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Enhancement of natural killer cell function by titanocenes in mice bearing Ehrlich ascites tumour

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Abstract

In the present work, we studied the effects of two titanocenes, biscyclopentadienyldichlorotitanium IV, (DDCT) and its derivative, biscyclopentadienylditiocianatetitanium IV (BCDT), on the activity of natural killer (NK) cells in Ehrlich ascites tumour (EAT)-bearing BALB/c mice. In order to investigate a more direct effect of these compounds on NK cell function, we performed experiments with severe combined immunodeficiency (SCID) mice, which exhibit a normal NK cell response in the absence of T and B cells. The treatment consisted of intraperitoneal (i.p.) administration of 15 mg/kg/day of DDCT for 2 days or 10 mg/kg/day of BCDT for 3 days. In addition, to verify whether the effects produced by the titanocenes were compound specific or related to a direct antitumour effect, we also investigated the effects of a 3-day treatment with 100 mg/kg of cyclophosphamide cyclophosphamide on NK cell activity. Our results demonstrated that, in BALB/c and SCID mice, NK cell function declined to subnormal levels after inoculation of the tumour. In these animals, although treatment with DDCT and BCDT significantly enhanced NK cell function, only DDCT restored NK cell activity to normal values in all stages studied. Conversely, treatment with cyclophosphamide reduced NK cell function in nontumour bearing SCID mice and was also unable to restore the decreased NK activity of tumour-bearing SCID mice, thus demonstrating that the enhancement of NK cell function by titanocenes is compound specific. The same effect of cyclophosphamide was observed with BALB/c mice. In the present study, the up-modulatory effects of these two compounds on NK cell function reveal a new aspect of the mechanism of antitumoural action of titanocenes.

Keywords: Natural killer cell; Titanocene; Antitumoural; Ehrlich ascites tumour

1. Introduction

The cytotoxic activity observed with the *cis*-diaminedichloroplatinum (II) (cisplatin) inorganic heavy-metal complex, which was introduced into clinical practice in the early 1970s, stimulated the search for other metal-containing complexes. This lead to the discovery of the tumour-inhibiting transition metal compounds, the metallocene dihalides, a group of substances characterised by carbon–metal bonds (Boyles et al., 2001).

The bis(cyclopentadyenil) metal (IV) (metallocene) dihalide complexes MIV(C_5H_5)X_2, with M = titanium, vanadium or molybdenum as central metal atom, were the first early transition metal compounds with antitumour activity (Kopf and Kopf-Maier, 1979; Kopf-Maier et al., 1980, 1986; Kopf-Maier, 1988; Modsksi et al., 2001). These substances exhibit marked antineoplastic activity against fluid and solid Ehrlich tumour, fluid and solid Sarcoma 180, B16 melanoma, Lewis Lung carcinoma, mouse mammary tumour TA3Ha and colon 38 adenocarcinoma (Kopf-Maier, 1988; Valadares et al., 1998; Valadares and Queiroz, 2002), and markedly inhibit the growth of xenografted human lung, breast, gastrointestinal and renal carcinomas (Kopf-Maier and Kopf, 1986; Kopf-Maier, 1989, 1999). The most active neutral metallocene complex, titanocene dichloride Ti IV $(C_5H_5)_2Cl_2$ is now

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undergoing clinical trials with promising results in patients with progressive advanced cancer (Christodoulou et al., 1988; Mross et al., 2000).

The mechanism of antitumoural efficacy of the titanocenes is related to the chemical changes produced in the general titanocene formula; results are conflicting. In this regard, in vitro studies of the structure–activity relationship of metallocene dihalides, using Ehrlich ascites tumour cells (EAT), demonstrated that the efficacy of these compounds is not affected by substitutions in the halides and is reduced by chemical modification of the cyclopentadyenil rings (Kopf-Maier et al., 1981). Boyles et al., (2001) observed increased effectiveness in vitro after the introduction of the electron-withdrawing carbomethoxy group into the cyclopentadyenil rings. We recently demonstrated that a substitution of the halide halogens in the general titanocene formula by pseudo-halogens, as in the biscyclopentadienylditiocianatetitanium IV (BCDT) compound, led to a lower up-modulatory effect of the haematopoietic response of Ehrlich tumour-bearing mice when compared to that of the parental compound biscyclopentadienyldichlorotitanium IV (DDCT) (Valadares and Queiroz, 2002).

Progressive Ehrlich ascites tumour growth is regularly accompanied by profound alterations of the immune response, leading to decreased immunocompetence. Studies have shown that Ehrlich ascites tumour development results in the down-regulation of cytotoxic cells, such as T lymphocytes and natural killer (NK) cells, mediated mainly by macrophages and natural suppressor cells (Parhar and Lala, 1985a,b; Subiza et al., 1989; Segura et al., 1997; Ruiz de Morales et al., 1999). In view of the importance of gaining more information on the antitumoural mechanisms of action of these agents, and the fact that NK cells are depressed by antitumoural drugs, such as cyclophosphamide cyclophosphamide, we designed the present study to investigate the effects of DDCT and its derivative, BCDT, on the activity of natural killer cells in Ehrlich ascites tumour-bearing mice.

2. Material and methods

2.1. Mice

The mice used in this study were bred at CEMIB-Unicamp Central Animal Facilities and raised under specific pathogen-free conditions. Male BALB/c mice, 8–10 weeks old, were matched for body weight before use. The animals were housed 10/cage and allowed free access to laboratory chow and water. Severe combined immunodeficiency (SCID) mice (C.B-17/Uni scid), 8–10 weeks old, were housed in individual positively ventilated cages and supplied with sterilised food and water ad libitum (Bosma et al., 1989). Evaluation of immunoglobulin levels by immunoblot test was performed to check the immunodeficiency (Gordon et al., 1991). Animal experiments were performed in accordance with the institutional protocols and guidelines of the Institutional Animal Care and Use Committee.

2.2. Tumour

Ehrlich ascites tumour (EAT) was maintained in BALB/c mice in the ascites form by serial transplantation. Tumour cell suspensions were prepared in balanced salt solution at pH 7.4 to a final concentration of 6×10^6 viable cells/ml. In all experimental protocols described, mice were inoculated intraperitoneally (i.p) on day 0 with 6×10^6 viable tumour cells per mouse in a volume of 0.1 ml. Viability, assessed by the Trypan blue dye exclusion method, was always found to be 95% or more.

2.3. Drug and mice treatment

The compounds were synthesized by the group of Dr. Stanley I. Klein, Instituto de Química, Universidade Estadual Paulista, UNESP, Araraquara, SP, Brazil. The DDCT and BCDT compounds were prepared and purified accord-

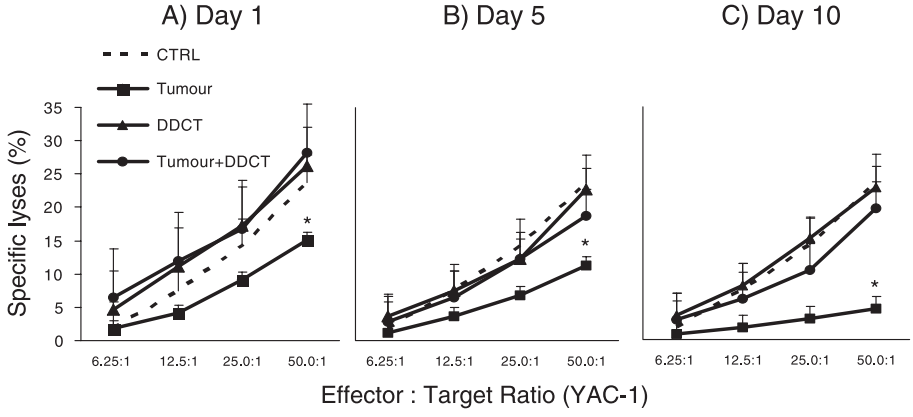

Fig. 1. NK cell activity in BALB/c mice bearing the Ehrlich ascites tumour. Mice were treated i.p. with 15 mg/kg/day of DDCT for 2 days and killed on days 1 (A), 5 (B) and 10 (C) after the last injection. Control mice received diluent only. *$P < 0.05$ compared to control group.

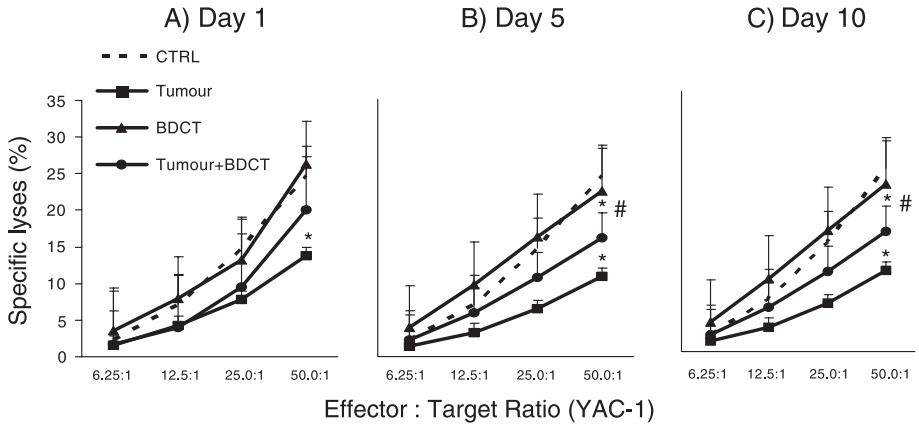

Fig. 2. NK cell activity in mice BALB/c bearing the Ehrlich ascites tumour. Mice were treated i.p. with 10 mg/kg/day of BCDT for 3 days and killed on days 1 (A), 5 (B) and 10 (C) after the last injection. Control mice received diluent only. *$P<0.05$ compared to control group; #$P<0.05$ compared to tumour group.

ing to Birmingham and Wilkinson (1954) (Pereira and Klein, 2000). These compounds were diluted into appropriate concentrations in dimethylsulfoxide/saline (1/10) immediately before use. Groups of normal and EAT-bearing mice received consecutive daily intraperitoneal (i.p.) doses of 15 mg/kg of DDCT for 2 days or 10 mg/kg of BCDT for 3 days (0.1 ml/mouse). These doses were selected based on previous studies performed in our laboratory (Valadares et al., 1998; Valadares and Queiroz, 2002). Treatment started 24 h after tumour inoculation and NK cell assays were performed on days 1, 5 and 10 after the last dose of the drug. Each experiment included parallel control groups of normal and EAT-bearing mice treated with the same volume of the diluents. Cyclophosphamide (ASTA Pharma, Frankfurt, Germany) was diluted in distilled water and administered daily as 100mg/kg i.p. doses for 3 days. NK cell activity was measured 24 h after the end of treatment. Since NK cell cytotoxicity in SCID mice is demonstrable only at a high effector/target ratio, we studied the NK response in these animals using the 50:1 effector/target cell ratio (Dorshkind et al., 1985).

2.4. Preparation of effectors cells for the Natural Killer cell assay

Spleen from mice were collected and passed through a stainless-steel mesh net to obtain single-cell suspensions. The resultant mononuclear cells were isolated from the cell suspensions by Ficoll-Hypaque (Ficoll-Sigma, St. Louis, USA; Hypaque-Pharmacia, Piscataway, USA) gradient separation, washed three times and resuspended in enriched RPMI-1640 (Sigma) culture medium supplemented with 10% fetal calf serum (FCS). Cell suspensions were placed in 150-mm tissue culture dishes and incubated at 37 °C in 5% CO_2 for 90 min to remove adherent cells. Nonadherent cells were then harvested by gentle pipetting. The cells were washed three times and the concentration was adjusted to 5×10^6 cells/ml.

2.5. Preparation of target cells for the Natural Killer cell assay

YAC-1, a Moloney virus-induced mouse T-cell lymphoma of A/SN origin, was used as target cell in the 4 h ^{51}Cr-release assay. Briefly, 5×10^6 pelleted YAC-1 cells were resuspended to 0.2 ml of fetal calf serum and labelled with 100-μCi of sodium chromate (^{51}Cr) (IPEN, Brazil) for 90 min at 37 °C in a shaking water bath. After labelling, the cells were washed twice with RPMI culture medium and resuspended at a concentration of 1×10^5 cells/ml in enriched RPMI culture medium supplemented with 10% of fetal calf serum.

2.6. Natural killer cell cytolytic assay

NK activity of the effector cells was measured with a 4 h ^{51}Cr-release assay with YAC-1 target cells. Effector and target cells were dispensed in triplicate into round-bottom microtiter plate wells (Corning, New York, USA), in effector to target ratios of 50:1; 25:1; 12.5:1 and 6.2:1. Plates were centrifuged at 800 rpm for 5 min and incubated 4 h at 37 °C in

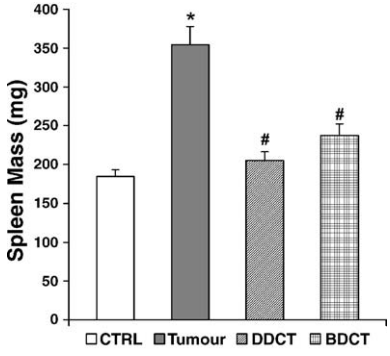

Fig. 3. Effects of 15 mg/kg/day of DDCT and 10 mg/kg/day of BCDT on spleen weight in Ehrlich ascites tumour-bearing mice. Control mice received diluent only. *$P<0.05$ compared to control group; #$P<0.05$ compared to tumour group.

a humidified CO_2 incubator. After the incubation period, the plates were centrifuged again at 1200 rpm for 10 min and 0.1 ml of the supernatants was collected for radioactivity counts in a Beckman Biograma Counting System (Beckman 5500 B, Irvine, USA). Spontaneous release was determined by adding 100 labelled target cells to 0.05% Tween-20. Percentage of cytotoxicity, as measured by specific ^{51}Cr release, was calculated by using the formula: (cpm experimental − cpm spontaneous)/(cpm maximal − cpm spontaneous) × 100.

2.7. Statistical analysis

Comparisons of data among all groups were done by analyses of variance (ANOVA). In cases of significant differences, Tukey test was used. Statistical significance was assigned when $P < 0.05$.

3. Results

The effects of treatment of normal and EAT-bearing BALB/c mice with 2 doses of 15 mg/kg DDCT or 3 doses of 10 mg/kg BCDT on NK cell activity are presented in Figs. 1 and 2, respectively. Treatment of normal mice with both compounds produced no change in NK cell activity when compared to that of the controls. After tumour transplantation, a sharp and progressive decrease in NK cell cytotoxicity, followed by increased tumour burden, was observed and culminated, in the last day of assessment, in an almost complete abolishment of NK cell activity ($P < 0.05$). Additionally, marked splenomegaly was observed in these mice. Treatment with DDCT significantly increased NK cell function, restoring to normal values the activity at all effector/target cell ratios on days 1, 5 and 10 of evaluation ($P < 0.05$). With BCDT, although NK cell activity was also increased at all stages of tumour development, only on the first day of evaluation did these values reach levels similar to those of control ($P < 0.05$). In addition, DDCT and BCDT reduced by 43% and 34%, respectively, the splenomegaly observed in tumour-bearing mice (Fig. 3). DDCT and BCDT also restored to normal values the impaired NK cell function induced by the tumour in SCID mice, as measured at the 50:1 effector/target cell ratio, without affecting the NK cell response of nontumour bearing SCID mice (Fig. 4). In spite of the increased NK cell activity, no protection was observed in tumour-bearing SCID mice, as evaluated by measuring survival time. Conversely, a dramatic reduction in NK cell activity in both nontumour bearing and tumour-bearing SCID (Fig. 4) and BALB/c mice (results not shown) was produced by the administration of 3 doses of 100 mg/kg of cyclophosphamide.

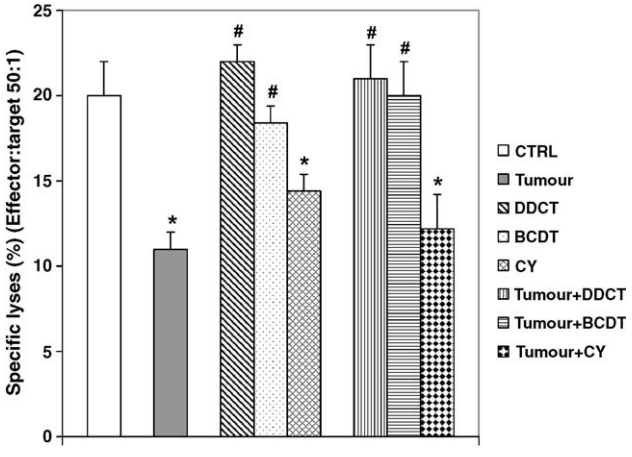

Fig. 4. NK cell activity in SCID mice bearing the Ehrlich ascites tumour. Mice were treated i.p. with 15 mg/kg/day of DDCT for 2 days, 10 mg/kg/day of BCDT for 3 days or 100 mg/kg/day of cyclophosphamide (CY) for 3 days and killed 24 h after the last injection. Control mice received diluent only. $^{*}P < 0.05$ compared to control group; $^{\#}P < 0.05$ compared to tumour group.

4. Discussion

In the present study, we observed that DDCT was more effective than BCDT in restoring the reduced NK cell function produced by tumour development in BALB/c and SCID mice. We have previously demonstrated that these compounds prolong, in a dose-dependent manner, the survival of BALB/c mice inoculated with Ehrlich ascites tumour (Valadares et al., 1998; Valadares and Queiroz, 2002). The effect of the titanocenes on NK cell activity was demonstrated to be compound-specific since an opposite response profile was found after treatment with cyclophosphamide.

Natural killer cells/or their precursors are highly sensitive to in vivo treatment with cyclophosphamide. Treatment of animals with this agent leads to low levels of NK cell activity and provides a valuable model for the in vivo study of the role of these cells in host defense against neoplasm. In this regard, the treatment with this standard chemotherapeutic agent reduced NK cell function in nontumour-bearing SCID mice, which lack T and B cells and display normal NK function. Moreover, cyclophosphamide was unable to produce any changes in the depleted NK cell function of tumour-bearing SCID mice. Conversely, treatment with DDCT and BCDT of tumour-bearing SCID and BALB/c mice demonstrated that both compounds maintained their ability to increase NK cell activity in these animals. The survival rate, which was previously demonstrated (Valadares et al., 1998; Valadares and Queiroz, 2002) to be increased by the administration of DDCT and BCDT in tumour-bearing BALB/c mice, was not affected when these compounds were given to tumour-bearing SCID mice (data not shown). This lack of protection, in spite of increased NK cell activity, might be explained by the lack of cooperation of the cytokines produced by T cells. Based on their defining function of causing spontaneous cytotoxicity with-

out prior immunisation, NK cells play a critical role in immune surveillance and cancer therapy. NK cells that infiltrate tumours may protect against tumour spread, which may be correlated with fewer metastases and less lymphatic invasion (Mihich and Ehrke, 2000; Miller, 2001). NK cells are thought to be important in innate immunity because of their potent capacity to produce cytokines, particularly interferon-gamma (Kim et al., 2000), which increases NK cell reactivity and activates macrophages (Yamamoto et al., 1995; Misawa et al., 2000).

During Ehrlich ascites tumour growth, the progressive loss of NK cell activity has been related to factors such as reduction of interferon-gamma levels, decreased interleukin-2 expression, and the presence of immunosuppressive factors, mainly prostaglandin E_2 produced by natural suppressor cells resident in the spleen (Parhar and Lala, 1985a,b, 1988; Subiza et al., 1989; Segura et al., 1997, 2000; Ruiz de Morales et al., 1999). Additionally, tumour progression in this tumour model is accompanied by marked splenomegaly and increased splenic haematopoiesis, which happen in a progressive manner, culminating in the death of the animal in about 15 days. Moreover, after the tumour is present for 13 days, a two-fold increase in spleen cellularity and weight is also observed (Queiroz et al., submitted for publication). In this regard, treatment of EAT-bearing mice with both compounds, DDCT and BCDT, produces a significant reduction of the EAT-induced splenic haematopoiesis followed by a significant reduction in spleen weight (Valadares and Queiroz, 2002).

The results presented here extend our previous findings of a greater effectiveness of DDCT in comparison to BCDT (Valadares and Queiroz, 2002). One explanation for these different behaviours in vivo may be related to the different hydrolytic activity of both compounds. In this regard, studies performed by Kopf-Maier et al. (1980) demonstrated that the presence of chloride in the DDCT formula allows for more extensive hydrolysis and a greater availability of Ti species in comparison to the pseudo-halogens of the BCDT compound.

The present findings support the hypothesis that NK cell up-regulation by titanocenes contributes to the antitumoural effect observed in EAT-bearing mice. Taken together, the up-modulatory effects on the function of NK cells and the complete recovery from the myelosuppression induced by the Ehrlich ascites tumour reveal a new aspect of the antitumoural mechanism of action of these compounds. Collectively, these results reinforce the trend in the literature that indicate the need for active immune surveillance as part of a mechanism of the titanocenes.

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