

Effect of testosterone on growth of P388 leukemia cell line *in vivo* and *in vitro*. Distribution of peripheral blood T lymphocytes and cell cycle progression *

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Received November 10, 2004

In transplanted mice, the P388 tumor grew better in castrated than in non castrated (NC) mice. The proportion of CD8+ in the blood was more numerous in NC mice. The T cell subsets (CD4+ and CD8+) were also high in the mice with small tumor tissue (<10 mg). The correlation observed between the tumor weight and T cell subset in PBL and in the mice with small tumors could confirm the important intervention of CD4+ and CD8+ cells to inhibit growth of tumor.

Depo-testosterone (DT) injection reduced strongly weight and tumor growth in mice. On top of that, DT administration induced a significant increase in the percentage of blood CD8+ cells in grafted mice.

The effect of DT was studied on the cell cycle progression, in tumor tissue of P388 tumor bearing BDF1 mice and in P388 murine leukemia cell line in culture. The cell cycle analysis in tumor tissue showed that DT decreased both the cells in S phase and the proliferating leukemic cells, with accumulation of cells in G0/G1 phase. The testosterone can inhibit the proliferation of leukemic cells with a pharmacological dose (10^{-7} M). This growth inhibition, dose and time dependent, was associated with cell cycle arrest; P388 cells accumulates in G0/G1 phase. We also observed a correlation between tumor weight and the percentage of cells in G0/G1 and the relative number of cells in proliferative state (S + G2/M).

To conclude, our experiments reported that testosterone prevents the growth of tumor: indirectly by modulation of subsets T cells distribution and directly by the alteration of the cell cycle.

Key words: testosterone, P388 leukemia cell line, peripheral blood lymphocyte, tumor tissue, subsets T cells, cell cycle.

Many works discussed the relation between the hormonal imbalance and the development of cancer. The data suggest that a decrease in serum level of Dehydroepiandrosterone (DHEA) may be associated with patients who have some clinical subtypes of ATL (adult-T-cell leukemia) [16]. The 17β -estradiol/testosterone ratio was considerably higher in men with CLL (chronic lymphocytic leukemia) [8, 9]. Among therapeutic agents, the sex steroids have been reported to have some effect on acute non-lymphocytic leukemia (ANLL) [19]. In fact, the influence of androgen on the long survival in acute myeloid leukemia has been reported [14].

The immune system plays an important role in controlling tumor cell growth [4]. The works of EVERAUS [8] confirmed

that the abnormalities of the immune system observed in CLL may be connected with an imbalance of endocrine regulation. The underlying mechanism for disordered T cell function in CLL is still unknown.

Cell cycle control in G1 phase has attracted considerable attention in recent cancer research, because many of important proteins involved in G1 progression, or G1/S transition have been found to play a crucial role in proliferation, differentiation, transformation and programmed cell death (apoptosis) [21]. A characteristic of acute leukemia cells is that these cells lose the ability to differentiate into mature, functional cells and remain in a high proliferative status over their normal counterpart. One possible approach to treat patients with acute leukemia is to use agents which can induce differentiation of leukemic cells and inhibit their proliferation [17, 22], or by cell cycle alteration that led to apoptosis [19]. Several results also suggest the possible involvement of

*This work was supported by a grant from Ligue nationale contre le cancer (Comités de l'Aude, du Gard et des Pyrénées Orientales).

sex steroids in the control of proliferation of leukemic [7] and in apoptotic phenomenon regulation [6]. However, the mechanisms implicated in this regulation were not clear. The works of GREGORY et al [12] in the transplanted mice with CWR22 cells (human prostate cancer) indicate that androgen receptor regulates cellular proliferation by control of CDK and cyclin that influence cell cycle protein activity.

Based on these findings, we investigated the effects of testosterone (DT) on growth of tumor of leukemia cell line (P388) *in vivo* and *in vitro*, and their impact on the distribution of T cells subsets in peripheral blood lymphocyte (PBL), and tumor tissue. To clarify the mechanism of this hormone, we followed the cell cycle progression in tumor tissue and in leukemia cell line in culture with or without DT.

Material and methods

Animals. BDF1 male mice (C57BL/6 x DBA/2)F1, 3 weeks old, (IFFA-CREDO, Les Oncins, France), were housed in air conditioned room (temperature 21 ± 2 °C) in standard cages, each housing 7 animals. Food and water were available *ad libitum*. The mice were kept on a 12 h light-dark cycle (L/D, 12:12 hours).

Castration. Male mice were castrated when four weeks old. They were anesthetized with ether and castrated by making an abdominal incision, removing the testes and closing the incision with 6-0 silk sutures. As controls there were two groups: sham-operated and non-operated (intact) animals. It was found that the sham-operated group was indistinguishable from non-operated controls, hence the data from these two groups were pooled.

Steroids. Depo-testosterone (DT, Testosterone 17beta-cypionate, Sigma-Aldrich, L'Isle D'Abeau Chesnes, France) in sterile peanut oil (PNO) was administered subcutaneously to mice at a dose of 0.5 mg/100 g body weight, in a final volume of 0.2 ml. The same volume of PNO was injected in castrated and in intact control group. DT was administered to animals on alternate days for a period of two weeks. Steroids were injected in treated animals one week after castration. Dose, route and frequency of DT administration was chosen according to the works of AHMED et al [3]. The different experimental groups are summarized in Table 1.

Cell line. P388 leukemia cell line from a DBA/2 mouse, was obtained from Flow, France. Cells were maintained in culture in Fisher medium supplemented with 5% fetal bovine serum, 1% glutamine (2 mM) and 1% penicillin-streptomycin, at 37 °C in a

humidified atmosphere of 5% CO₂. The cells were counted with an automate (Coulter Agency, France). Trypan blue exclusion test indicated that the viability always exceeded 90%.

Transplanted mice. The P388 cells were injected subcutaneously at 10^5 cells per mouse and the mice were examined daily to monitor survival and tumor growth. The P388 tumors could only be followed for approximately two weeks after which the animals developed leukemia and died [1]. For that reason, the mice were distributed in two batches and killed after 15 days or 30 days after grafting. Control mice were injected with Fisher medium, or with peanut oil, no difference was observed between the two batches.

Plasma testosterone. Mice were killed by cardiac exsanguination. The blood was collected in heparinized syringes and the plasma was separated by centrifugation. The plasma testosterone concentration was measured by radio-immunoassay (Pr. Baudin, Department of Nuclear Medicine, CHU-Nimes).

Cell preparation. The tumor tissue and seminal vesicle were removed from each mouse and weighed. The tumor tissue was divided into two groups, one group having weight less than 10 mg (small tumor), other group have more than 10 mg (big tumor). The single-cell suspensions were prepared by fine needle cytopunction [26]. The cells were washed twice in cold PBS, and counted in a Coulter counter. The viability was determined by trypan blue exclusion.

T-cell phenotype studies. The cells were washed twice in cold PBS containing 2% bovine serum albumin, 0.01 sodium azide, and later resuspended in an appropriate volume. Erythrocytes were lysed by adding 2 ml of lysing solution to blood samples. The cell suspension of tumor tissue and peripheral blood lymphocytes (PBL) were labeled by incubation with 4 µl monoclonal antibody (MoAb) conjugated to fluorescein isotiocyanate (FITC) plus 4 µl MoAb conjugated to phycoerythrin (PE) for 20 min at 4 °C. The samples were then washed in cold PBS and the cells suspended in 1 ml PBS. The

Table 1. Effect of DT on the plasma testosterone concentration (nmol/l), seminal vesicle (mg/100 g) and tumor (mg) weights of castrated or non castrated (NC) mice

		Seminal vesicle mg/100g ± sem	Testosterone nmol/l ± sem	Tumor weight mg ± sem	Tumor appearance %	Survival %
NON CASTRATED (NC)	NC	372 ± 63.2	22.4 ± 1			
	TM	374.6 ± 68.9	20.5 ± 2	9.5 ± 2.8	63.3	85
	DT	514.8 ± 72.0 p<0.01	50.8 ± 11.1 p<0.01	6.5 ± 2.3 p<0.05	8.3	83.3
CASTRATED	Castrated	228.1 ± 67.9	0.03 ± 0.01 p<0.001			
	TM	230.0 ± 65.2	0.02 ± 0.008	12.5 ± 4.5	70	50
	DT	480.3 ± 76.3 p<0.01	20.5 ± 7.5 p<0.001	8.5 ± 3.5	40	40

Values are the means ± SEM. Each group contained ten mice. The transplanted mice were compared to animals without tumor. The transplanted DT treated-mice were compared to grafted untreated mice. TM – castrated or NC transplanted mice, DT – castrated or NC transplanted mice treated with DT.

monoclonal antibodies used for the phenotypic analysis were Thy 1.2-FITC (CD2, clone 30H12, Becton Dickinson, Mountain View, CA, USA) which recognizes immature and mature T cells; Lyt-2-FITC (CD8, Clone 53-6.7, Becton Dickinson) which recognizes cytotoxic/suppressor cells; and L3T4 (CD4, Clone GK1.5, Becton Dickinson) which recognizes helper/inducer cells.

Cell cycle analysis. The cells of tumor tissue and leukemia cell line were stained with propidium iodide (IP) by the 3 steps method of VINDELOV et al [27].

Flow cytometric analysis. Samples were run in a flow cytometer (cytofluograph IIS, OrthoDiagnostic Instruments, Westwood, Massachusetts, USA) equipped with an innova 90-5 argon ion laser (Coherent PaloAlto, California, USA) operating at 488 nm and 500 mW in light-regulated mode. The cytometer was calibrated with fluorescent microbeads before each run. Data from the forward angle scatter and fluorescence parameters (peak and area signals of fluorescence emission) were collected by user-defined protocol and stored in an Ortho 2151 computer system (Ortho Diagnostic Instruments) in list mode.

The phenotypic analysis, considering the proportion of positive cells, was determined from histograms, and the red (for L3T4) and green (for Lyt-2 and Thy1,2) fluorescences were used to determine the numbers of cells per channel based on 20,000 events gated in the lymphocyte area of cytograms: forward angle scatter versus right angle scatter.

The DNA content histograms (red fluorescence emission area versus number of cells per channel) were established from 20,000 events gated on "red fluorescence area peak" cytograms in order to discriminate doublets. The cells in G0/G1 were considered to be non-proliferating cells, S and G2/M were considered to be proliferating [23].

Statistical analysis. The data were analyzed with Student's t-test. A p value of less than 0.05 was considered significant.

Results

Tumor growth (Tab. 1). The percentage of mice that developed tumors was high in castrated mice (70%), counter only 63% of the non castrated (NC) mice developed tumors. The NC mice survived longer (85%) than castrated mice (50%). Compared to non treated mice, the DT injection reduced strongly the tumor growth in NC (63.3 to 8.3%) mice and in castrated mice (40%).

Tumor weight (Tab. 1). In castrated mice the tumor weight was more important than NC grafted mice (12.5 ± 4.5 against 9.5 ± 2.3 , $p < 0.05$). The injection of DT reduced significantly ($p < 0.05$) the tumor weight in NC mice. The decrease of tumor weight in castrated DT treated mice was not significant.

Seminal vesicle weight (Tab. 1). The weight of the seminal vesicles was initially determined in intact mice and in castrated mice to confirm the success of castration. In fact, the

castration reduced significantly ($p < 0.01$) the weight of the seminal vesicles compared to control. DT treatment induced a significant ($p < 0.01$) increase in the weight of seminal vesicle.

Testosterone plasma level (Tab. 1, Fig. 1). The plasma testosterone concentration was significantly decreased ($p < 0.001$) in castrated mice. Compared to control, the plasma testosterone level was increased after DT injection in NC ($p < 0.01$) and castrated mice ($p < 0.001$). Regressive curve is significant between tumor weight and the testosterone level ($r = 0.9$, $p < 0.01$) (Fig. 1).

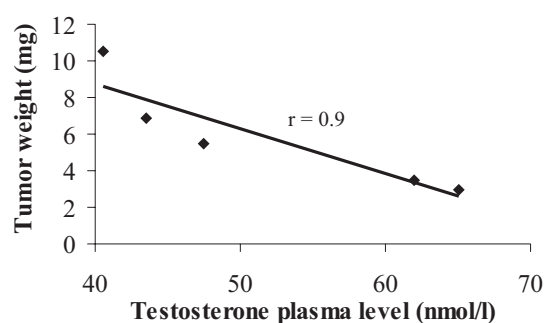


Figure 1. Correlation between tumor weight and plasma testosterone level in DT treated non castrated mice.

Analysis of T cell subsets

Blood (Figs. 2, 3). In mice bearing tumor, an increase is observed in the percentage of CD8+ ($p < 0.05$) and CD2+ ($p < 0.01$) cells in NC mice and the percentage of CD4+ cells ($p < 0.05$) in castrated mice compared to control. The CD4+/CD8+ ratio was lower ($p < 0.01$) in NC mice with tumor than control (Fig. 2).

Positive correlation is observed in NC mice between the tumor weight and the percentage of CD8+ ($r = 0.74$, $p < 0.05$), CD4+ ($r = 0.88$, $p < 0.01$) and CD2+ cells ($r = 0.82$, $p < 0.05$) (Fig. 3a).

DT injection increased the percentage of CD8+ cells in grafted animals ($p < 0.01$). The percentage of CD4+ cells increased ($p < 0.05$) in castrated mice with DT (Fig. 2).

Tumor tissue (Figs. 3, 4). The percentage of CD4+, CD8+ cells increase significantly ($p < 0.01$) in animals with small tumors, compared to big tumor (Fig. 4). Positive correlation is observed in tumor tissue between the small tumor weight and the percentage of CD2+ ($r = 0.8$) and CD4+ ($r = 0.72$) (Fig. 3b).

Cell cycle analysis

Tumor tissue. Compared to untreated animals, the DT administration increased significantly the proportion of G0/G1 cells. The accumulation of cells in G0/G1 phase was compensated by a diminution in S phase. The percentage of proliferating cells showed a significant reduction in both NC

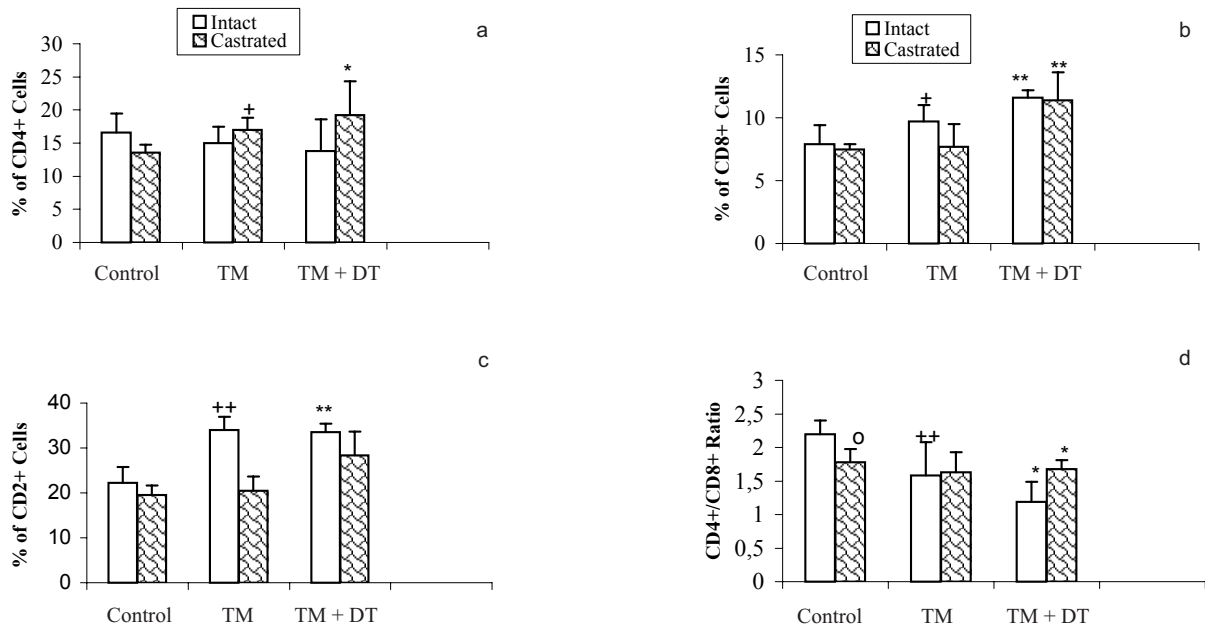


Figure 2. Effect of testosterone in the percentages of CD4+ (a), CD8+ (b), CD2+ (c) and CD4+/CD8+ ratio (d) in the peripheral blood lymphocytes (PBL) of intact (NC) and castrated mice with or without tumor cells. Data are means \pm SEM. o $p < 0.05$ compared to non transplanted animals (control). + $p < 0.05$; ++ $p < 0.01$ grafted mice compared to control. * $p < 0.05$; ** $p < 0.01$ grafted mice treated with DT compared to mice bearing tumor without DT treatment.

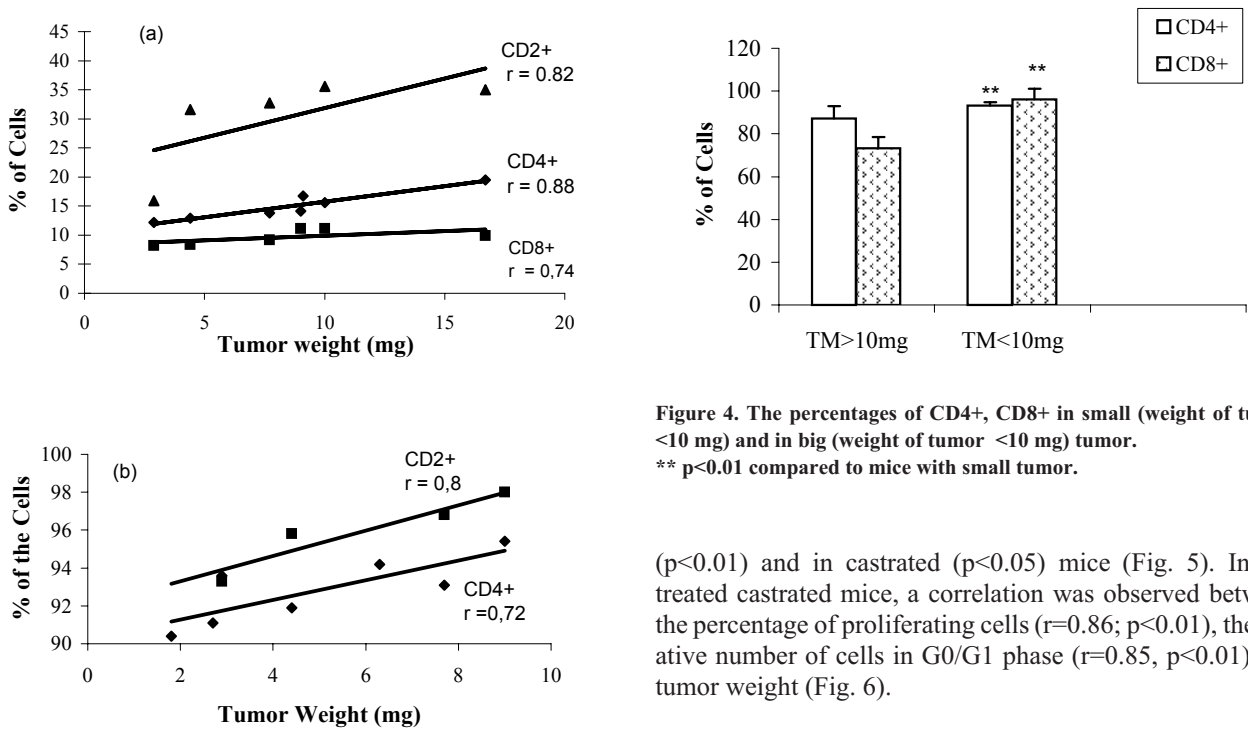


Figure 3. Correlation between tumor weight and: a) The percentage of CD8+, CD4+, and CD2+ cells in the blood, b) the percentage of CD4+, CD8+ in tumor tissue.

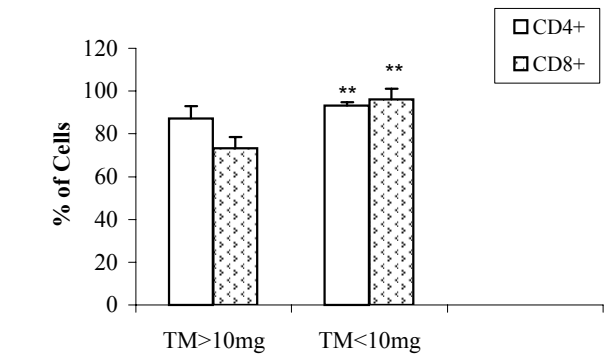


Figure 4. The percentages of CD4+, CD8+ in small (weight of tumor < 10 mg) and in big (weight of tumor > 10 mg) tumor. ** $p < 0.01$ compared to mice with small tumor.

($p < 0.01$) and in castrated ($p < 0.05$) mice (Fig. 5). In DT treated castrated mice, a correlation was observed between the percentage of proliferating cells ($r = 0.86$; $p < 0.01$), the relative number of cells in G0/G1 phase ($r = 0.85$, $p < 0.01$) and tumor weight (Fig. 6).

In vitro study (P388 cell culture)

Cell growth. Figure 7a show the classical growth curve of P388 cells, with a doubling time of approximately 24 hours. DT treatment greatly reduced the cell growth, its action was

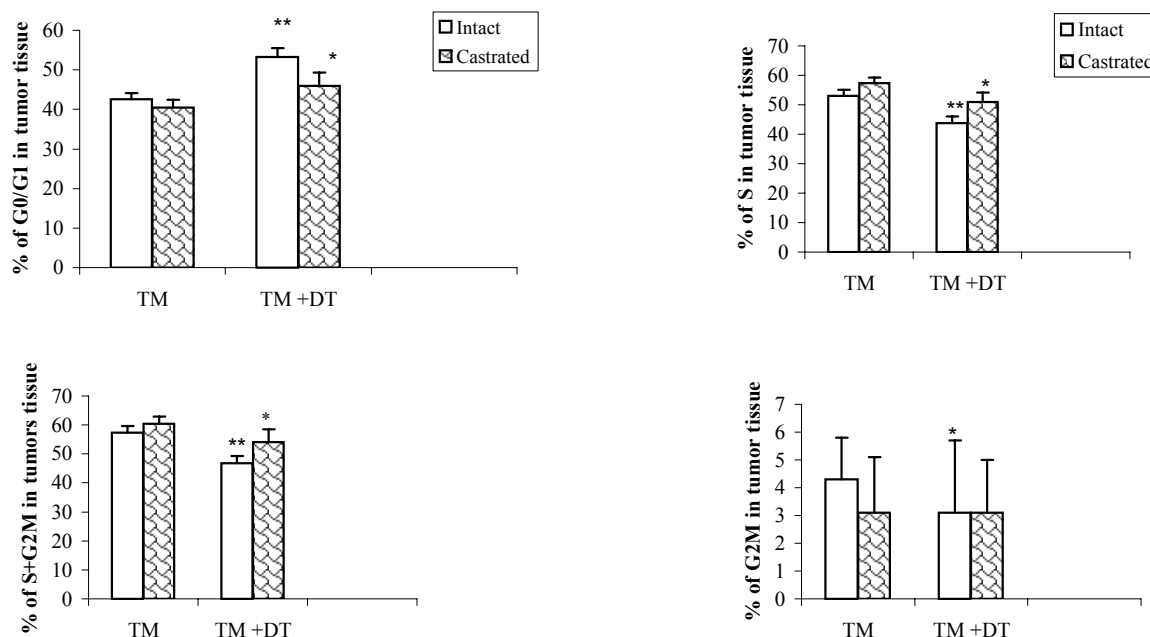


Figure 5. Effect of DT on the repartition of cells among the phases of the cell cycle (G0/G1 (a), S (b), G2/M (c) and proliferative phase (d) in tumor tissue of intact (NC) and castrated transplanted mice. Data are means \pm SEM.

* $p < 0.05$, ** $p < 0.01$, compared to tumor-bearing mice without treatment.

time and dose dependent. The growth inhibition started after 24 hours of cultivation for the two high DT concentrations (10^{-6} and 10^{-4} M).

Cell cycle analysis. In exponentially growing culture, P388 cells were mainly in G0/G1 and S phase (57.9 ± 3.1 and $35.9 \pm 3\%$, respectively), the proportion of cells in G2/M phase was low ($7.6 \pm 2\%$) (Fig. 7b, control).

According to growth inhibition, DT treatment induced a significant decrease of cells in S and G2/M phases and an increase of cells in G0/G1 phase. The effect of DT was seen when concentration was up to 10^{-7} M (Fig. 7b).

Discussion

These works were designed to investigate the influence of androgens (by castration and testosterone treatment) on growth and development of murine leukemia cell line P388 *in vitro* and *in vivo*. To understand the main mechanism of this hormone, the distribution of T cell subsets was studied in peripheral blood and tumor tissue. The cell cycle was analyzed in tumor tissue of grafted mice and in murine leukemia cell line (P388) in culture.

P388 tumor grew better in castrated than in NC mice, the CD8+ cells in the blood were more numerous in NC tumor bearing mice. The T cell subsets were also high in small tumor tissue (< 10 mg).

The lymphocyte cytotoxic activity of CD8+ cells seem to be the more important and seems to be a crucial effector system to inhibit tumor growth *in vivo* [11]. The level of CD8+

CTL activity was directly correlated with the degree of inhibition of tumor [25].

In our results, the correlation observed between the tumor weight and T cell subset, both in PBL and in small tumor could confirmed the important intervention of CD4+ and CD8+ cells to inhibit growth of tumor.

In big tumor tissue, the low proportion of CD4+ and CD8+ observed could be the consequences of losing antigenic markers and it could be an infiltration of the peripheral blood by P388 cells.

HOGGHE and BONIVER [15] have shown that thymic lymphomas lose either antigenic markers and their function. At opposite, the lymphoma T cells acquire other differentiation markers after transformation [19]. The finding of OHSHIMA et al [19] reported that CD8 and/or CD56 positivity confer no cytotoxic function on adult-T-cell leukemia/lymphoma (ATLL), and it is possible that CD8 and CD56 may be simply aberrant surface markers in ATLL.

The subset of T cells in PBL of tumor bearing castrated mice, remain unchanged. In fact, our previous works [2] showed that testosterone injection was involved in the distribution of some T-lymphocyte subsets. As the relative numbers of mature T cells (CD4+CD3+ and CD8+CD3+) were decreased in the spleens of castrated animals [28], a reverse effect is observed in testosterone treated mice.

Our results show that the DT treatment reduced strongly the weight and the rate of tumor in BDF1 mice grafted with murine leukemia cell line (P388). CD8+ cells are increased in the PBL of tumor bearing treated mice.

Moreover testosterone inhibits the *in vivo* tumorigenic properties of the 1246-3A cells. Castrated male mice receiving injections of 1246-3A cells developed larger tumor at a higher frequency than sham-operated animals. Administration of testosterone to castrated male mice resulted in a dramatic decrease in tumor development [25].

In fact, the recent work [24] demonstrated that IL-10 producing CD4⁺ T cells can manifest anti-tumor functions. The IL-10 maintain the number of anti-tumor CD8⁺ T cells. LIVA and VOSKUHL [18] showed that testosterone can act directly via androgen receptor on CD4⁺ T-lymphocyte to increase IL-10 gene expression.

Thus we suggest, that testosterone can inhibit tumor growth by modifying CD8⁺ and CD4⁺ cells distribution. To verify if testosterone has a direct effect on the leukemia cells, the cell cycle was analyzed in tumor tissue of grafted mice and in murine leukemia cell line (P388) in culture.

In the present finding, the cell cycle analysis in tumor tissue show that DT decreased both the cells in S phase and the

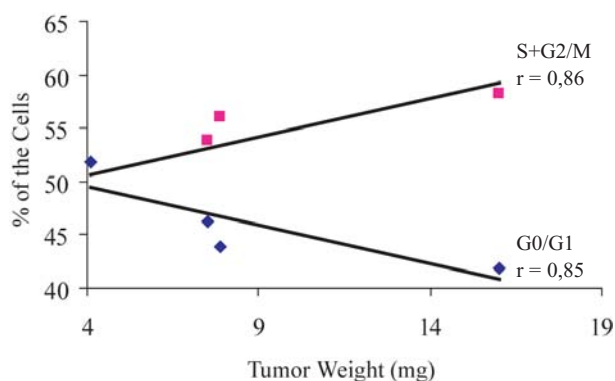


Figure 6. Regression Curve between the tumor weight and the percentage of cells in G0/G1 phase ($r=0.86$, $p<0.01$) and in proliferative phase ($r=0.85$, $p<0.01$), in castrated P388 tumor-bearing mice treated with DT.

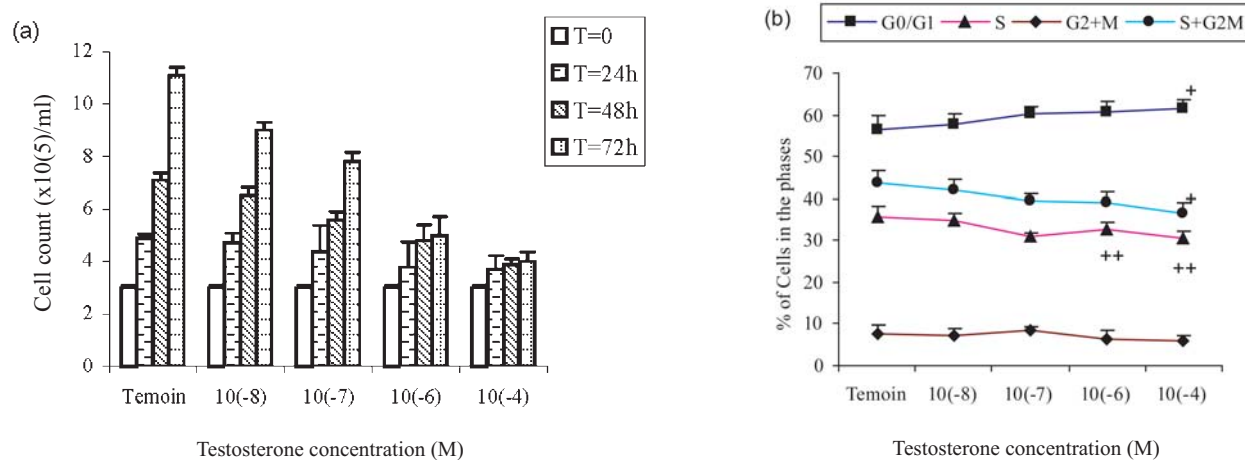


Figure 7. Effect of DT concentration on P388 cell growth (a) and on the proportion of P388 cells in the different phases of the cell cycle. DT was added in the medium at the beginning of the culture. Culture was followed during 3 days. Data are mean \pm SEM.

+ $p<0.05$, ++ $p<0.01$ compared to untreated cells.

proliferating leukemic cells, with accumulation of cells in G0/G1 phase. On top of that, our experiments showed that testosterone can inhibit the proliferation of leukemic cells with a pharmacological dose (10^{-7} M). This growth inhibition was dose and time dependent. Lower dose was ineffective.

In fact, the novel aminosteroid 5 α -androstane (HY) inhibit the proliferation of the human promyelocytic leukemia cell line HL-60. This aminosteroid HY was a derivative of 5 α -androstane and posses some structural similarities with an androgen steroid [21]. The association of testosterone and methotrexate (MTX) (anti-proliferative) compared to MTX alone suggests possible synergistic actions [6].

In our results, the growth inhibition exerted by DT was associated with cell cycle arrest; P388 cells accumulated in G2/M phase. We also observed a correlation in tumor tissue between the percentage of cells in G0/G1, the relative number of cells in proliferative state (S + G2/M) and tumor weight.

Our finding are in agreement with MOSSUZ et al [19] who reported that the testosterone can inhibit the proliferation and the clonogenic potential of the human monoblastic leukemic cell line U937. The growth inhibition was associated with cell cycle arrest, and accumulation of U937 cells in G2/M phase.

Others works [10], reported that in P388 murine leukemia cells, cell cycle analysis revealed a potent anti-tumor marine steroid (YTA0040) blocked the cells into S phase leading to arrest in the late G1 phase.

These data confirm that testosterone prevent the proliferation of leukemic cells both in tumor tissue and P388 murine leukemia cell line in culture.

To conclude, our experiments reported that testosterone prevent the growth of tumor: indirectly by modulation of subsets T cells distribution and directly by the alteration of the cell cycle. In fact, the T cells subset can modify the cell cycle by their mediators: the cytokine. CAMILLO et al [5],

showed that IL2 exerts anti-proliferative effect on three human malignant cell lines *in vitro*. The exposition to higher doses of IL2 induced a reduction of malignant cells in the S phase of the cell cycle with an accumulation in G0/G1. The alteration of the cell cycle kinetics is followed by apoptosis.

We thank Dr G. BAUDIN for dosage of testosterone in Department of Nuclear Medicine.

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