effect[2] and that, in certain situations, snake venoms induce cytokine release;[3–5] in others, they either did not induce[7,8] or degraded[6] the produced cytokines. For some cytokines evaluated in this study, an antitumor effect[13,19] or that they are used as growth factors by tumor cells[9,18] has been reported.

The results observed in our previous paper, in which we reported inhibition of EAT cell growth[1], associated with modulation of the cytokine profile observed in the present paper, mainly for TNF-α, IL-6, and IL-10 production, suggest a effective inter-relation among cytokines and inhibition of EAT cell growth. Since IL-6 was produced in significantly higher quantities than TNF-α and IL-10, we believe that IL-6 is the main cytokine associated with EAT growth, either as a growth factor or a host suppressor factor. We suggest that tumor growth inhibition was largely due to the suppression of IL-6 production and that this suppression may be associated in part with proteolysis induced by *Bj*V.

Comparing the results obtained for the cytokine levels with those obtained for the tumoral growth,[1] we can note a correlation between the increase in TNF-α, IL-6 and IL-10 levels and the tumor growth in the non-venom EAT group, which was the group where a high rate of tumor growth was observed. On the contrary, no significant levels of these cytokines was observed in EAT groups that were treated with *Bj*V. In our previous observation, in this latter group, the tumor growth was inhibited.[1] We can suggest that the tumor cells are producing or inducing production of these cytokines. These results are in agreement with the literature on the production of TNF-α[9,10] and IL-6[16,17] by tumor cells and also induction of IL-10[18] by cells of the host, the latter stimulating tumor growth.

Although the treatment of mice bearing EAT cells with *Bj*V did not induce a significant difference in the total number of mononuclear leukocytes,[1] we do not rule out the possibility that different subpopulations may be involved in tumor growth inhibition. At the moment, we are evaluating through flow cytometry which populations of mononuclear cells are involved in the response of inhibition of the tumor growth, so that we will better be able to understand the relationships among the tumor evolution, the inflammatory influx, and the cytokine profile in mice bearing EAT cells treated with *Bj*V.

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