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Titanocene modulation of cytokine imbalance induced by Ehrlich ascites tumour progression

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Abstract

In the present work, we have studied the effects of two titanocenes, biscyclopentadienyldichlorotitanium IV (DDCT) and its derivative, biscyclopentadienylditiocianatetitanium IV (BCDT), on the production of cytokines [interferon-gamma (IFN-γ), interelukin-1, interleukin (IL) 2, IL-4, and IL-10] by concanavalin A (Con A)-stimulated T cells obtained from Ehrlich ascites tumour (EAT)-bearing BALB/c mice. The treatment consisted of intraperitoneal (i.p) administration of 15 mg/kg/day DDCT for 2 days or 10 mg/kg/day BCDT for 3 days. We observed that the levels of IFN-γ, but not IL-2, were dramatically increased in the early phase of EAT development. With tumour evolution, however, a sharp and progressive decrease in the levels of both IFN-γ and IL-2 was found concomitantly to an enhancement in the levels of IL-10. Treatment of these mice with both titanocene compounds demonstrated that DDCT is more effective in modulating the cytokine imbalance induced by the tumour since it could prevent the early enhancement of IFN-γ, the late decline of IFN-γ and IL-2, and the increase in the IL-10. The administration of BCDT, in spite of preventing early IFN-γ enhancement and increase in IL-10, did not produce any change in the IL-2 levels and did not prevent the decline of IFN-γ levels during tumour evolution. Collectively, these results reveal that the ability of titanocenes to reverse tumour-induced immunosuppression and delay tumour growth is more evident in the DDCT compound, thus indicating that the substitution of the halides halogens by pseudohalogens, present in the molecular structure of BCDT, leads to a less effective antitumoral compound.

Keywords: Titanocene; Cytokine; Ehrlich ascites

1. Introduction

Tumours are characterised by their ability to avoid the host immune system (Segura et al., 1997, 2000; Piemonti et al., 2003; Queiroz et al., 2004). Ehrlich ascites tumour (EAT) is a rapidly growing carcinoma with a very aggressive behaviour. Mice inoculated with 6×10^6 tumour cells have a life span of 18 ± 2 days (Valadares and Queiroz, 2002) and avoid the host immune response by secreting a variety of immunosuppressive cytokines, resulting in a dysfunction of cytotoxic cell immunosurveillance (Segura et al., 2000). This tumour has early effects on T lymphocytes of the host, accounting for a dramatic decrease in the number of T helper (Th) cells in the spleen of tumour-bearing mice, represented by a reduction in the number of $CD4^+$ cells expressing interferon-gamma (IFN-γ) after in vitro stimulation by mitogens (Segura et al., 2000).

It has been proposed that tumours induce a T helper 2 (Th2)-type response, which does not negatively affect the tumour, but depresses the T helper 1 (Th1)-type response in the host, which would promote tumour progression (Fallarino et al., 2000; Hung et al., 1998; Nishimura et al., 1999). It is likely that EAT cells disturb the Th1/Th2 balance through cell interactions or the production of soluble factors.

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Evidence supports that a beneficial immunological control of tumour growth is associated with the development of antitumour cell-mediated cytotoxicity. Observations that imbalance of cytokine network was associated with tumour evolution set up the basis for therapeutic approaches to modify cytokine production to favour tumour rejection (Contasta et al., 2003).

Titanocene compounds are a new class of organometallic substances with marked antitumoural properties. Titanocene dichloride, the most studied compound, has been shown to possess antitumour activity in vitro on doxorubicin- and cisplatin-resistant human ovarian carcinoma cell lines, without cross-resistance (Christodoulou et al., 1998). In xenografted models of human cancer cell lines, titanocene dichloride affected growth suppression in more than 50% (Kopf-Maier, 1989, 1999; Melendez, 2002). The biological activity of titanocene dichloride was superior to that achieved with equitoxic doses of cisplatin, 5-fluorouracil, and cyclophosphamide (Kopf-Maier, 1999; Friedrich et al., 1998; Melendez, 2002).

In vivo, titanocene dichloride induced a very pronounced growth inhibition of experimental ascites tumour (e.g., EAT and sarcoma 180) and solid animal tumours (e.g., B16 melanoma, colon 38 carcinoma, and Lewis lung carcinoma) (Melendez, 2002; Valadares et al., 2003).

The exact mechanisms of action for the titanocenes are still under discussion. Spectroscopic studies and the synthesis of different model complexes suggested a Ti-mediated biding to macromolecules such as DNA and RNA (Kopf-Maier, 1999). We have recently demonstrated using two different titanocenes—biscyclopentadienyldichlorotitanium IV (DDCT; titanocene dichloride) and its derivative, biscyclopentadienylditiocianatetitanium IV (BCDT)—that the substitution of halide halogens by pseudohalogens, in the general formula of DDCT, leads to a less therapeutically effective antitumoral compound (Valadares and Queiroz, 2002; Valadares et al., 2003). Similarly, as demonstrated in this work, the presence of pseudohalogens in the BCDT compound makes it less effective to counterbalance the Th1→Th2 polarisation produced with EAT temporal evolution. The understanding of the mechanism of action of these novel chemotherapeutic agents, including their effectiveness and limitations, may contribute to the development of more effective anticancer drugs.

2. Materials and methods

2.1. Mice

The mice used in this study were bred at Unicamp Central Animal Facilities and raised under specific pathogen-free condition. Male BALB/c mice, 8–10 weeks old, were matched for body weight before use. The animals were housed 10 per cage and allowed free access to laboratory chaw and water. Animal experiments were done in accordance with institutional protocols and the guidelines of the Institutional Animal Care and Use Committee.

2.2. Tumour model

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 final concentrations 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 prepared and purified by Dr. Stanley I. Klein (Instituto de Química, Universidade Estadual Paulista, UNESP, Araraquara, SP, Brazil) according to Birmingham and Wilkinson (1954) (Pereira and Klein, 2000). The compounds were diluted in dimethylsulfoxide/saline (1/10) immediately before use in appropriate concentrations. Groups of normal and EAT-bearing mice received two 15-mg/kg doses of DDCT or three 10-mg/kg doses of BCDT (0.1 ml/mouse), given daily by i.p. route. These doses were chosen based on previous dose schedule studies in our laboratory (Valadares et al., 1998). Drug injections started 24 h after tumour inoculation, and on days 1, 5, or 10 after the last injection, induction of mouse cytokine secretion in vitro by concanavalin A (Con A) was investigated by enzyme-linked immunosorbent assay (ELISA). Each experiment included parallel control groups of normal and EAT-bearing mice, which were treated with equivalent volumes of the diluents.

2.4. Purification of T-cell populations from spleen and induction of mouse cytokine secretion in vitro

Suspensions of spleen cells from all mice were prepared by gently pressing aseptically removed spleen through a stainless steel mesh net. T cells were purified by immunomagnetic separation, a multistep procedure, according to a commercially available kit (Miltenyi, Auburn, CA, USA). The purity of the blood T-cell populations was monitored by incubation with anti-CD4 fluorescein isothiocyanate and anti-CD8 phycoerythrin (Becton Dickinson, CA, USA) followed by analysis with a flow cytometer (FACSCalibur; Becton Dickinson). The purified populations were found to contain at least 97% $CD4^+$ cells and 94% $CD8^+$ cells. The cells were suspended in enriched RPMI 1640 culture medium (Sigma, St. Louis, MO, USA) supplemented with 5% foetal calf serum (FCS) and seeded into 24-well culture plates (Corning, New York, USA) in the presence of 2.5 μg/ml Con A (Sigma). Cell-free supernatants were collected after 24 h of incubation at 37 °C in 5% CO_2, and cytokine levels were detected by ELISA.

2.5. Quantification of cytokine levels

Cytokines [interleukin (IL)-2, IL-4, IL-10, and IFN-γ] were quantified by sandwich ELISA using the following monoclonal antibodies (mAbs) purchased from Pharmingen (CA, USA): purified antimouse IL-2 mAbs (code: JES6-1A12-Rat ImmunoglobulinG_{2a}), purified antimouse IL-4 mAbs (code: BVD-1D11-Rat ImmunoglobulinG_{2b}), purified antimouse IL-10 mAbs (code: SXC-1 Rat-ImmunoglobulinG_1), and purified antimouse IFN-γ mAbs (code: R4-6A2). Antimouse IL-10 mAbs (code: SXC-1-Rat ImmunoglobulinG_1), antimouse IL-4 mAbs (code: BVD6-24G2-Rat ImmunoglobulinG_1), antimouse IL-2 mAbs (code: JES6-5H4-Rat ImmunoglobulinG_{2b}), and antimouse IFN-γ mAbs (code: XMG1.2-Rat ImmunoglobulinG_1) were biotinylated. Recombinant mouse IL-4 (code: 19231W), recombinant mouse IL-10 (code: 19291V), recombinant mouse IL-2 (code: 19211T), and recombinant mouse IFN-γ (code: 19301T) were used as standard. Cytokine determinations were done according to Pharmingen's cytokine ELISA protocol. Briefly, microtiter plates (96-well flat-bottom maxisorp microplate; NUNC, Roskilde, DM) were coated overnight with 2.0 μg/ml anticytokine mAbs in a coating buffer of 1.0 M Na HCO_3, pH 8.2, at 4 °C. A blocking step was performed for 2 h at room temperature (phosphate buffer saline/10% FCS). After washing, the recombinant standard and the supernatants were added to the coated plate and incubated overnight at 4 °C. The plates were washed and then incubated with biotinylated anticytokine-detecting antibodies (2.5 μg/ml) for 45 min. After incubation with avidin peroxidase (Sigma) for 30 min, the substrate consisting of 4.0 mg/ml *o*-phenylenediaminidihydrochloride (Sigma) and 0.003% H_2O_2 in citrate buffer, pH 4.35, was added. Reaction was determined by measuring optical density at 492 nm in a Labsystem Immunoreader (Finland) after stopping the reaction using 1 N H_2SO_4. Cytokine titers were expressed as picograms per milliliter, calculated by reference to standard curves constructed with known amounts of recombinant cytokines.

3. Results

The effects of the administration of two doses of 15 mg/kg DDCT and three doses of 10 mg/kg BCDT on the production of IFN-γ, IL-2, IL-4, and IL-10 were measured at three stages of tumour development (i.e., 1, 5, and 10 days after the onset of treatment).

As we can see from Fig. 1, in the early phase of tumour progression, there is a dramatic enhancement of IFN-γ

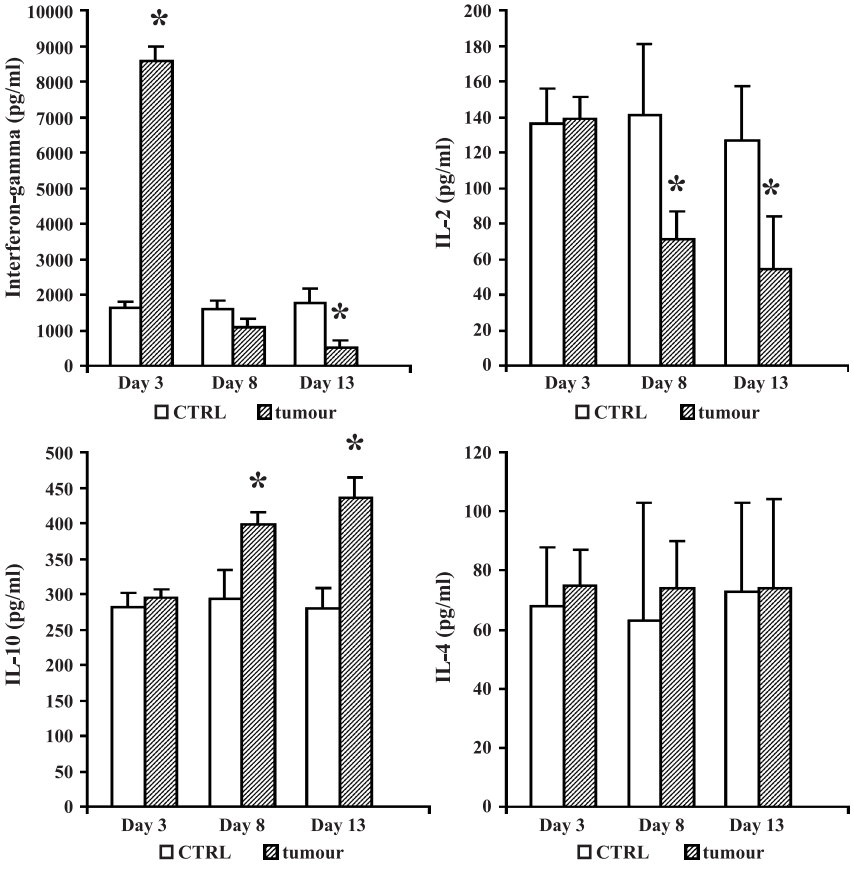

Fig. 1. Kinetics of IFN-γ, IL-2, IL-10, and IL-4 (pg/ml) produced by spleen cells of mice bearing the EAT. Cytokines were quantified by ELISA on days 3, 8, and 13 of tumour development. Results represent the mean±S.D. of eight mice per group *P<0.05 compared to control (CTRL) group.

concentration, reaching values that are almost fivefold higher then those found in control mice. At this stage, no changes were observed in the concentration of IL-2 or IL-10. On the other hand, during tumour exponential growth, we detected a sharp and progressive decline in IFN-γ levels of approximately 40% on day 8 and 70% on day 13 after tumour inoculation ($P<0.05$, Kruskal–Wallis, Wilcoxon test). In these same periods, IL-2 levels decreased by 50% and 42% ($P<0.05$, Kruskal–Wallis, Wilcoxon test) and IL-10 increased by 35% and 75%, respectively ($P<0.05$, Kruskal–Wallis, Wilcoxon test). No changes in IL-4 were produced by tumour development ($P<0.05$, Kruskal–Wallis, Wilcoxon test).

Similar profiles for IL-10 and IL-4 in the EAT were reported by da-Silva et al. (2002). In addition, our findings corroborate others reported by Segura et al. (2000), suggesting a progressive loss of T-cell functionality in the EAT model. Treatment with both compounds, particularly with DDCT, led to increased IL-2 levels in normal mice (Fig. 2) ($P<0.05$, Kruskal–Wallis, Wilcoxon test). In EAT-bearing mice, both compounds prevented the dramatic enhancement of IFN-γ levels at the initial stage of tumour development. However, only DDCT was able to prevent the reduction in the levels of this cytokine produced by tumour evolution ($P<0.05$, Kruskal–Wallis, Wilcoxon test) (Fig. 3). Similarly, in relation to IL-2 levels, DDCT, but not BCDT, was able to produce a significant increase in the levels of this cytokine, which, although progressively reduced with the temporal evolution of the tumour in the treated animals, reached normal control values at the last measurement taken ($P<0.05$, Kruskal–Wallis, Wilcoxon test) (Fig. 2). In relation to BCDT treatment, the levels of IL-2 presented a similar profile to that observed in nontreated tumour bearers ($P<0.05$, Kruskal–Wallis, Wilcoxon test). The pronounced enhancement of IL-10 levels produced by tumour development was completely abrogated by both compounds ($P<0.05$, Kruskal–Wallis, Wilcoxon test) (Fig. 4). The titanocene treatments pro-

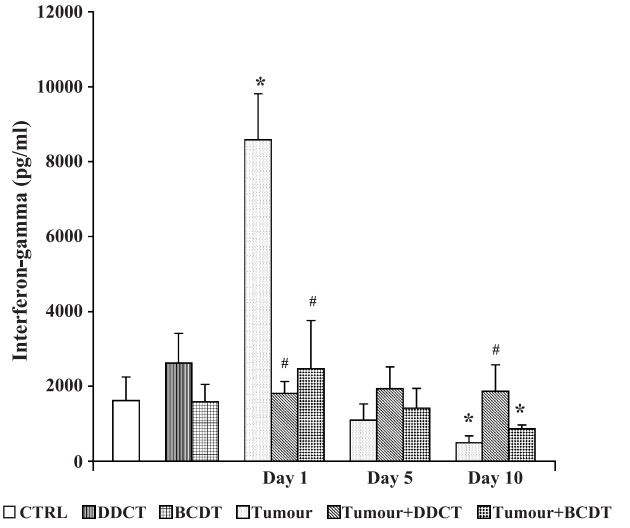

Fig. 3. Production of IFN-γ (pg/ml) by spleen cells of mice bearing the EAT. Mice were treated with i.p. injections of 10 mg/kg/day BCDT for 3 days, or with 15 mg/kg/day DDCT for 2 days, starting 24 h after i.p. inoculation of 6×10^6 EAT cells. Control mice received diluent only. Cytokines were quantified by ELISA. Results represent the mean±S.D. of eight mice per group. *$P<0.05$ compared to control group; #$P<0.05$ compared to tumour group.

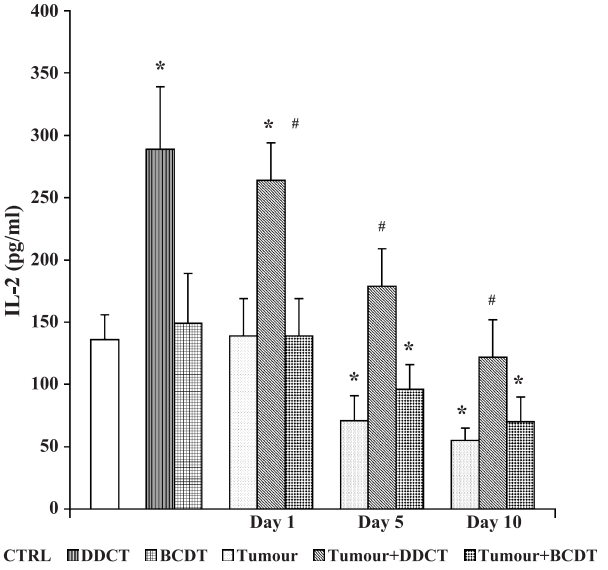

Fig. 2. Production of IL-2 (pg/ml) by spleen cells of mice bearing the EAT. Mice were treated with i.p. injections of 10 mg/kg/day BCDT for 3 days, or with 15 mg/kg/day DDCT for 2 days, starting 24 h after i.p. inoculation of 6×10^6 EAT cells. Control mice received diluent only. Cytokines were quantified by ELISA. Results represent the mean±S.D. of eight mice per group. *$P<0.05$ compared to control group; #$P<0.05$ compared to tumour group.

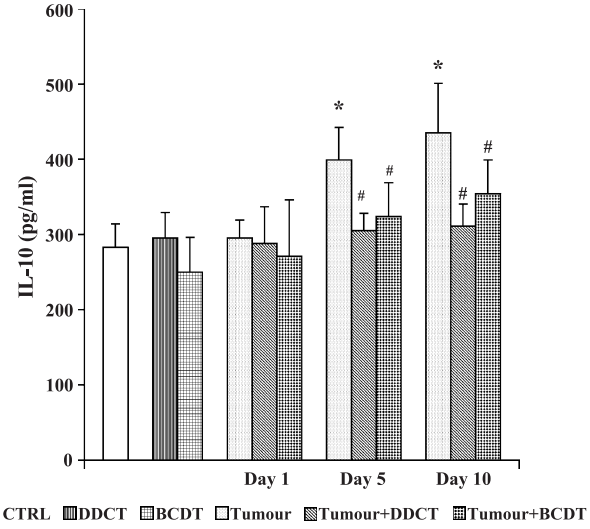

Fig. 4. Production of IL-10 (IL-10) (pg/ml) by spleen cells of mice inoculated (i.p.) with EAT cells (6×10^6 viable cells/ml) and treated (i.p.) with 10 mg/kg/day BCDT for 3 days, or with 15 mg/kg/day DDCT for 2 days starting 24 h after tumour inoculation. Control mice received diluent only. Cytokines were quantified by ELISA. Results represent the mean±S.D. of eight mice per group. *$P<0.05$ compared to control group; #$P<0.05$ compared to tumour group.

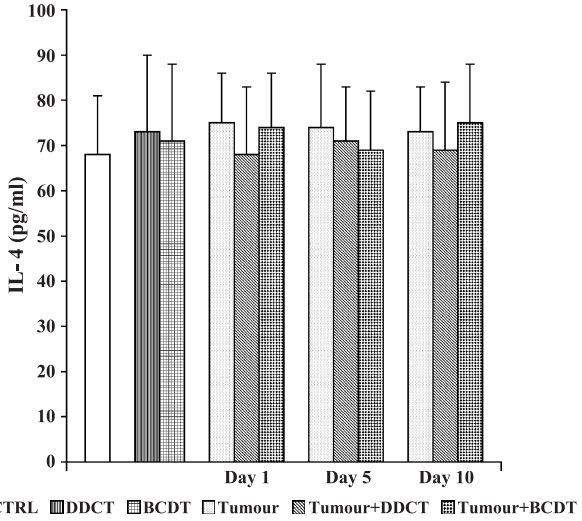

Fig. 5. Production of IL-4 (pg/ml) by spleen cells of mice inoculated (i.p.) with EAT cells (6×10^6 viable cells /ml) and treated (i.p.) with 10 mg/kg/day BCDT for 3 days, or with 15 mg/kg/day DDCT for 2 days starting 24 h after tumour inoculation. Control mice received diluent only. Cytokines were quantified by ELISA. Results represent the mean±S.D. of eight mice per group.

duced no changes in IL-4 levels ($P<0.05$, Kruskal–Wallis, Wilcoxon test) (Fig. 5).

4. Discussion

We have previously demonstrated a more effective response of DDCT, as compared to BCDT, in the recovery of both myelosuppression and reduced natural killer cell activity produced by tumour development. In addition, the greater antitumoral efficacy of DDCT was also demonstrated by its ability to enhance, in a dose-dependent manner, the rate of survival of mice inoculated with a lethal dose of Ehrlich tumour cells. With BCDT, however, there was only an extension in the period of time for the onset of death after tumour inoculation (Valadares et al., 1998). In this work, using the same experimental model, we demonstrate a greater ability of the parental compound DDCT to prevent the Th1→Th2 switch produced during tumour evolution.

The tumour-bearing state is regarded as a representative of a pathophysiologic condition in which two lines of modulation of cytokine production are induced. At the first stages of tumour development, Th1 cytokines (i.e., IL-2 and IFN-γ) play a crucial role in the accomplishment of T-cell-mediated antitumour immune responses and subsequent tumour regression (Abe et al., 1998; Schuler et al., 1999). However, tumour and immune cells implement different strategies against the generation/action of cytotoxic T lymphocytes (CTLs) and Th1 cells. First, tumour cells downmodulate the expression of major histocompatibility complex (MHC) molecules to avoid specific recognition by cytotoxic T cells (Levitsky et al., 1994; Maeda and Shiraishi, 1996). Moreover, immunosuppressive cytokines such as IL-4, IL-10, and transforming growth factor-β are produced by tumour cells and coinfiltrating lymphocytes. These cytokines induce a downmodulatory effect on Th1 cell generation. In addition, IL-10 also decreases the expression of MHC molecules on tumour cells and reduces the capability of dendritic cells to produce antigen-specific Th1 cells (Yamamoto et al., 1995; Kundu and Fulton, 1997). Similarly, the overexpression of transforming growth factor-β by tumour cells strongly downmodulates the proliferation of T cells, especially Th1-type cells (Hayakawa et al., 2001).

In this context, Segura et al. (2000) demonstrated that EAT progression produces a state of reduced responsiveness to stimulation of T cells, diminishes IFN-γ expression, and continuously induces the production of some non-T-cell-derived cytokines, such as transforming growth factor-β, which exhibit downmodulatory effects on the immune surveillance of the host. Elexpuru et al. (1997) showed that tumour cells present a transforming growth factor-β-like activity and that antibodies against this cytokine prevent Ehrlich tumour proliferation. Similarly, other cytokines such as IL-6, tumour necrosis factor-α, and IL-10 are increased in the peritoneal cavity of EAT bearers at the latest stages of tumour development (da-Silva et al., 2002).

In line with these findings, we observed a dramatic enhancement in IFN-γ in the early phase of tumour development over control levels. At this stage, no changes in the concentration of IL-2 were observed. On the other hand, during tumour exponential growth, there was a sharp and progressive decline of IFN-γ and IL-2, concomitant to a pronounced enhancement in the levels of IL-10.

The dramatic enhancement of IFN-γ levels produced at the initial stages of tumour development was abrogated by treatment with both compounds, whereas the progressive reduction in the levels of this cytokine observed during tumour evolution was prevented only by the treatment with DDCT. In relation to IL-2, a upmodulatory effect on the levels of this cytokine was induced mainly with the DDCT treatment, and was observed also in the normal nontumour-bearing mice. In addition, only DDCT was able to prevent the reduction in the levels of this cytokine produced by tumour evolution. Extensive data in the literature have shown that the anticancer characteristics of IL-2 include stimulation of the proliferation of helper and cytotoxic T cells primed by antigen, induction of secretion of lymphokines, and enhancement of cytotoxicity of natural killer cells (Wagstaff et al., 1995). The increased production of IFN-γ followed by a drastic decrease of IL-2 in EAT seems to be a tumour escape mechanism since the overproduction of IFN-γ could decrease the sensibility of the host's immune system. In this regard, Hodsdon et al. (2001) showed in HIV-positive patients an elevated production of IFN-γ and IL-10 in association with high proliferation of *Mycobacterium tuberculosis*.

The pronounced enhancement in the levels of IL-10 produced by tumour development was downmodulated by treatment with both compounds, in all stages studied. Data

on the literature demonstrate that tumour-induced IL-10 can block the generation of a tumour-specific type 1 immune response and subvert attempts to elicit a type 1 immune response to a nontumour antigen at the tumour site (Halak et al., 1999).

Taken together, these results demonstrate that substitution in halides by pseudohalogens in the general titanocene formula leads to a lower upmodulatory effect in association with a less effective antitumoral activity.

Acknowledgements

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