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Chlorpheniramine inhibits the ornithine decarboxylase induction of Ehrlich carcinoma growing in vivo

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The antihistaminic (±)-chlorpheniramine significantly reduced the progression of Ehrlich carcinoma when it was administered at 0.5 mg/mouse/day from the third day on, after tumour inoculation. The ODC activity of tumour cells was diminished by 70% on day 7 after tumour transplantation, when maximum ODC activity is detected in non-treated tumour growing 'in vivo'. Northern blot analyses indicated that the inhibitory effect of this 1,4-diamine takes place at a post-transcriptional level. Results obtained from serum-free cultured cells indicated that chlorpheniramine inhibits the ODC synthesis rate.

Ornithine decarboxylase; Chlorpheniramine; Polyamine; Tumor

1. INTRODUCTION

Polyamines, putrescine, spermidine and spermine are organic cations which play an important role in cell proliferation [1]. Ornithine decarboxylase activity (ODC, EC 4.1.1.17), the main regulatory step for polyamine biosynthesis, is induced by different growth stimuli at the transcriptional and translational level [2–4]. However, polyamines inhibit ODC expression at the translational level and/or by post-translational modifications of the protein [5,6]. In addition, an ODC-inhibitory protein named 'antizyme' is induced by poly- and diamines [7]. Actually, some non-natural diamines (i.e. 1,3-diaminopropane, bis-ethyl-spermidine), have been described as polyamine analogs also inhibiting ODC induction at translational and post-translational level [8,9].

In 1989, Matés et al. [10] added histamine and serotonin to the list of diamines controlling ODC activity. Both 1,4-diamines were able to inhibit the short-term induction of ODC activity caused by ornithine in perifused Ehrlich carcinoma cells, showing a clear dose–response effect. Recently, authors have reported that this effect can also be exerted by other non-natural amines which have some common structural characteristics: 1,4-diamine structure, N–N distance around 6 Å, and at least one charge amino group [11]; among these 1,4-diamines, the histamine analog (±)-chlorpheniramine was able to inhibit by more than 90% the induction of ODC activity when Ehrlich tumour cells were perifused in saline buffer supplemented with 0.5 mM ornithine and 55 μM chlorpheniramine.

In this report, the effect of (±)-chlorpheniramine on the ODC expression and tumour progression is studied. Results indicate that the presence of chlorpheniramine in cell cultures causes an inhibition of 'de novo' synthesis of the protein. 'In vivo' experiments resulted in a decrease of both ODC activity and tumour proliferation.

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2. MATERIALS AND METHODS

2.1. *Ehrlich ascites cells and animal treatments*

A hyperdiploid Lettré strain of Ehrlich carcinoma was maintained as previously reported [12]. For Fig. 1, groups of 8 mice were inoculated with 5×10^6 tumour cells and treated as described in section 3. The animals' weights were determined daily until death. To determine the total number of cells, ascitic fluid volumes, ODC activities and levels of ODC mRNAs, two groups of 16 mice were inoculated with 5×10^6 tumour cells; 8 animals of each group were daily injected i.p. with 0.2 ml of 6.4 mM of maleate solution in phosphate saline buffer pH 7.4 (6.16 mM KCl, 154 mM NaCl, 1.65 mM NaH_2PO_4, 9.35 mM Na_2HPO_4, pH 7.4), and 8 animals with 0.2 ml of 6.4 mM chlorpheniramine (maleate salt) solution in the same buffer, pH 7.4; (±)-chlorpheniramine was purchased from Sigma Química (Spain). Animals were sacrificed on the 7th and 11th days after tumour transplantation by cervical dislocation. Immediately, cells were separated by centrifugation at 2,000×*g* for 5 min, frozen and stored until used at −20°C (ODC assay) or −80°C (total RNA isolation). Cell viability and the total number of tumour cells were determined by the erythrosine exclusion test. For synthesis and degradation experiments, cells were collected 10–11 days after tumour transplantation and cultured in a 1:1 mixture of Ham's F12 and Dulbecco's Modified Eagle's medium supplemented with 0.2% bovine serum albumin and antibiotic (50 IU/ml penicillin and 50 μg/ml streptomycin) at 37°C and 5% of CO_2. After 4 h of culture, (±)-chlorpheniramine was added to different concentrations, and cells were further cultured and collected 4 h later. In control samples, the antihistaminic addition was replaced by the culture medium.

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2.2. ODC mRNA levels

ODC mRNA was determined by Northern blot analysis. Total RNA (10 μg), isolated as described by Chomczynski and Sacchi [13], was fractionated as described by Lehrach et al. [14] in formaldehyde-containing 1.4% agarose gels (2.2 M formaldehyde). After being transferred to S&S NYTRAN nylon membranes (Schleicher and Schuell Inc., Germany), the RNA was hybridized to the ODC cDNA insert of the clone pODC934 [15] labelled by Multiprime DNA Labelling System with [α-^{32}P]dCTP (Amersham Iberica, Spain). As an internal standard, mRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected [16], using a 1.3 kb GAPDH cDNA cloned in pUC9 (obtained by Dr. Bos, State University of Leiden, and kindly provided by Dr. van Steeg, National Institute of Public Health and Environmental protection, The Netherlands). Autoradiographs were analyzed by transmittance densitometry, using a Hoeffer GS 300 scanning densitometer (Hoeffer Scientific, USA).

2.3. *ODC synthesis, degradation and activity*

After 8 h of culture, cells were collected by centrifugation. They were resuspended at 4 × 10^6 cells/ml in growth medium lacking methionine and preincubated for 5–10 min at 37°C. Then, 200 μCi/ml Tran^{35}Slabel (ICN, USA) was added and cells were incubated for different times up to 30 min. The incorporation of radiolabel into protein was stopped and cell extracts were obtained as described by Wallon et al. [17]. Duplicates of 2 μl from each cell extract were precipitated using final 10% (w/w) trichloroacetic acid, and filtered as described by Holm et al. [5]; and the acid-insoluble radioactivity was counted. Total protein synthesis rate is the slope obtained after representing the ^{35}S incorporation into acid-insoluble fraction (cpm)/μg of extract protein versus labelling time (min). For measuring relative ODC synthesis, aliquots of the supernatant (containing equal amounts of acid-insoluble radioactivity) were incubated with an excess of polyclonal monospecific antibody raised against mouse ODC (kindly provided by Dr. Persson, University of Lund, Sweden). The ODC–antibody complex was collected by precipitation with insoluble Protein A [5,17]. Proteins were separated by SDS-PAGE and visualized by fluorography using EN^{3}HANCE (Du Pont, USA). A 53 kDa band (ODC) was detectable when using specific ODC-antibodies, but it was absent in lanes loaded with parallel samples treated with non-immune rabbit serum. Gel lanes were cut from 50–55 kDa and counted for radioactivity (cpm incorporated in ODC). Non-immune serum treated lanes were used as blanks. Relative ODC synthesis is expressed as (cpm incorporated in ODC/total cpm used for immunoprecipitation) × 100. The half-life of ODC was determined by measuring the decline in enzyme activity after adding 50 μg/ml of cycloheximide to the medium after the 8th hour of culture [17]. Ornithine decarboxylase assays were carried out as reported previously by Matés et al. [10,11].

3. RESULTS

Fig. 1A shows the evolution of the total body weight of non-treated inoculated animals. The weight increase correlates to total tumour volume [18]. Tumour proliferation has a 3-day lag phase after inoculation into animals; seven days after transplantation, cells are in the exponential phase of growth, and the maximum number of intact cells occurred between the 9th and the 11th day after tumour inoculation [19]. Commercial (±)-chlorpheniramine is available as maleate salt; thus, groups of control animals were treated with daily i.p. injections of 0.75 mg of maleic acid neutralized at pH 7.4. Maleate had no effect on the evolution of total animal weight, tumor proliferation and animal life span (16 ± 1 day), neither when injected from day three after tumour inoculation (Fig. 1A) nor when injected from

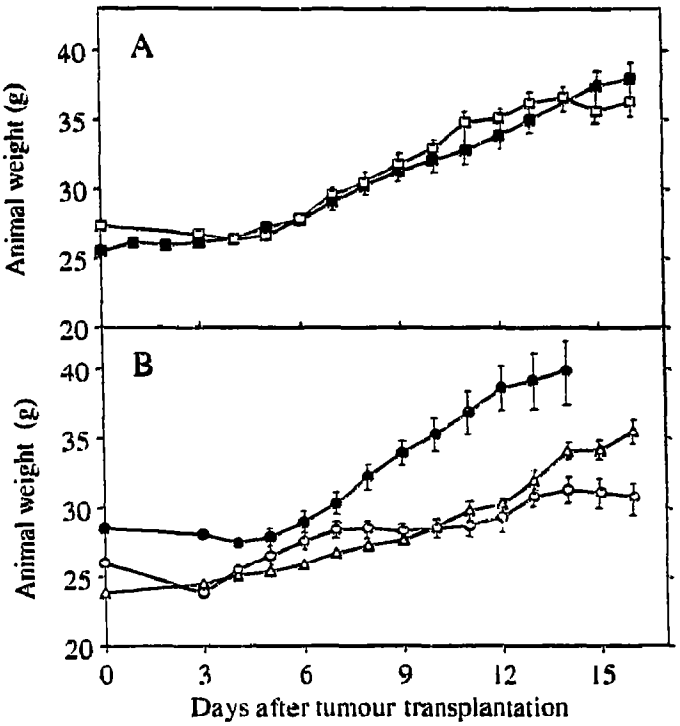

Fig. 1. Evolution of total body weight of animals inoculated with Ehrlich ascitic carcinoma. A: non-treated animals (■), and animals treated daily with 0.75 mg of neutralized maleic acid (□); B: (±)-chlorpheniramine treated animals, 0.2 mg/day (△), 0.5 mg/day (○), and 1.5 mg/day (●).

the first day on. Daily i.p. injections of 1.52 mg/day of (±)- chlorpheniramine starting from day 3 after inoculation had no effect on the total body weight of tumour-bearing mice, but led to a shortage in the animal life span (14 ± 1 day). In contrast, lower i.p. doses 0.2–0.5 mg/day of the antihistamine significantly reduced animal body weight during the second part of the tumour progression (Fig. 1B). However, the life-span values (17 ± 2 days) were not significantly different from those of maleate-treated controls. Similar results were obtained when animals were treated with equal doses of chlorpheniramine from the first day after tumour transplantation (results not shown).

The differences observed in total animal weights between maleate- and chlorpheniramine-treated animals clearly matched those in ascitic fluid volume and total cell number (Table I). Cell viability was the same in chlorpheniramine-treated and control animals. However, the increase in the tumour cell number observed in control mice from day 7 to day 11 after tumor inoculation [18] is avoided in chlorpheniramine-treated animals (0.5 mg/day, starting from day 3 after tumour inoculation). The maximum ODC activity in Ehrlich ascitic carcinoma growing 'in vivo' is detected at day 7 [20]; (±)-chlorpheniramine was able to reduce this 'in vivo' ODC induction by 70% (Table I). However, at day 11 after tumour inoculation, when tumour growth has

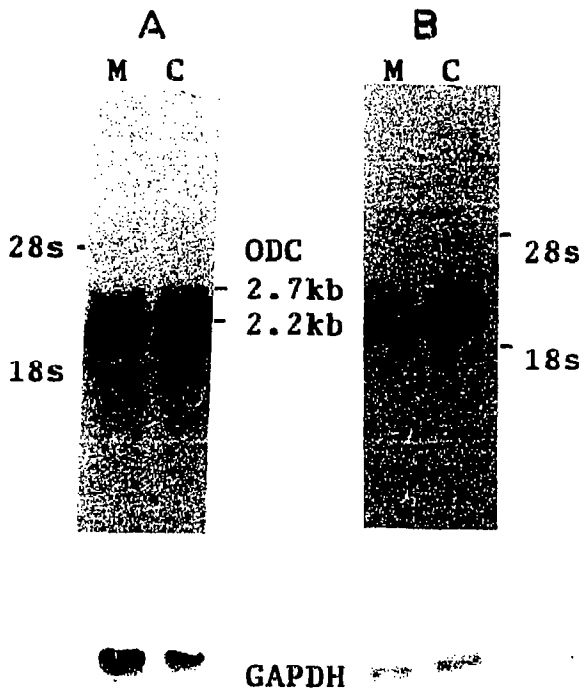

Fig. 2. RNA blot analysis of ODC mRNAs of Ehrlich tumour cell growing in vivo, (A) day 7, and (B) day 11 after tumour inoculation. Experimental conditions are explained in section 2; (M) RNA of cells growing in maleate-treated animals; (C) RNA of cells growing in chlorpheniramine-treated animals from day 3 after tumour inoculation. The signal for control mRNA (GAPDH) is shown below each ODC autoradiogram, and it was obtained by rehybridization of the same filter.

stopped and ODC is the lowest detected 'in vivo' [19,20]; the antihistamine had a very weak effect.

ODC induction by natural growth stimuli is related to increased transcription and translation rates of their

Table I

Total tumour cells, ascitic fluid volume and total tumour ODC activity in mice treated daily with 0.2 mg of maleate (control) or 0.5 mg of (±)-chlorpheniramine from day 3 after tumour inoculation

Parameters	Control	(±)-chlorpheniramine
Total tumour cells (10^6 cells)		
Day 7	992 ± 93	1013 ± 96
Day 11	1857 ± 175	975 ± 148*
Ascitic fluid volume (ml)		
Day 7	1.93 ± 0.19	2.13 ± 0.26
Day 11	7.04 ± 0.47	4.29 ± 0.71**
Total tumour ODC activity (nmol CO_2/h)		
Day 7	10.49 ± 2.99	2.99 ± 0.51*
Day 11	4.38 ± 1.04	1.81 ± 0.36*

The Mann-Whitney *U* non-parametric test was used to estimate the degree of statistical significance.
*$P<0.005$ vs. maleate control values
**$P<0.01$ vs. maleate control values

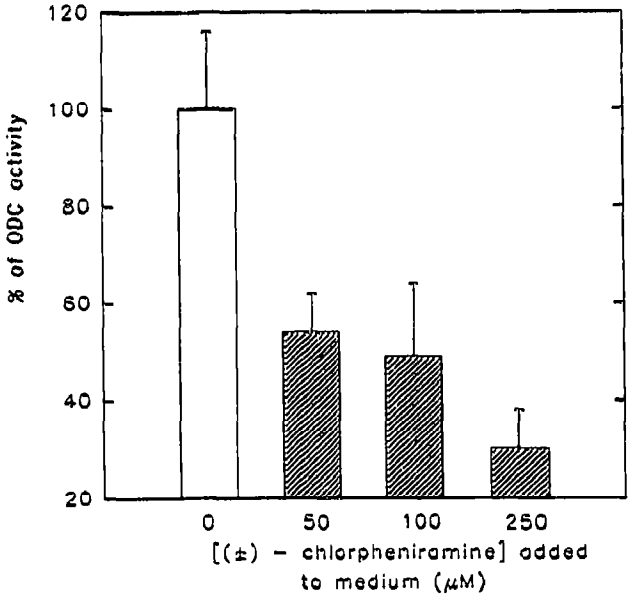

Fig. 3. Effect of (±)-chlorpheniramine on the ODC activity of cultured Ehrlich carcinoma cells. At 4 h after seeding, chlorpheniramine was added to cultures reaching final concentrations of 50, 100 and 250 μM. Cells were further cultured for 4 h, and then removed for ODC activity assays. Results are expressed as the percentage of ODC activity in respect to control cultures (without chlorpheniramine added). Data are mean of 4 different assays ± S.E.M.

mRNAs [2–4]. Since chlorpheniramine showed a more marked effect on ODC during the 'in vivo' exponential phase of growth, the effect of the antihistamine on the tumor ODC mRNA levels was tested. A representative result is shown in Fig. 2. The signal intensities for ODC mRNA versus control mRNA calculated from several Northern blot experiments did not show differences in ODC mRNA levels between controls and chlorpheniramine treated animals at day 7 or 11 after tumour transplantation.

The effect of the antihistamine on the ODC synthesis and degradation was tested on Ehrlich cultured cells. Control cells were maintained for 8 h in serum-free medium. After this time, ODC activity (0.38 ± 0.09 nmol CO_2/h/10^6 cells) was 164-fold induced with respect to seeded cells. The presence of 50–250 μM (±)-chlorpheniramine from the 4th hour of culture (lag phase of growth) to the 8th hour (the beginning of the exponential phase of growth) led to a marked reduction in the ODC activity ranking from 50–80% of the control values (Fig. 3). The estimated ODC half-life values were very similar for controls and 250 μM chlorpheniramine-treated cultures. After 4 h of treatment, 250 μM (±)-chlorpheniramine did not affect either total protein content or total protein synthesis rate. However, differences are observed in specific ^{35}S incorporation into ODC between controls and chlorpheniramine treated cultures (Table II). As Heby and Persson pointed out, ODC protein represents a minimum percentage of the total

protein content [1]. The decrease in relative ODC synthesis caused by 250 μM chlorpheniramine agreed with the observed decrease in ODC activity (Fig. 3).

4. DISCUSSION

The present work validates results obtained from chlorpheniramine with perifused tumour cells [11]. In previous works, it was suggested that histamine and other 1,4-diamines, at micromolar concentrations, mimic the effect of natural polyamines suppressing the ODC induction caused by ornithine in perifused Ehrlich ascites tumour cells; these 1,4-diamines were more effective for ODC induction inhibition than other amines having 1,3- and 1,5-diamine structures [10,11]. Multiple mechanisms have been proposed to take place during the ODC feedback regulation by natural polyamines: inhibition of the protein synthesis initiation and/or elongation [5,21], increase in ODC inactivation and/or degradation [6,22], and induction of antizyme [23]. Nevertheless, Kahana and Nathans conclude that these effects are not specific for polyamines, other diamines are also able to mimic natural polyamines inhibiting ODC synthesis [8].

In agreement with the mechanism proposed for other diamines, the inhibitory effect of chlorpheniramine must be related to post-transcriptional regulatory mechanisms, since ODC mRNA levels were not changed by chlorpheniramine. The results obtained after inhibition of protein synthesis with cycloheximide do not support an effect of (±)-chlorpheniramine on ODC degradation. In this sense, chlorpheniramine differs from natural polyamines when used at micromolar range concentration on culture cells [5,6]. However, results obtained from ^{35}S incorporation seem to indicate that chlorpheniramine inhibits the ODC induction by affecting specifically de novo synthesis of the enzyme, as natural polyamines do at translational level [5,8,17].

On the other hand, the shortage in life-span caused by higher doses of chlorpheniramine could be related to secondary effects of the antihistaminic on the host: decrease in vascular permeability and immunosuppressor [24,25]. In spite of the secondary effects reported for the antagonists of H_1-receptors, these data could be useful for the design of new and less toxic histamine analogs [26] with inhibitory effects on ODC. Actually, treatment with histamine or serotonin has an inhibitory effect on the progression of several experimental and human tumors [24,27]. The delay between the inhibition of ODC by chlorpheniramine and the reduction of tumour growth agrees with data reported by other authors for this and other tumour models, since the treatment with an ODC inhibitor induces multiple compensatory mechanisms in polyamine uptake and biosynthesis [28,29]. Nevertheless, the characterization of ODC inhibitors is interesting not only for therapeutic purposes, but they are also considered valuable tools in basic Sciences [30].

Table II

Changes in cell viability, total protein content and synthesis, and ornithine decarboxylase half life and synthesis caused by 250 μM (±)-chlorpheniramine in Ehrlich cultured cells after 4 h of treatment

Parameters	Control	(±)-chlorpheniramine	Effect (%)
% Cell viability	88.3 ± 2.6	83.3 ± 3.6	94
Total protein content (μg/10^6 cells)	272 ± 33	281 ± 20	103
Total protein synthesis rate[a] (cpm/μg prot./min)	1389 (r = 0.96)	1341 (r=0.99)	97
ODC half life[b] (min)	49 (r = −0.96)	54 (r = −0.95)	110
Relative ODC synthesis (%)[c]			
20 min labelling	5.59×10^{-3}	0.96×10^{-3}	17
30 min labelling	3.65×10^{-3}	1.61×10^{-3}	44

r = correlation coefficient obtained from linear regression analysis.
[a] Measured by the radiolabel incorporation in total extract protein.
[b] Obtained after representing the percentage of ODC activity remaining vs. time.
[c] Ornithine decarboxylase synthesis is expressed as the percentage of the specific radiolabel incorporation in ODC relative to the total protein synthesis (see section 2 for details).

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