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Subpopulations of mononuclear leukocytes associated with inhibition of Ehrlich ascites tumor growth by treatment with *Bothrops jararaca* venom

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Snake venoms have been used as antineoplastic substances in several experimental models. We demonstrated in previous studies that *Bothrops jararaca* venom (BjV) induces inhibition of Ehrlich ascites tumor (EAT) growth accompanied by an increase of mononuclear (MN) leukocytes in all groups inoculated with EAT and/or venom. The objective of the present study was to characterize the subpopulations of MN leukocytes involved in the inhibition of EAT growth by treatment with BjV. Swiss mice were inoculated with 1.0×10^3 EAT cells by the intraperitoneal route and treated with 0.4 mg/kg of BjV by the same route (Group TV). Treatment was started 24 h after tumor cell inoculation and consisted of five intraperitoneal injections performed at 72 h intervals. After 2, 8 and 14 days, groups of animals were sacrificed and the number of B, TCD4 and TCD8 lymphocytes, macrophages and natural killer cells present in the peritoneal cavity was determined by flow cytometry. The control group consisted of animals inoculated with EAT and treated with 0.1 ml of saline under the same conditions as the experimental group (Group T). Two additional control groups consisted of animals not inoculated with EAT and treated with saline or venom. Data were analyzed statistically by the Kruskal–Wallis nonparametric test for independent samples. On the 2nd and 8th day we observed a difference between groups T and TV (group T > group TV) for all cell types, except natural killer cells, that only differed on the 2nd day. However, on the 14th day there was no difference in MN cells among groups. These data suggest that the inhibition of EAT is related to the toxic action of BjV on tumor cells and/or to the proteolytic effect of the venom on the mediators produced by the cells for growth modulation.

Key words: Mononuclear leukocytes, Ehrlich ascites tumor, Venom, *Bothrops jararaca*

Introduction

Snake venoms have been used as antitumor substances in several experimental protocols.[1] In previous studies we demonstrated that the venom of *Crotalus durissus terrificus* and *Bothrops jararaca* venom (BjV) have an important inhibitory effect on the growth of Ehrlich ascites tumor (EAT).[2,3]

The inhibition of EAT growth in the animals treated with BjV is accompanied by alteration in the cytokine profile in the peritoneal cavity of the animals[4] and by an increase of mononuclear (MN) leukocytes in all groups inoculated with EAT and/or venom.[3] Although no difference was detected in the increased quantity of MN cells in the peritoneal cavity of these groups, we do not rule out the possibility that different subpopulations may be involved in tumor growth inhibition.

There are no reports in the literature that demonstrate the cell types involved in the inhibition of EAT growth. This aspect is important because it will allow one to infer whether the inhibition of the tumor growth is associated with a certain cell population and/or with the direct effect of the venom on these cells. The aim of the present study was determine the cell subpopulation involved in EAT growth inhibition after treatment with BjV.

Materials and methods

Animals

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

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Venoms

BjV 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 200 × g, filtered through a GSWP00250 Millipore filter, and 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. Preliminary protocols have shown that 0.4 mg/kg of BjV is effective for tumor growth inhibition.

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 $200 \times g$, and washed twice with phosphate-buffered saline (pH 7.2). Cell viability was evaluated by the Trypan blue exclusion test, and only cell suspensions that presented more than 95% viability were used.

Peritoneal cells

Cells were harvested by peritoneal washing using 3 ml of sterile phosphate-buffered saline. The number of cells was determined with a hemocytometer. Differential counts were performed on fixed and stained cell suspensions (0.5% crystal violet dissolved in 30% acetic acid).

Analysis of the MN leukocyte subpopulations by flow cytometry

In this protocol we evaluated the number of five types of MN leukocytes: TCD_4, TCD_8 and B lymphocytes (CD22), macrophages (Mac3), and natural killer (NK1.1) cells. The total number of cells present in the peritoneal cavity was determined with a hemocytometer and the number of MN leukocytes was determined by flow cytometry. The peritoneal suspensions were adjusted to 1×10^6 cells/ml, and 100 µl of this solution were incubated with a monoclonal antibody labeled with fluorescein isothiocyanate (FITC). The protocols, antibodies and reagents employed were as per the manufacturer's recommendations (Pharmigen). The percentage of labeled cells was obtained by analysis of 10,000 cells/sample with a FACSCalibur flow cytometer (Becton-Dickinson, Palo Alto, CA, USA) using the Cell-Quest software.

Experiment design

Mice were inoculated by the intraperitoneal route (i.p.) with 1×10^3 EAT cells and treated with BjV (0.4 mg/kg) or saline (0.1 ml, i.p.). The first BjV dose was administered 24 h after tumor implantation and repeated five times at 72 h intervals. Additional non-tumor-bearing control groups were treated with BjV or saline according to the same protocol as used for the experimental groups. Ten animals from each group were sacrificed by sulfur ether inhalation 2, 8, and 14 days after EAT implantation, and the MN leukocyte number present in the peritoneal cavity was evaluated by flow cytometry.

Statistical analysis

Data were analyzed statistically by the Kruskall–Wallis test or by analysis of variance for independent sets, and by the Tukey or Student–Newman–Keuls test for differences between groups, with the level of significance set at 5%.

Results

Analysis of the results obtained on the 2nd and 8th day of tumor growth showed that the group inoculated with tumor and treated with BjV presented a smaller amount of TCD4, TCD8 and B lymphocytes, and of macrophages, compared with the group inoculated with EAT cells and not treated with venom. For natural killer cells, a decrease was observed only on the 2nd day. However, on the 14th day the amounts of all cell types were similar in the groups inoculated with tumor, whether or not they were treated with venom (Table 1).

We also observed that the group not inoculated with EAT cells and treated with BjV showed a reduction in the number of TCD4 lymphocyte, B lymphocyte, macrophage and tural killer cells on the 2nd day of tumor growth, an increase in TCD8 cells on the 8th day and an increase in macrophages on the 14th day compared the control group inoculated with saline (Table 1).

Discussion

The present study was carried out to characterize the cell subpopulation involved in the evolution of EAT treated with BjV, because in a previous study we demonstrated that treatment with this venom induces an increase in MN leukocytes in all groups inoculated with EAT cells and/or venom compared with the saline control group.[3] However, in this previous study, we did not detect a difference in the amount of MN leukocytes present in the peritoneal cavity peritoneal among the groups inoculated with tumor and/or venom. Nevertheless, we did not exclude the possibility that different subpopulations could be involved in the inhibition of tumor growth. The

Table 1. Kinetics of inflammatory influx in the peritoneal cavity of mice inoculated with EAT cells and treated with *Bj* venom

Days	Cell types	Cell numbers ($n \times 10^5$/ml) in each experimental group				Statistics
		Saline (G1)	Venom (G2)	EAT† (G3)	EAT+venom‡ (G4)	
2	$M\varnothing$	1.95 (0.99)*	0.19 (0.32)	1.88 (0.75)	0.13 (0.14)	$H = 17.657$, $p < 0.001$, (G1 = G3) > (G2 = G4)
	TCD_4	0.40 (0.25)	0.04 (0.12)	0.26 (0.14)	0.02 (0.04)	$H = 19.303$, $p < 0.001$, (G1 = G3) > G2 > G4
	TCD_8	0.33 (0.13)	0.06 (0.18)	0.30 (0.10)	0.01 (0.01)	$H = 15.036$, $p = 0.002$, (G1 = G2 = G3) > G4
	B	1.89 (1.27)	0.02 (0.41)	1.44 (0.72)	0.02 (0.17)	$H = 18.075$, $p < 0.001$, (G1 = G3) > (G2 = G4)
	NK	1.08 (0.65)	0.23 (0.21)	1.54 (0.21)	0.08 (0.01)	$H = 16.450$, $p < 0.001$, (G1 = G3) > (G2 = G4)
8	$M\varnothing$	0.68 (0.53)	0.56 (0.98)	6.75 (5.03)	4.23 (6.15)	$H = 18.343$, $p < 0.001$, (G1 = G2) < G4 < G3
	TCD_4	0.28 (0.03)	0.20 (0.23)	3.72 (1.53)	1.02 (0.84)	$H = 22.217$, $p < 0.001$, (G1 = G2) < G4 < G3
	TCD_8	0.23 (0.14)	0.39 (0.51)	2.53 (0.97)	1.18 (1.60)	$H = 15.159$, $p = 0.001$, G1 < G2 < G4 < G3
	B	1.63 (1.12)	0.43 (0.38)	6.21 (1.58)	0.67 (0.69)	$H = 17.496$, $p < 0.001$, G3 > (G1 = G2 = G4)
	NK	0.53 (0.24)	0.50 (0.76)	5.28 (4.88)	5.18 (6.25)	$H = 19.799$, $p < 0.001$, (G1 = G2) < (G3 = G4)
14	$M\varnothing$	1.57 (1.64)	4.95 (2.03)	45.08 (39.68)	25.99 (36.85)	$H = 9.137$, $p = 0.028$, G1 < (G2 = G3 = G4)
	TCD_4	0.49 (0.27)	1.16 (0.16)	5.87 (3.55)	3.10 (2.81)	$H = 6.132$, $p = 0.105$, G1 = G2 = G3 = G4
	TCD_8	0.41 (0.33)	1.76 (0.45)	6.88 (16.13)	11.37 (12.41)	$H = 7.005$, $p = 0.072$, G1 = G2 = G3 = G4
	B	3.46 (2.60)	4.65 (2.43)	3.10 (4.65)	5.69 (3.68)	$H = 0.572$, $p = 0.903$, G1 = G2 = G3 = G4
	NK	1.01 (0.52)	2.69 (1.07)	50.59 (38.22)	24.81 (35.48)	$H = 10.898$, $p = 0.012$, (G1 = G2) < (G3 = G4)

* Data presented as median (semi-interquartile range). $M\varnothing$, macrophages; TCD_4, TCD_4 lymphocytes; TCD_8, TCD_8 lymphocytes; B, B lymphocytes; NK, natural killer cells. † Animals inoculated with 1.0×10^3 EAT cells, i.p., treated with saline solution. ‡ Animals inoculated with 1.0×10^3 EAT cells, i.p., treated with 0.4 mg/kg of *BjV*.

literature shows that MN leukocytes can have an effective participation in the elimination of tumor cells,[5–13] and therefore it would be important to evaluate the participation of this cell type in the evolution of EAT.

However, analysis of the results obtained in this stage of the study showed that there was no difference in the amounts of TCD4 lymphocytes, TCD lymphocytes, B lymphocytes, macrophage and natural killer cells between the groups inoculated with tumor, treated or not with BjV. Thus, we believe that tumor growth inhibition is related to a direct effect of the poison on tumoral cells and/or for to the proteolytic effect of the venom on the mediators produced by the tumor cells for growth modulation. These data agree with the results reported by Rizzo and Tuchiya[14] and Silva *et al*.,[3] who showed a toxic effect of BjV both on tumor cells and on peritoneal cells.

Analysis of the results obtained by evaluation of the MN leukocyte subpopulations during this stage of the study showed that, 24 h after the first venom inoculation (2nd day of tumor growth), the numbers of all cell types studied (i.e. TCD4 lymphocytes, TCD8 lymphocytes, B lymphocytes, macrophages and natural killer cells) were significantly reduced compared with the control group inoculated with saline, indicating a toxic effect of the venom on these cells.

The present results did not show an effective participation of MN cell in the process of tumor growth inhibition observed in the previous experiments. These results strongly suggest that this inhibition is related to the direct effect of the venom on tumor cells and/or to the proteolytic effect of the venom on the mediators produced by the tumor cells for growth modulation.

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