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Effect of Thyroxine and Propylthiouracil in Ehrlich Ascitic Tumor Cells

Efecto de la Tiroxina y Propiltiouracilo en las Células del Tumor Ascítico de Ehrlich

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SUMMARY: The main purpose of this study was to investigate the effect of thyroxine and PTU in ascitic Ehrlich tumor cells. Tumor was implanted in 30 female mice distributed in three groups: treated with PTU, treated with thyroxine and control. Each group received an intraperitoneal injection of neoplastic cells, pre-incubated with sterile solutions of PTU, thyroxine and distilled water, respectively. On the fifth and seventh days after inoculation, animals received an intraperitoneal injection of the respective solutions. On the tenth day after inoculation, animals were sacrificed. Volume of ascitic liquid, number of neoplastic cells/ml and percentage of viable cells were determined. Ascitic liquid smears were carried out for tumor cytological evaluation. There was no difference among groups regarding ascitic liquid and as for the number and viability of tumor cells. However, cells under the effect of thyroxine presented significantly larger mean of nuclear diameter, size and number of nucleolus organizer regions. In this group, there was a predominance of clear, round cells with abundant eosinophilic and very vacuolated cytoplasm with little defined edges. Under the PTU effect, tumor cells were small with hyperchromatic nucleus and the same number of NORs as the control group. It was concluded that PTU and thyroxine have not changed the number and viability of cells after 10 days of tumor inoculation but they changed significantly cell characteristics. Whilst thyroxine increases cell size and the number of NORs of ascitic Ehrlich tumor cells, PTU causes an opposite effect.

KEY WORDS: Cytology, Ehrlich tumor; Mouse; NORs; Propylthiouracil; thyroxine.

INTRODUCCIÓN

Transplantable experimental tumors have been used in studies of physical, chemical, viral and hormonal carcinogeneses (Bonamin, 1990). Ehrlich tumor is a transplantable neoplasia from a malign epithelium, which corresponds to mammary adenocarcinoma in female mice. When inoculated intraperitoneally it grows in an ascitic form and when inoculated subcutaneously it grows in the form of a solid tumor (Ehrlich, 1906).

This tumor has been used as a model for several studies, such as those to verify the influence of stress on cancer (Palermo-Neto *et al*., 2001; Palermo-Neto *et al*., 2003) and host's immunological response to tumor (Segura *et al.*, 2000; Pinto, 2003), in the evaluation of tumor growth under the effect of ophidic toxins (Mady, 2002), vegetable extracts (Rajeshkumar *et al.*, 2002), antiinflammatory drugs (Pal et *al.,* 2001), chemical agents derived from fluoracil and cisplatin (Yoneda *et al.*, 1999; Valadares & Queiroz, 2002) and vitamines A and B1(Oloris *et al*., 2002).

Thyroid hormones are important cell growth, development and differentiation regulators. In order to assess the effect of thyroid dysfunctions on breast cancer prognosis, Ehrlich tumor has also been proposed as an experimental model. Studies have shown that hypothyroidism slows tumor growth even without changing malignity cell characteristics (Silva *et al*., 2004) and that hyperthyroidism promotes this growth (Ferreira, 2004). But it is not clear whether this effect is caused by thyroid dysfunction or by direct action of drugs used to experimentally induce hypo and hyperthyroidism.

There are no studies in the literature studied regarding direct effect of thyroxine, drug used to experimentally indu-

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ce hyperthyroidism or of propylthiouracil, used to induce hypothyroidism, on Ehrlich tumor. The purpose of this study was to evaluate the effect of these drugs on characteristics of ascitic Ehrlich tumor cells.

MATERIAL AND METHOD

Thirty 2-month-old female Swiss mice were used, kept in plastic cages (10 animals/cage), where they received commercial diet and water ad libitum and were maintained in a regimen of 12-hour light/12-hour dark. Animals were separated in three groups of 10 animals each. Group 1 treated with thyroxine; Group 2 treated with propylthiouracil (PTU) and Group 3 treatd with placebo (control group).

Tumor cell preparation included washing, counting, viability test and standardization of the cells. With sterile disposable syringes and needles, 3.0 mL of ascitic fluid was collected from a pre-inoculated mouse with Ehrlich tumor for ten days in cavidade peritonial. This fluid was centrifuged, supernatant was disregarded and cells were resuspended in physiological saline until a dense and clear liquid was obtained, corresponding to cell suspension with a minimum of fibrin and erythrocytes. Then tumor cells were counted and their viability test was performed, with a 95% of viability. The formula to determine the number of viable cells was deduced in accordance with Guerra (1983).

Before inoculation, tumor cells were pretreated with drugs for 30 minutes, in incubator at 37ºC, according to each group. Each ml of tumor cell suspension was pre-incubated with 1mL of sterile thyroxine solution (10mg/mL), PTU (1mg/mL) and bidistilled water, as placebo, according to each treatment. At the end of incubation period, tumor cells were counted and cell viability test was performed again, exactly as it was described previously, with the purpose of evaluating the effect of incubation on neoplastic cells exposed to drugs. Mice received an abdominal injection containing 0.3mL of cell suspension with $2.5x10^6$ tumor cells, in order to obtain tumor in the ascitic form, remaining 10 days with this tumor. At days five and seven after inoculating tumor cells, animals from groups 1, 2 and 3 received 0.3mL thyroxine (10mg/animal), 0.3ml PTU (1mg/animal) and 0.3mL distilled water, all of which were sterile, intra-peritoneally, respectively. In order to evaluate tumor growth curve, abdominal circumference was measured with the use of a measuring tape and animals were weighed immediately before and every two days after tumor inoculation, with a total of six measurements.

After 10 days of tumor implantation, animals were necropsied. Ascitic liquid was also collected in order to count tumor cells and determine their viability. Smears were carried out from the cell suspension obtained from each animal, then they were submitted to a panotico stain, in order to determine cell characterisctics, dark cells/clear cells ratio, nucleus/cytoplasm ratio and mean diameter of neoplastic cell nuclei, in immersion objective. Mean nucleus diameter, was only evaluated in mononucleated neoplastic cells. Nucleolus organizer region was stained with silver (AgNORs), in accordance with a technique described by Ploton et al. (1986), modified by Aubele *et al.* (1994). Incubation was carried out in a humid and dark chamber at 40ºC for 15 minutes. Characterizing and counting the number of AgNORs were carried out in immersion objective.

The number of cells and nuclei (to determine nucleus/cytoplasm ratio and the number of NORs) and fields (to quantify the number of clear and dark cells) was determined using the technique of studying mean value instability variation regarding the sample where they came from. Standard deviation and variation coefficient of the variables studied were stable in about thirty cells and thirty fields/slide/animal respectively.

Experimental delineation was completely at random with three treatments and ten repetitions/treatment. Data were submitted to variance analysis using test SNK to compare means (Sampaio, 1998).

RESULTS

In the beginning of the treatment, that is, before inoculating tumor cells, up to the end of the experiment, there was no significant difference of weight among animals (Table I). However, PTU-treated animals presented significantly smaller abdominal circumference on the second day of tumor growth evaluation, but this result has not repeated in the following measurements (Table II). There was no significant difference regarding ascitic liquid volume, cell number and viability among groups (Table III).

Characteristics of Ehrlich tumor cells differed among groups. In the group treated with thyroxine there was a significantly smaller number of dark cells. In this group, a predominance of clear and round cells with abundant, eosinophilic, quite vacuolated cytoplasm with little defined edges, large nuclei, sometimes being multinucleated (Figure 1b). In the group treated with PTU, the opposite occurred, that is, a significantly smaller number of clear cells with predominance of dark cells. Most cells were round with moderate, basophilic cytoplasm, with a few vacuoli, defined

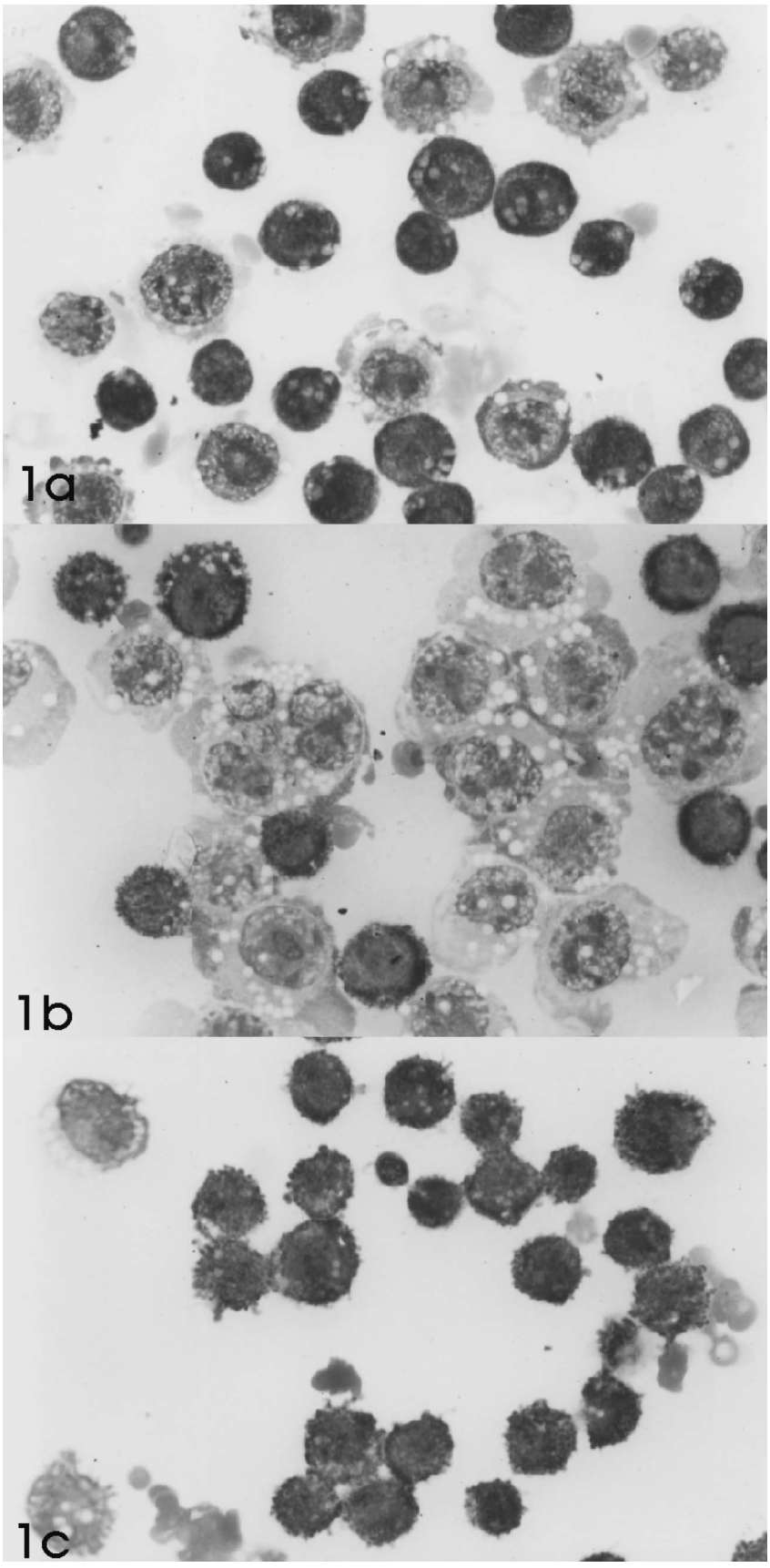

Fig. 1. Smears of the ascitic liquid cells of ascitic Ehrlich tumor in female mice. Panotico stain, 1071´.
a) Control group. Prevalence of cells with moderate cytoplasm and nuclear volume and few vacuoles.
b) Group treated with thyroxine. Cells round, with abundant cytoplasm and very vacuolated, bigger nuclei.
c) Group treated with propylthiouracil. Cells round, with smaller cytoplasm with few vacuoles.

edges and hyperchromatic nuclei varying from oval to round ones (Figure 1c). Neoplastic cells from the control group presented cytoplasmic and nuclear volume smaller when compared to the group treated with thyroxine and larger than the group treated with PTU (Fig. 1a). Morphometric analysis corroborated differences among groups as for cell and nucleus size (Table IV).

Nucleolus organizer regions (NORs) were small and distributed among nuclei and nucleoli in cells both from the control group and in those under thyroxine effect (Figs. 2a and b). In PTU-treated cells, NORs were larger and concentrated in the nucleolus (Fig. 2c). The number of NORs was significantly higher only in cells under thyroxine action (Table IV).

In tumor cells treated with thyroxine, no increase was observed in nucleus/cytoplasm ratio, as there was an increase, not only in the nucleus but also in the entire cell (Table IV).

Regardless the group, no metastases were observed in spleen, liver, heart, lungs or even in abdominal lymph nodes. There was only a large amount of neoplastic cells under these organs capsule, which does not characterize metastasis.

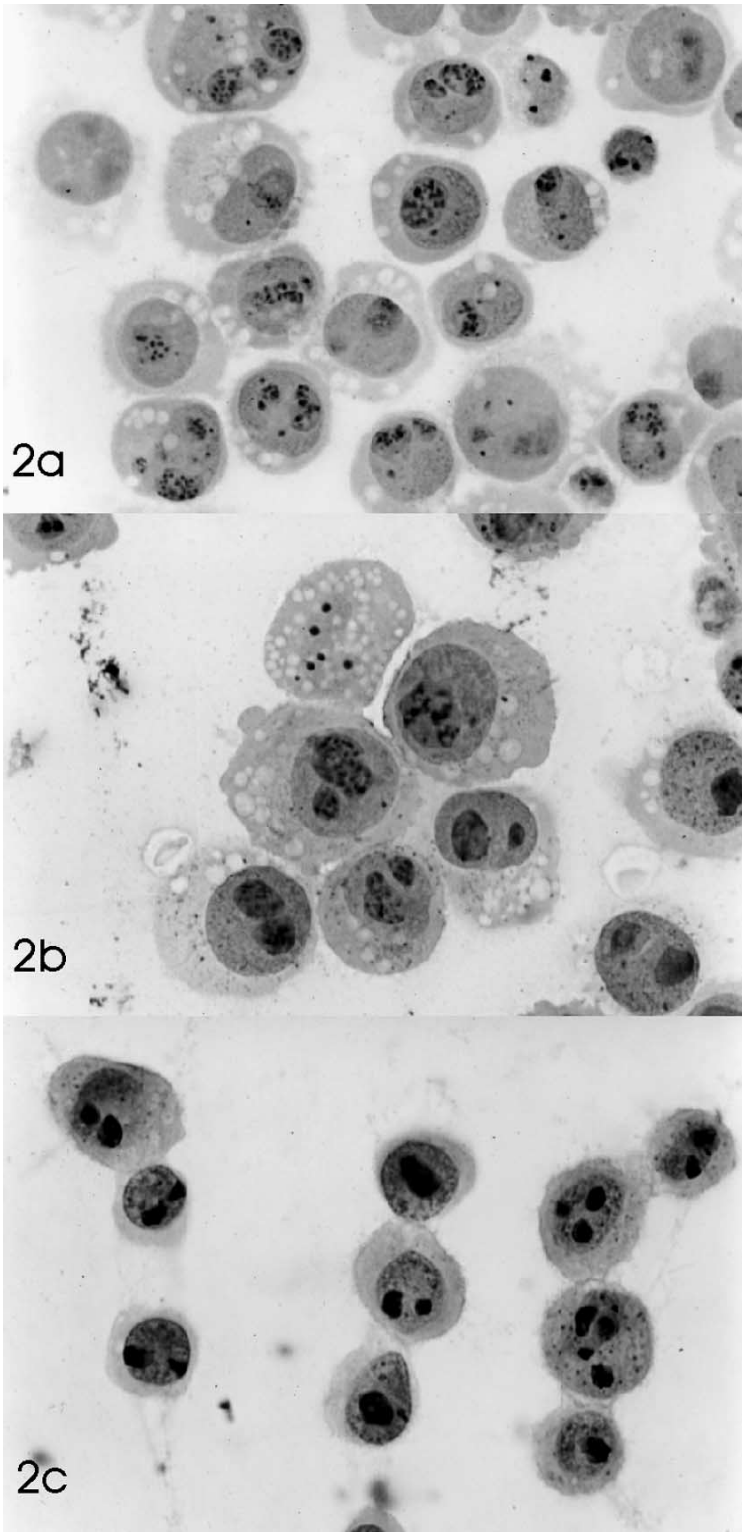

Fig. 2. Smears of the ascitic liquid cells of ascitic Ehrlich tumor in female mice. Impregnation of AgNOR, 1607´. a) Control group. Nucleoli organizer regions (NORs) small thoroughly filling the nucleus and nucleolus. b) Group treated with thyroxine. NORs similar the control group but in smaller number. c) Group treated with propylthiouracil. NORs larger, more concentrated in the nucleolus and in small number.

DISCUSSION

PTU-treated animals presented significantly smaller abdominal circumference only on the second day of tumor growth evaluation. This is probably because only after incubating tumor cells with the respective drugs, which was carried out before inoculation, there was a decrease in the viability of cells treated with PTU when compared to those incubated with thyroxine and with placebo (data not shown). This fact possibly explains why growth of tumor cells treated with PTU decreased on the second day after inoculation. However, PTU only decreased cell viability in the period of drug incubation, with no difference among groups regarding viable cells in the end of the experiment.

The production of ascitic liquid in Ehrlich tumor is said to occur due to increased capillary permeability present in peritoneal cavity (Senger *et al*., 1983). This vascular change occurs due to increased receptor expression for autocrine motility factor (AMF). AMF link to its receptor induces angiogenesis and changes endothelial cell morphology, causing subsequent increase of vascular permeability with increased amount of ascitic liquid (Funasaka *et al*., 2002). This mechanism does not seem to be affected by treatments, as there was no significant difference among groups regarding ascitic liquid volume. Number of NORs was significantly larger in cells under thyroxine action. NORs quantification, chromosomal DNA loops where ribosomal genes are coded, have been widely used as an indicator of cell proliferation, presenting significant value, both diagnostic and prognostic, in tumor pathology (Rüschoff *et al.,* 1994; Cia *et al*., 1999; Metze *et al.,* 1999; Metze *et al*., 2000). NORs have awakened interest as its number in the nucleus has been significantly larger in neoplastic cells when compared to normal, reactive or benign neoplastic cells (Derenzine, 2000).

Cell pleomorfism and the presence of several nucleoli and binucleated cells are also important characteristics of a tumor malignity (Brasileiro Filho, 2004). Although they have all been present in tumor cells treated with thyroxine, no increase was observed in nucleus/cytoplasm ratio, another important characteristic of malign neoplastic cells. In cells treated with thyroxine

Table I. Weight (g) (mean ± SD) in female mice, control, treated with thyroxine and treated with propylthiouracil with ascitic Ehrlich tumor in day 0 until day 10.

Weigth	Group		
	Control (n=10)	Thyroxine (n=10)	Propylthiouracil (n=10)
Day before inoculation	32.81 ± 1.58a	33.20 ± 2.36a	31.93 ± 1.71a
Day 2 after inoculation	29.42 ± 9.09a	31.83 ± 1.99a	31.45 ± 1.81a
Day 4 after inoculation	33.25 ± 1.76a	34.10 ± 2.01a	33.83 ± 2.49a
Day 6 after inoculation	34.82 ± 1.99a	37.41 ± 3.03a	37.18 ± 3.27a
Day 8 after inoculation	39.41 ± 1.94a	40.12 ± 2.20a	39.54 ± 4.39a
Day 10 after inoculation	39.84 ± 2.63a	38.80 ± 2.95a	38.81 ± 4.75a

* Different letters in the same row indicate a statistically significant difference (p<0.05)

Table II. Abdominal circumference (cm) (mean ± SD) in female mice, control, treated with thyroxine and treated with propylthiouracil with ascitic Ehrlich tumor in day 0 until day 10.

	Group		
Measurements day	Control (n=10)	Thyroxine (n=10)	Propylthiouracil (n=10)
Day 0 before inoculation	8.11 ± 0.31a	8.00 ± 0.29a	7.88 ± 0.25a
Day 2 after inoculation	7.87 ± 0.21 a	7.65 ± 0.17a	7.49 ± 0.33b
Day 4 after inoculation	8.07 ± 0.22 a	7.94 ± 0.20a	8.10 ± 0.26a
Day 6 after inoculation	8.29 ± 0.34 a	8.35 ± 0.57a	8.53 ± 0.41a
Day 8 after inoculation	9.58 ± 0.32 a	9.44 ± 0.32a	9.31 ± 0.49a
Day 10 after inoculation	10 ± 0.37 a	10.16 ± 0.52a	9.79 ± 0.66 a

* Different letters in the same row indicate a statistically significant difference (p<0.05)

Table III. Measured variables in ascitic Ehrlich tumor (mean ± SD) in female mice, control, treated with thyroxine and treated with propylthiouracil with ascitic Ehrlich tumor in day 0 until day 10.

	Group		
Variable	Control (n=10)	Thyroxine (n=10)	Propylthiouracil (n=10)
Ascitic liquid volume (ml)	6.75 ± 1.97 a	8.09 ± 2.28 a	7.54 ± 2.54 a
N° of tumor cells/ml	143.25 ± 41.9 a	116.55 ± 32.05 a	127 ± 35.47 a
% of viable tumor cells/ml	95.98 ± 2.59 a	96.39 ± 1.50 a	96.31 ± 1.85 a

*Different letters in the same row indicate a statistically significant difference (P<0.05)

Table IV. Nucleoli organizer regions (NORs) number, ratio dark/clear cells, mean nuclear diameter (mm), mean cell diameter (mm) and ratio nucleus/cytoplasm (mean ± SD) in smears of ascitic Ehrlich tumor in female mice, control, treated with thyroxine and treated with propylthiouracil

	Group		
Variable	Control (n=10)	Thyroxine (n=10)	Propylthiouracil (n=10)
N° of NORs/nucleus	10.95 ± 5.34b	19.16 ± 5.16a	9.63 ± 3.17b
N° of dark cells/field	28.59 ± 13.14a	19.22 ±12.23b	35.76 ± 13.18a
N° of clear cells/field	13.27 ± 4.59a	11.09 ± 5.042a	7.35 ± 2.99b
Ratio of dark/clear cells/field	2.751 ± 2.225bc	2.023 ± 1.327c	5.747 ± 3.690a
Mean nuclear diameter (_m)	21.18 ± 1.21b	24.56 ± 2.37a	15.65 ± 4.02c
Mean cell diameter (_m)	32.15 ± 1.98b	38.99 ± 2.33a	28.87 ± 1.12c
Ratio of nucleus/cytoplasm	0.66 ± 0.04a	0.63 ± 0.08a	0.54 ± 0.15a

*Different letters in the same row indicate a statistically significant difference (p<0.05)

there was an increase, not only in the nucleus but in the whole cell. Another intriguing factor is why there was no significant difference among groups regarding the number of neoplastic cells. Whether NORs quantification has been pointed out as a good indicator of cell proliferative activity and whether thyroxine treated cells presented an increased number of NORs, it should be investigated whether there was a simultaneous increase of proliferation rate and cell apoptosis.

Neoplastic cell exposure to thyroxine changed cell parameters, pointed out as important malignity characteristics. This may be the explanation for a higher speed of solid Erhlich tumor growth in the hyperthyroid state (Ferreira *et al*., 2004) . But, PTU action on tumor cells was in contrast to hypothyroidism, induced by this drug (Silva *et al.*). Tumor cells treated with PTU were smaller and with larger NORs and in a smaller number, exactly in contrast to

what was observed in Ehrlich tumor in hypothyroid mice(Silva *et al.*). In the study of Silva *et al.* , thyroid hypofunction delayed tumor growth, without decreasing tumor cells malignity. Thus, the effect of direct PTU administration on Ehrlich tumor cells differs from the effect caused by hypothyroidism.

Several types of antineoplastic drugs, such as, antimetabolic, alkaloid and antibiotic have been developed and used against malign tumors in humans. Among antimetabolic drugs, BOF-A2, a 5-fluoracil derivative, PTU analog, has been used to treat carcinomas and its action decreases tumor growth by inhibiting DNA synthesis and inducing apoptosis (Yoneda *et al.*).

In conclusion, PTU and thyroxine even without changing the number and viability of cells after 10 days of tumor inoculation changed significantly cell characteristics. Whereas thyroxine increases cell size and the number of NORs of ascitic Ehrlich tumor cells, PTU causes an opposite effect.

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RESUMEN: El propósito principal de este estudio ha sido investigar el efecto de la tiroxina y del propiltiouracilo (PTU) en las células del tumor de Ehrlich. El tumor fue implantado en 30 ratones hembras distribuidas en tres grupos: tratado con PTU, tratado con tiroxina y control. Cada grupo recibió una inyección intraperitoneal de células neoplásicas, pre-incubadas con las soluciones estériles de PTU, tiroxina y agua, respectivamente. En el quinto y séptimo días después de la inoculación, los animales recibieron una inyección intraperitoneal de las soluciones respectivas. En el décimo día después de la inoculación, se sacrificaron los animales. Fueron determinados el volumen de líquido ascítico, el número de células/ml y el porcentaje de células viables. Además se realizaron frotis del líquido ascítico para la evaluación de la citología del tumor. No hubo ninguna diferencia entre los grupos con respecto al volumen del líquido ascítico y el número y viabilidad de las células del tumor. Sin embargo, las células bajo el efecto de la tiroxina presentaron una media significativamente superior del diámetro nuclear, tamaño y número de regiones organizadoras de nucleélos. En este grupo, había un predominio de células claras, redondas con citoplasma abundante, eosinofílico y vacuolado con poca definición de los bordes. Bajo el efecto del PTU, las células del tumor eran pequeñas con el núcleo hipercromático y el mismo número de NORs como el grupo control. Se concluye que el PTU y tiroxina no afectaron el número y viabilidad de las células después de 10 días de inoculación del tumor, pero sí cambiaron las características celulares. Aunque la tiroxina aumenta el tamaño celular y el número de NORs de las celulas del tumor de Ehrlich, PTU causa efectos opuestos.

PALABRAS CLAVE: Citología; Tumor de Ehrlich; Ratón; NORs; propilthiouracil; Tiroxina.

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