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Sensitivity of the Hormone Dependent MXT-Mouse Mammary Carcinoma to Estradiol during Tumoral Growth. An Autoradiographic Study

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Abstract—*The MXT transplantable mammary tumor is an ovarian sensitive carcinoma of the B6D2F1 mouse strain. The present work documents the mitogenic effect of a single near-physiological dose (pulse) of estradiol (E2) in castrated mice bearing MXT neoplasms 2–7 weeks after their inoculation. Histological sections of tumors and uteri (as control E2 target organs) were processed and nuclear thymidine labeling indices (TLI) were determined by autoradiography. Tumor estrogen receptors contents were evaluated in parallel. The results indictate that the MXT tumor keeps a constant sensitivity to E2 6 weeks after implantation.*

INTRODUCTION

THE MXT mammary tumor of the mouse is a cancer model initially developed by Watson *et al.* [1]. This tumor arose on a urethane-treated female mouse of the C57BL × DBA2f/F1 strain (B6D2F1) carrying a pituitary isograft under the renal capsule. It can be maintained by serial transplantation in adult female mice and contains significant amounts of estrogen receptors (ER) [2].

Growth of this tumor is diminished markedly if animals are castrated a few days prior to their implantation [3]; administration of estradiol (E2) alone [4] or combined with medroxyprogesterone acetate (MPA) [5] abolishes this effect. On the contrary, if castration is performed 4 weeks after tumor implantation, tumor growth seems less affected [6]. The present study was undertaken in order to characterize this apparent loss of hormone sensitivity.

The test for studying E2-sensitivity is the measure of the increase of the nuclear incorporation of tritiated thymidine produced by a single injection of a 1×10^{-6} m E2, 6 days after castration. It was shown in previous experiments that maximum stimulatory effect of E2 on cell proliferation occurred 24 hr after its injection [7]. In the present study, this challenge test was performed on tumors arising from the same transplant, from 2 to 7 weeks after grafting. The ER tumoral content was measured in parallel.

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MATERIALS AND METHODS

Chemicals

Estradiol 17-beta was obtained from Sigma, MO. All solutions used for injection were prepared extemporaneously by appropriate dilution with sterile saline (NaCl, 9.0 g/l) of a stock ethanolic solution containing 2.7 mg E2 per ml and maintained at 4°C.

Tritiated thymidine (methyl 3H-thymidine: SA 48 Ci/mmol) was purchased by Amersham International, U.K. The solutions to be injected were prepared extemporaneously by adequate dilution with sterile saline.

Animals and tumor transplantation procedure

The original MXT mouse mammary tumor was obtained from Dr. A.E. Bogden (Mason Research Institute, Worcester, MA). The tumor was maintained in our laboratory by regular transplantation performed every 4 weeks in 8–10-week-old female B6D2F1 mice. At each passage, several (± 10) tumors of about 1 cm^3, without visible areas of necrosis, were selected and dissected under sterile conditions. Systematically, fragments of each tumor were taken for histological examination and

estrogen receptor (ER) measurement. Remaining mouse tissues were pooled and minced into 15 mm^3 pieces. In each mouse, two fragments were inoculated subcutaneously, one into each flank, through a trochar (ga. 13).

The present experiments were performed on the 10th generation of transplants grown in our laboratory. All tumors displayed the characteristic histological pattern of a well-differentiated ductal papillary adenocarcinoma [8]. All mice were housed throughout the study in cages at constant temperature (24 ± 1°C) and light exposure (14 hr of light and 10 hr of dark). Food and water were provided *ad libitum*.

Experimental schedule

One hundred female adult mice weighing approx. 25 g each underwent tumor inoculation on the same day and were randomly allocated into 10 groups of 10 animals each.

Two, four, five, six and seven weeks after tumor inoculation, the body wt of the animals was recorded and tumors were measured with a caliper. Their individual size was expressed as a surface (mm^2) which corresponded to the product of the largest two perpendicular axes.

All animals were ovariectomized 6 days before evaluating the effect of E2 on the 3H-thymidine uptake. Twenty-four hours prior to killing, the controls (groups A) received an intraperitoneal (i.p.) injection of 0.1 ml saline, whereas the E2-treated mice (groups B) received 0.1 ml i.p. of a saline solution containing 0.25 μg E2. One hour prior to being killed, all animals received 0.1 ml (25 μCi) of 3H-TdR by i.p. injection. At the scheduled time for killing (i.e. 2, 4, 5, 6 and 7 weeks post-inoculation), animals were killed within a short time by cervical dislocation. All MXT tumors were subsequently removed and processed for histological examination. Uteri were also systematically taken and similarly processed as normal estrogen target organ and control of the methodology.

Histological procedure and autoradiography

Immediately after sacrifice, mammary tumors and uteri were removed and fixed in EFA [ethanol (96°) 75 vol., neutral formol (40%) 20 vol. and glacial acetic acid 5 vol.]. After histological processing and paraffin embedding, 4 μm sections were sliced. Tumor sections were systematically made through the center of the tissue and assembled by two or three per slide, depending on their size. For uteri, four transversal sections always passing through the midportion of the horns were assembled on a same slide. Subsequently, slides were dipped into Ilford K5 photoemulsion diluted 1 : 1 (v/v) in twice distilled water, air dried and stored at 4°C in a light-tight box for 2 weeks. After development with Dektol (3 min) and fixation in sodium thiosulfate (2 × 15 min), autoradiographs were stained by the methyl-green pyronine method and mounted.

For each tumor and uterus, two slides were selected for the assessment of the thymidine labeling index (TLI) which represented the percentage of cells of a given type with labeled nuclei. Nuclear labeling was considered positive when a nucleus contained at least five silver grains. Counts were made on a fixed number of cells taken in representative regions of the tissue. In tumors, microscopic fields were randomly selected, three in the periphery and three in the center, amounting to an average of 3000 neoplastic cells analyzed per tumor. In uteri, the TLI of luminal epithelium, glands and stroma were separately recorded. For the former two regions (epithelium and glands), 300 cells of each type were analyzed whereas for the latter (stroma) which exhibited a lower average degree of labeling, counts were made on 1000 cells. All slides were identified only by a code number. They were examined by the same investigator (R.K.), who ignored the corresponding experimental conditions.

Estrogen receptors measurement

ER concentrations were determined by measuring the binding capacity of the tumor cytosols for 3H-estradiol by the dextran-coated charcoal method previously described [9]. It was expressed in femtomoles per mg of protein. Histology of the tumors was always checked, to ensure the presence of neoplastic cells.

Statistical analysis

Results are given as mean ± Standard Error on the Mean (S.E.M.). Statistical comparisons of data were performed by using the Fischer F test (variance analysis) or the 'test for trend' [10].

RESULTS

Growth pattern of the tumors and histologic features

Mean tumor growth was about 25 mm^2/week from week 2 to 4, 40 mm^2/week from week 4 to 5, 140 mm^2/week from week 5 to 6 and 30 mm^2 from week 6 to 7. So, growth accelerates from week 2 to 6 and deccelerates during the last week (Chart 1).

During the entire experiment, no tumor regression was observed and all animals remained in good condition except in the group taken 7 weeks after implantation where two controls and two E2-treated animals died. The mean wts (± S.E.M.) registered on the sacrifice day were almost identical in the five groups, i.e. 2 weeks: 24.2 ± .6 g; 4 weeks: 23.8 ± 0.8 g; 5 weeks: 23.9 ± 0.7 g; 6 weeks; 24.4 g ± 0.9 and 7 weeks: 24.5 g ± 0.7.

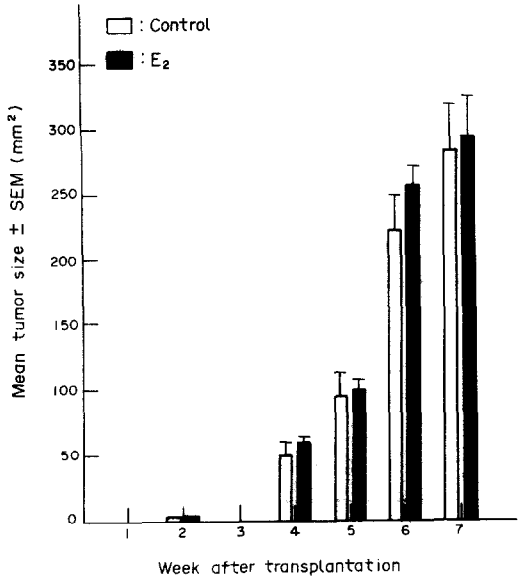

Chart 1. Growth pattern of the 10th *MXT transplant. Each block represents the mean (± S.E.M. = bars) tumor size recorded in group of* 10 *mice killed at various times after tumor implantation. All animals were ovariectomized* 6 days *prior to sacrifice. Open columns = control animals* (0.1 ml *saline* 24 h *prior to sacrifice); block columns = E2-treated animals* (0.25 μg *E2 in* 0.1 ml *saline, i.p.* 24 hr *prior to sacrifice).*

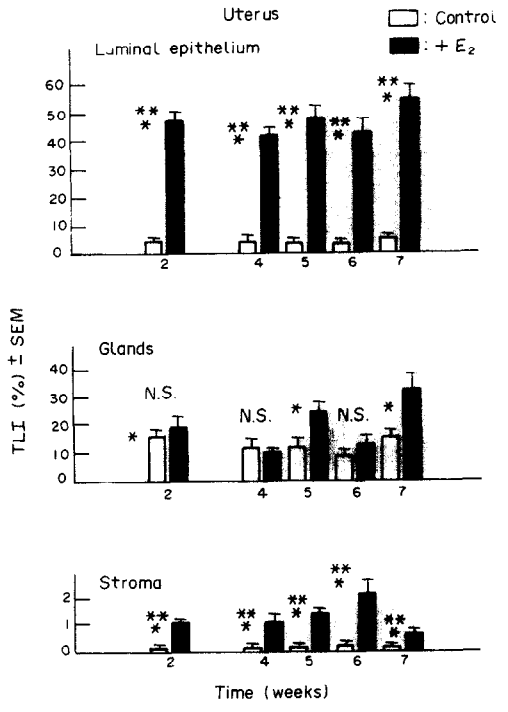

Chart 2. Mean TLI values recorded in uteri *from controls and E2-treated mice. Counts were separately recorded for luminal epithelium, glands and stroma. Each block represents mean (± S.E.M. = bars) TLI of* 10 *uteri from* 10 *animals subjected to identical experimental conditions. Controls received i.p. saline, whereas 'stimulated' animals received* 0.25 μg *i.p.* (0.1 ml) 17*-beta-estradiol. Variance analysis (Fischer F test) was used to compare mean controls and mean E2-stimulated values recorded in tumors of the same age (N.S.: not significant;* *: $P < .05$; **: $P < .01$; ***: $P < .001$).

Tumors younger than 2 weeks were almost undetectable during careful dissection. The histological picture exhibited by the tumors from the 2nd to the 6th week after their transplantation was that of an adenocarcinoma not different as compared to the parent tumor. At the seventh week after transplantation, all tumors contained widespread areas of necrosis; this necrotic tissue often extended to the periphery of the tumor and in larger tumors caused ulceration through the skin. Therefore TLI from these elderly tumors was not included in the data.

Effects of E2 pulse on uteri

As shown in Chart 2 the mean basal nuclear incorporation of 3H-TdR (TLI) were consistently low in castrated mice and marked differences in sensitivity to E2 exist among the three endometrial tissues. As illustrated by Figs. 1A and B, the most E2-sensitive tissue was the luminal epithelium where a dramatic increase of TLI was consistently observed 24 hr after estradiol injection. This phenomenon occurred at each time throughout the whole experiment with almost the same intensity (i.e. ± 10-fold E2-induced increase in TLI over basal values). The stroma was also highly significantly stimulated by E_2 but to a lower extent than the luminal epithelium. The glands were weakly or not at all stimulated, the level of statistical significance of the differences observed between the castrated- and E2-stimulated conditions never exceeding 5%.

Effects of E2 pulse on tumors

As illustrated in Figs. 2A and B, TLI in tumor cells was significantly higher in E2-stimulated mice than in controls. In both groups (A and B), mean values gradually increased from 2 to 6 weeks after inoculation (Chart 3). A plateau was reached at 6 and 7 weeks (B6 vs B7: $P > 0.05$). Moreover, the TLI ratio between E2-stimulated and non-stimulated tumors, aged from 2 to 6 weeks, remained almost constant (± 1.6) as for the uterine luminal epithelium (± 10).

In Chart 4, we have classified the control and E2-treated tumors into four arbitrary classes corresponding to low (I: TLI < 5%), intermediate (II: 5% ≤ TLI < 10%), high (III: 10% ≤ TLI < 15%) and very high (IV: TLI ≥ 15%) labeling indices respectively. Hence, E2 dramatically reduces the number of tumors belonging to class I (46–14%, $P < 0.01$), keeps constant the number of tumors of class I (48–54%, NS) and increases about 4 times the number of tumors of class III (6–23%, $P < 0.02$) (Table 1). Moreover E2 led to the appearance of tumors with very high TLI not observed in the controls (class IV: 0–9%, $P < 0.05$). On the other hand, E2-stimulated and unstimulated tumors appeared continuously distri-

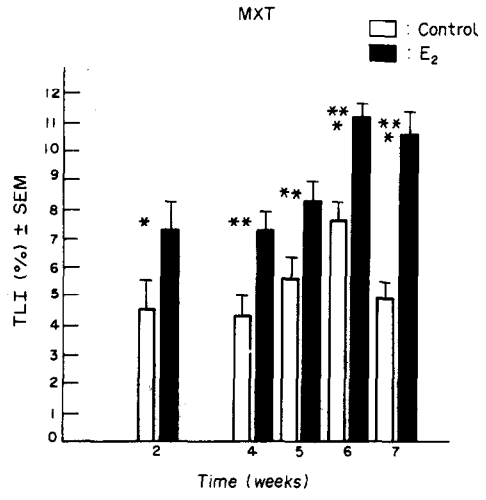

Chart 3. *Mean TLI values recorded in MXT mouse mammary tumors in controls and E2-treated mice from* 2 *to* 7 weeks *after transplantation. The experimental groups comprise* 10 *animals each and correspond to those described in Chart* 1.

buted suggesting that the hormone increased TLI in every tumor, whatever its basal value.

ER content of MXT tumors

On the average, pooled fragments of the tumors used as source of implants for the present experiments (10th transplant generation) contained 40 fmol ER/mg protein. This concentration was similar to that found in the former (9th: 55 fmol ER/mg protein) and subsequent (11th: 64 fmol ER/mg protein) transplant generations. The latter was used to evaluate the possibility of ER variation among tumor growth (up to 7 weeks after implantation). For that purpose, ER levels were assessed on pooled tumors taken 3, 4, 5, 6 and 7 weeks after their implantation; assays were not feasible at an earlier time in view of the lack of palpable lumps. During this period, ER levels remain constant (39 ± 4 fmol/mg protein).

Table 1. *Distribution of tumors according to TLI classes * in control and E2-treated animals*

Class	I		II		III		IV		Total
Control	30	75%	31	45%	4	20%	0	0%	65
+ E2	10	25%	38	55%	16	80%	6	100%	70
Total	40	100%	69	100%	20	100%	6	100%	135

* Class I: 0% < TLI < 5%; II: 5% ≤ TLI < 10%; III: 10% ≤ TLI < 15%; IV: TLI ≥ 15%.

Statistical significance ('test for trend'): Control vs E2: $P < 0.05$; I control vs IE2: $P < 0.01$; II control vs IIE2: $P > 0.05$ (NS), III control vs IIIE2: $P < 0.02$, IV control vs IVE2: $P < 0.05$.

DISCUSSION

In the present work, the sensitivity of the MXT mammary tumor to a physiological dose of E2 was investigated throughout its growth by means of a tritiated thymidine autoradiographic technique. As control for the methodology, we used the uterus, a well-known estrogen target organ. The low TLI values found in all three endometrial regions, i.e. the luminal epithelium, the glands and the stroma, in spayed mice along with the marked TLI increase in the luminal epithelium and to a lesser degree in the stroma after a 24-hr E2 injection

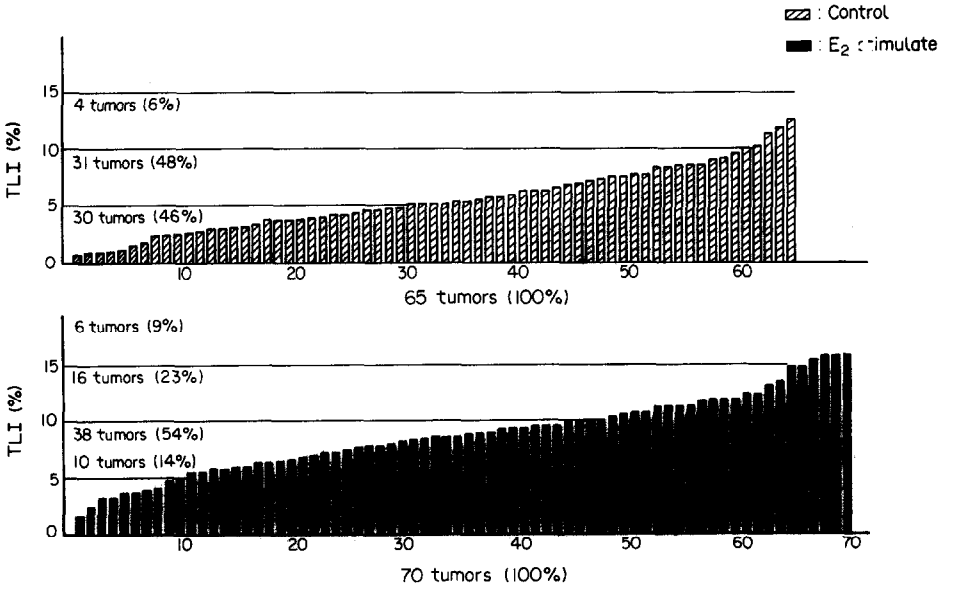

Chart 4. *Individual MXT tumors of various ages* (2–7 weeks) *are ordered by increasing TLI values, in controls* (*top figure*) and E2-*treated mice* (*bottom figure*). *The horizontal lines allow to separate arbitrarily these tumors in four classes, according to their TLI* (*class* I: *TLI* < 5%; *class* II: 5% ≤ *TLI* < 10%; *class* III: 10% ≤ *TLI* < 15%; *class* IV: *TLI* ≥ 15%).

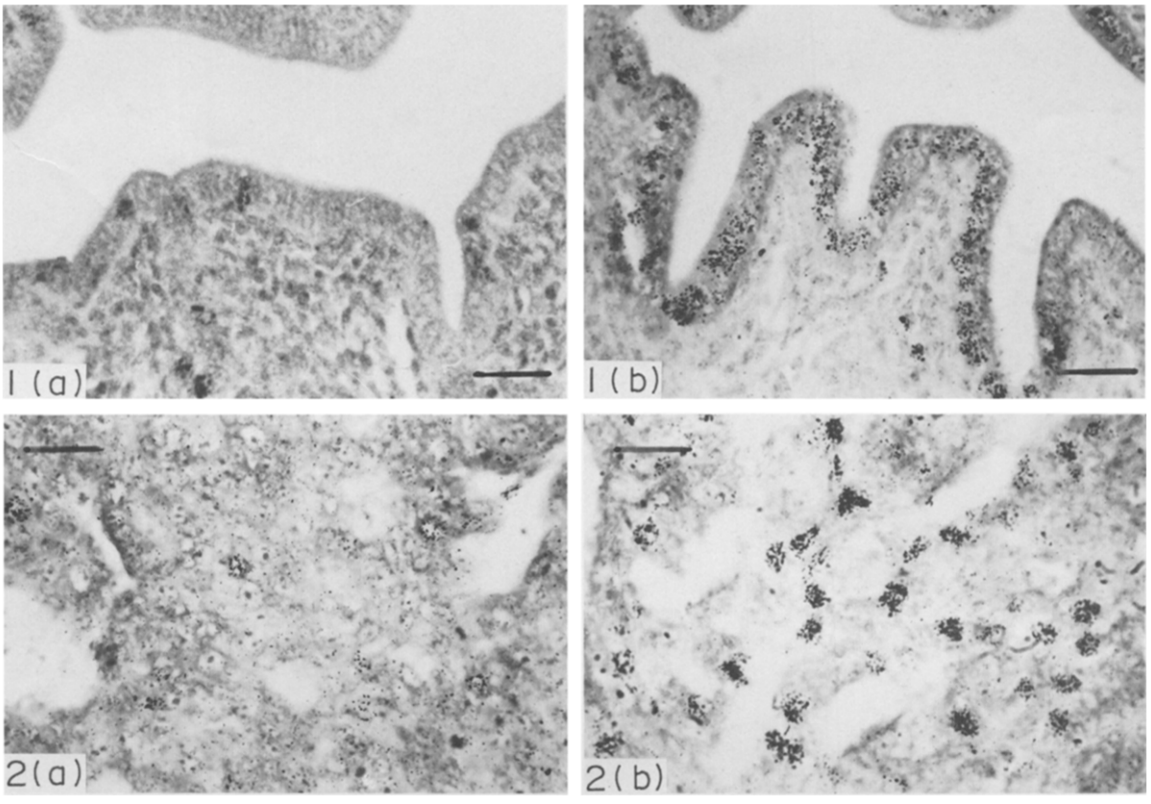

Fig. 1. *Autoradiographs of the endometrium in two spayed mice, killed* 1 hr *after i.p. injection of* 3H-*thymidine* (1 μCi/g *animal*). A = *control animal* (0.1 ml, *saline i.p.* 24 hr *prior to sacrifice*); B = *estradiol-treated animal* (0.25 μg *E*2 *in* 0.1 ml *saline i.p.*) 24 hr *prior to cervical dislocation. Bar* = 10 μm.

Fig. 2. *Autoradiographs of MXT mammary tumors in two spayed mice killed* 1 hr *after i.p. injection of* 3H-*thymidine.* A = *control animal*; B = *E*2-*treated animal* (0.25 μg*E*2 *i.p.*, 24 hr *prior to killing*). *Bar* = 10 μm.

document the trophic action of the hormone on this organ (Chart 2). This trophic effect, in agreement with observations from our group (6) and others (11–14), ensures the validity of our autoradiographic methodology.

MXT growth seems to be characterized by several phases: a latency located around the first 4 weeks (A2 vs A4: N.S.), an exponential proliferation phase between the 4th and the 6th week and a plateau at the end of the 6th week. Indeed at the 7th week, the mean basal TLI is significantly lower than that observed at 6 weeks (A6 vs A7: $P < .05$).

In tumors aged 2–6 weeks, the mean TLI ratio (stimulated/basal) remains constant (Chart 3). This ratio is approx. 5-fold higher in the luminal epithelium. Therefore, it appears that the MXT neoplasm keeps its estrogen-sensitivity constant during this period of growth. This assumption is in good agreement with the finding of stable ER concentrations in these tumors during the process of ageing. Whether or not this characteristic is also valid for younger (< 2 weeks) or older (> 6 weeks) tumors is unknown. Indeed, tumors younger than 2 weeks could not be studied because they were macroscopically undetectable. Similarly, wide necrotic areas in 7-week-old tumors provided unsuitable material for TLI assessment. In the latter, proliferative indices were highly variable among different areas, their TLI values being positively correlated with distance from necrotic areas.

Tannock summarized evidence which suggests three possible causes for the development of necrosis in tumors. Cells may die from lack of glucose, from lack of oxygen, or from an injurious effect of high concentration of lactic acid [15]. Laird [16,17] suggests that growth of tumors in a host animal meets active resistance rather than a failure of the blood supply, an accumulation of toxic by-products [18,19] or chalone [20]. The latter might consist of an immune response, by a 'feedback' control exerted by the whole organism on the growth of its parts, to which the tumors are still responsive to a greater or lesser extent [21].

Our observations clearly indicate that the sensitivity to E2 of MXT tumors transplanted in intact mice is not affected by the process of ageing. This feature is quite different from what we observed previously when tumors were implanted in castrated animals [6,7]. Indeed, under this condition, intact and castrated mice develop two distinct tumor phenotypes, the one hormone-sensitive, the other unsensitive. These two phenotypes arising from a same tumor transplant, one may logically assume that the latter could be composed of at least two different cell populations.

Under light microscopy, the MXT tumor strain used here (previously called 'MXT BOG. I') [8], appears homogeneous and different cell subpopulations are not identified. However, these apparently similar cells should possess different characteristics regarding their differentiation stages [22–24]. As a matter of fact, at least three distinct MXT tumor types characterized by distinct morphological and biochemical (ER content) features have already been described in our laboratory [8]. One of these ('MXT BOG. II') consists of a mixture of two cell populations, i.e. polygonal cells sensitive to E2 and spindle-shaped cells, insensitive to E2 [25].

All these observations led to the conclusion that the MXT mouse mammary neoplasm, although apparently homogeneous from the morphological point of view, contains in fact estrogen-dependent and -independent cells. This cancer keeps its sensitivity to estradiol during the first 6–7 weeks (comprising latency, exponential phases and the plateau) of its growth. This neoplasm might consist of a mixture in variable proportion of E2-dependent and E2-independent cancer cell populations, both co-existing in a dynamical equilibrium during the whole experiment.

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