Influence of Pituitary Grafts or Prolactin Administrations on the Hormone Sensitivity of Ovarian Hormone-independent Mouse Mammary MXT Tumors

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ABSTRACT

The sensitivity of MXT mouse mammary tumors to ovarian hormones was assessed by the following parameters: growth in vivo; presence or absence of estrogen receptors and progesterone receptors; and histological differentiation. A spontaneous evolution from hormone sensitivity (HS) to hormone independence (HI) was observed when the tumors underwent monthly transplantation onto intact recipient mice, with the tumors fulfilling the criteria of HI tumors after the 12th transplantation. In contrast, the tumors recovered most of the criteria of hormone sensitivity when pituitary isografts were placed under the kidney capsules of HI tumor-bearing animals or when these animals received daily administrations of prolactin over several months. Sensitivity to 17β-estradiol, progesterone, or prolactin was further assessed by actinomycin binding on the nucleus and thymidine labeling index, both measured by autoradiography. These technical approaches revealed that 17β-estradiol and prolactin induced a stimulation of thymidine labeling index of both HI (despite the lack of detectable estrogen receptors) and HS MXT tumors whereas progesterone influenced only that of HS cancers. The three hormones significantly stimulated [3H]actinomycin D binding within HS tumors but not within HI ones. However, such “HI” tumors were characterized by increased actinomycin binding and thymidine labeling index in comparison with HS neoplasms. Thus, all the data presently reported strongly suggest that prolactin is able to restore the hormone-sensitive phenotype within so-called MXT hormone-independent tumors.

INTRODUCTION

About one-third of the patients with advanced breast cancer respond to hormonal manipulations (1, 2), and “hormone-sensitive” tumors have thus been the subject of considerable interest, particularly since their growth could be controlled. However, as argued by Briand (3), although HS4 tumors may often regress upon deletion of the correct hormone(s), most regressing tumors spontaneously resume growth after partial regression (4, 5), and the regrowing tumor is “hormone independent.” The late regrowth of a tumor may be explained in terms of the result of the cell heterogeneity of the original tumor (6, 7) or by the fact that dormant HS tumor cells may convert to HI cells that proliferate without hormonal stimulation (3, 8). Furthermore, sometimes during aging, HS tumors may also spontaneously evolve into HI tumors (9, 10), a feature also observed in the MXT model (5) the HS variants of which were sensitive to estradiol (11, 12), progesterone (13, 14), prolactin (15), and gonadoliberin-releasing hormones (16).

In the experiments described in this work we tried to restore HS conditions in HI tumors by pituitary isografts, thus expecting to supply continuous stimulation by prolactin. The daily administration of prolactin instead of pituitary isografts was also tried. Our initial hope was to stimulate “dormant” HS cells in the HI tumors and thus to transform HI tumors into HS tumors. Hormone sensitivity was assessed by the following parameters: the influence of hormones on the growth of a tumor in vivo; histological typing; and the assay of ER and PgR. In another series of experiments, sensitivity to ovarian hormones (17β-estradiol and Pg) and prolactin was confirmed by the autoradiography of tritiated [3H]AMD fixation onto the genomic material; and the autoradiography of [3H]dThd nuclear labeling.

MATERIALS AND METHODS

Chemicals

17β-Estradiol and Pg were purchased from Sigma Chemical Co. (St. Louis, MO) and prolactin (P-5-3 ovine) was obtained from the NIH (Bethesda, MD). All the solutions used for administration were prepared extemporaneously by appropriate dilution with sterile saline (9.0 g NaCl/liter).

[3H]dThd (specific activity, 42 Ci/mm mol), [3H]AMD (specific activity, 8 Ci/mm mol), tritiated ORG-2058 (specific activity, 50 Ci/mm mol), and tritiated estradiol (specific activity, 100 Ci/mm mol) were purchased from the Radiochemical Centre (Amersham, England). The solutions to be injected ([3H]dThd) were prepared extemporaneously by means of adequate dilutions of sterile saline.

Tumors and Animals

The original MXT mammary tumor of the C57BL x DBA/2F1 (hereafter called B6D2F1) mouse strain is a transplantable s.c. model initially developed by Watson et al. (17) on an urchen-treated female carrying a pituitary isograft under the renal capsule. The HS-MXT variant used here was obtained from Dr. M. Schneider (Naturwissenschaftliche, Fakultät IV, Universität Regensburg, West Germany); it displayed the feature of a well-differentiated adenocarcinoma and was identical (histopathology, growth pattern, ER and PgR contents, etc.) to those of the MXT BOG.1 T1-T10 strain described previously (5). The tumor was maintained in our laboratory by regular transfers performed monthly on female B6DF1 mice between 8 and 12 weeks old (21–23 g; Iffa-Credo, Lyon, France). In each transfer 10 tumors about 1 cm2 each were pooled and minced into 10-mm3 pieces under sterile conditions.

For each passage, over a 2-year period corresponding to 24 passages, MXT tumor size (measured weekly with calipers and expressed as “area” by multiplying the two perpendicular diameters), histopathology (Bouin’s fluid fixation and hematoxylin-eosin stain) and ER and PgR measurement (assessed on material stored in liquid nitrogen; see “ER and PgR Determinations”) were determined on tumors bilaterally grafted onto 5 intact mice (ITC) and 5 mice ovariolectomized 15 days postgraft (OO). It must be pointed out that all the “intact” mice underwent a sham operation, i.e., a bilaterally surgical operation without castration. At the 12th passage (T-12) the tumors possessed HI properties (see “Growth of the Tumors”) and the same biological markers as above were determined not only on 5 ITC and 5 OO animals but also on 5 animals carrying pituitary isografts placed under the renal capsule 1 week prior to the transplantation (IPI) and 5 IPI-OO animals. Finally, between the 30th (T-30) and the 34th (T-34) passages we...
studied the evolution of the growth and histopathology of HI MXT tumors grafted onto intact mice receiving 10 μg of prolactin/mouse daily. Sensitivity to ovariectomy was studied on tumors (arising from PRL-treated mice) grafted onto intact mice that were not given injections of PRL but castrated 15 days postgraft.

ER and PgR Determinations

The estrogen and progesterone receptors were assayed on tumor samples of 0.5-1 cm³. The binding capacity of the cytosolic fraction of the tumors for [3H]ORG-2058 and [3H]estradiol was measured according to the traditional dextran-coated charcoal method (18, 19). All the results were expressed in fmol/mg protein.

Fixation of [3H]AMD onto the Genomic Material

Experimental Schedule. Two hundred forty adult female mice were randomly allocated into one group of 120 OVX-I mice and one group of 120 OVX-II mice. All the mice in group OVX-II immediately underwent bilateral oophorectomy (“early-spayed mice”). Six days later, MXT tumors for the T-I passage (see “Tumors and Animals”) were bilaterally implanted in all the animals of the two groups on the same day (= day 0) and each group was further divided into four subgroups (Ct, 17β-estradiol, Pg, and PRL) of 30 mice each. The mice in group OVX-I were spayed 36 days (= day 36) later (“late-spayed mice”) in order to eliminate the endogeneous influence of ovarian hormones. These treatments were performed to promote the development of HS tumors in the OVX-I mice and according to a procedure described previously (11) HI tumors in the OVX-II mice. All the tumors thus corresponded to the same passage and were of the same age. The Ct mice received the vehicle alone i.e., an i.p. injection of 0.1 ml saline (NaCl, 9.0 g/liter); the animals in the 17β-estradiol and Pg groups received an i.p. injection of 0.1 ml of saline solution containing 0.25 μg 17β-estradiol or 125 μg Pg, respectively, and those in group PRL received 10 μg of ovine prolactin. The 30 mice in each group were killed in lots of 5, 5, 20, 40, 60, 180, or 360 min, respectively, after the placebo or the hormonal injection. Immediately after death (cervical dislocation occurring on day 42), the MXT tumors were dissected, rinsed in saline, and fractionated into two parts: one was stored in liquid nitrogen while awaiting dosage procedure; and the other was fixed for histology and processed as described below.

Histological Procedure and Autoradiography. Immediately after removal the MXT tumors were fixed for 45 min at 4°C in ethanol:acetic acid, 5:1 (v/v). The material was dehydrated, embedded in paraffin, and sliced into 3-μm sections: three samples of each tumor were placed on the same slide; and two slides were analyzed per tumor. A sample intended for routine histology was stained with hematoxylin and eosin. Histological slides were kept in the dark for 1 h. 4-fiin sections were prepared and dipped into Ilford K5 photoemulsion, diluted 1:3 (v/v) in bidistilled water, air dried, and stored at 4°C in a lightproof box for 20 days. The autoradiographs were then developed, stained, and mounted as described under the previous section called “Histological Procedure and Autoradiography.” Four sections were systematically made for each tumor piece. TLIs, which represent the percentage of neoplastic cells labeled with nuclei, were assessed in 250 neoplastic cells counted per tumoral section. Thus, a total of 1000 neoplastic cells was counted per tumoral piece, and a total of 5000 neoplastic cells per tumor was counted per experimental condition. Nuclear labeling was considered positive when a nucleus contained at least 5 silver grains.

Statistical Analyses

Results are given as mean ± SEM; statistical comparisons of data were performed by using the Fisher F test (one-way analysis of variance). Normal parameter distribution fitting was assessed by the χ² test and variance homogeneity was verified by the Hartley test.

RESULTS

Growth of the Tumors. Data are given in Figs. 1 and 2. Fig. 1 shows that, at the first passage (T1), tumor growth was slower in animals that were ovariectomized 15 days postgraft than in intact controls. The T-1 hormone-sensitive group grafted onto intact animals appeared to grow larger at each point (Fig. 1). Each “intact” animal nonetheless underwent a sham operation (see “Materials and Methods”) that did not influence the growth of the MXT model since we observed no statistically significant differences between the group of MXT tumors grafted onto the sham-operated animals and the tumors grafted onto the really intact mice, i.e., the animals not sham-operated (data not shown in Fig. 1 for the sake of clarity). Such evidence of hormone sensitivity was no longer observed either at the 12th passage (T-12) or later (T-18, T-24) except in animals carrying a pituitary isograft placed under the kidney capsule 1 week before tumor transplantation. In these, tumor growth was slower than in the controls and was further depressed by ovariectomy. The apparent recovery of HS properties was observable from the 15th passage, i.e., the 3rd passage on the IPI animals. According to growth patterns, the reversal of HI into HS behavior was also observed with daily administration of PRL instead of a pituitary isograft (Fig. 2). Indeed, this experimental schedule restored significant HS properties within HI MXT tumors as early as two passages following the beginning of prolactin administration (Fig. 2). This recovery of HS properties within HI tumors is consistent with both the slow rate of growth in HI MXT tumors grafted onto intact PRL-treated bearing-mice and the significant influence of castration; this can be compared with HI tumors not treated by prolactin (Figs. 1 and 2).

Histology and Steroid Hormone Receptor Contents. The MXT T-1 tumors grafted onto ITC or OO animals displayed the
**RETURN OF HORMONE-INDEPENDENT TUMORS TO HORMONE SENSITIVITY**

Fig. 1. Comparison of MXT tumor growths (10 tumors/experimental condition) transplanted monthly: T-1 and T-12 passages were grafted s.c. onto intact B6D2F1, mice (●●●, ITC) or onto mice ovariectomized 15 days postgraft (●●●, OO). From the 12th passage, MXT tumors were further grafted on intact animals carrying a pituitary isograft placed under the renal capsule 1 week prior to the transplantation procedure (□□□, IPI) or onto IPI animals castrated 15 days postgraft (□□□, IPI-OO). Statistical comparisons (Fisher F test) versus corresponding control value: T-1 OO versus ITC, P < 0.01 at the 6th week and P < 0.001 at the 7th week; T-12 OO versus ITC, P > 0.05 from the 2nd to the 6th week (also true for T-18 and T-24); T-18 IPI-OO versus IPI, P < 0.01 at the 6th week and P < 0.001 at the 7th week; T-24 IPI-OO versus IPI, P < 0.001 at the 6th and 7th weeks.

**Fig. 2.** Comparison of MXT tumor growths (10 tumors/experimental condition) transplanted monthly (T-31 to T-34) and grafted onto intact B6D2F1, mice (●●●●, ITC) or onto mice ovariectomized 15 days postgraft (●●●●, OO). MXT tumors were further grafted onto intact (□□□□, PRL-ITC) animals treated daily with 10 ng of prolactin from the day of transplantation until death. Tumors arising from the first generation of mice treated with PRL (MXT PRL T-31) were subsequently grafted onto intact PRL-treated mice, defined as MXT PRL T-32, etc. For each passage 10 tumors were also grafted onto intact animals that were not given injections of prolactin but oophorectomized 15 days postgraft (○○○○, CT-OO). The mean value recorded in the CT-OO group was statistically compared (Fisher F test) to the corresponding control value (PRL-ITC). *, P < 0.05; **, P < 0.01. No significant differences were observed for ITC versus OO, whatever the passage used.

Histological features of well-differentiated adenocarcinomas that showed acini varying in size and lined by a single layer of cuboidal or columnar epithelium, as was also the case for all the tumors grafted onto OVX-I and OVX-II mice, i.e., those used for SGN and TLI experiments. The T-12, T-18, and T-24 transplants grafted onto ITC or OO mice presented a characteristic of poorly differentiated adenocarcinoma showing intermingled small glandular elements and poorly differentiated polygonal cells, with the acinar aspect being lost. The T-18 and T-24 transplants, grafted onto IPI or IPI-OO mice, showed the same histopathology as the T-1 tumors, i.e., well-differentiated adenocarcinomas, as was also the case for the T-32 to T-34 passages grafted onto intact mice receiving a daily injection of prolactin. The T-30 passage corresponded exactly to the pattern of the T-12, T-18, or T-24 passages, i.e., a HI pattern, whereas the T-31 passage showed an intermediate HI-HS pattern, with some acini being present and its growth slowly decreasing as compared to that of the T-30 passage. The dedifferentiation of MXT tumors grafted onto IPI animals was observable from the 3rd passage on such IPI recipient mice, i.e., MXT T-15, and from the 2nd passage on MXT T-32 animals given daily injections of PRL.

ER and PgR contents are shown in Table 1. The T-1 passage borne by the intact animals contained significant amounts of ER and PgR; postgraft castration decreased the ER and PgR concentrations while pregraft castration induced ER and PgR loss. The transformation of well-differentiated (T-1) into poorly differentiated (T-18 and T-24) adenocarcinoma was accompanied by a progressive ER and PgR disappearance. Since T-15 ER, but not PgR, appeared again in MXT tumors grafted onto animals carrying a pituitary gland placed under the renal capsule [T-18 and T-24 IPI animals (Table 1)], the daily administration of PRL did not restore significant amounts of ER or PgR within MXT tumors even after several months of treatment (data not shown).

**Influence of 17β-Estradiol, Pg, and PRL on the SGN in HI and HS MXT Tumors.** The binding of [3H]AMD onto the genomic material of histologically fixed tissue submitted to an autoradiographic procedure allows the assessment of SGN. With regard to [3H]AMD binding, the silver grains were always located in the cell nucleus, this feature being consistent with a specific nuclear “staining” of the drug. The background was negligible (<1 grain/nucleus). No correlation was found between nuclear size and [3H]AMD binding.

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Table 1: Estrogen and progesterone receptors in MXT tumors

<table>
<thead>
<tr>
<th>Transplant no.</th>
<th>Experimental condition</th>
<th>Mean ± SEM (range)</th>
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<tr>
<td>T-1</td>
<td>ITC</td>
<td>47 ± 2 (26-71)</td>
<td>90 ± 7 (70-129)</td>
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<tr>
<td></td>
<td>OO</td>
<td>20 ± 4 (9-33)</td>
<td>28 ± 6 (8-45)</td>
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<td></td>
<td>OVX-I</td>
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<td>23 ± 3 (10-38)</td>
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<tr>
<td></td>
<td>OVX-II</td>
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<td>0 ± 0 (0-0)</td>
<td></td>
</tr>
<tr>
<td>T-12</td>
<td>ITC</td>
<td>27 ± 3 (22-36)</td>
<td>3 ± 2 (0-8)</td>
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<tr>
<td></td>
<td>OO</td>
<td>7 ± 3 (4-16)</td>
<td>0 ± 0 (0-0)</td>
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</tr>
<tr>
<td>T-18</td>
<td>ITC</td>
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<td>0 ± 0 (0-0)</td>
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</tr>
<tr>
<td></td>
<td>IPI</td>
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<td>0 ± 0 (0-0)</td>
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</tr>
<tr>
<td></td>
<td>IPI-OO</td>
<td>18 ± 7 (8-35)</td>
<td>0 ± 0 (0-0)</td>
<td></td>
</tr>
<tr>
<td>T-24</td>
<td>ITC</td>
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</tr>
<tr>
<td></td>
<td>IPI</td>
<td>24 ± 6 (13-30)</td>
<td>0 ± 0 (0-0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IPI-OO</td>
<td>17 ± 3 (6-23)</td>
<td>0 ± 0 (0-0)</td>
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As shown in Fig. 3, a highly significant SGN increase was induced by 17β-estradiol or Pg in HS MXT tumors (MXT tumor-OVX-I-bearing mice). A biphasic response seemed to be induced by 17β-estradiol (at 20 and 180 min) in these tumors, whereas Pg or PRL seemed only to induce a transient effect from the 5th to the 40th min after its injection into the mice. This response reached a maximum SGN value 20 mins after the administration of 17β-estradiol, Pg, or PRL (Fig. 3).

In contrast, no hormone-induced modifications were observed in HI tumors (MXT tumor-OVX-II-bearing mice). Moreover, in the case of these tumors, the mean SGN values recorded in the control groups were always significantly higher (P < 0.01) when compared with the corresponding control value of the HS MXT tumors.

As expected, granulocytes were unaffected by 17β-estradiol, Pg, or PRL. As shown in Fig. 4, a marked TLI increase over basal (control) values was recorded in the castrated OVX-I animals from 12 to 36 h after 17β-estradiol and Pg administration and from 12 to 48 h after PRL injection, with a maximum reached for all the hormones at the 24th h. Surprisingly, estradiol exerted a significant effect on the HI tumors borne by the castrated OVX-II mice from 24 to 36 h after its injection, whereas Pg was without any apparent effect. Prolactin also weakly but nevertheless significantly stimulated the TLI of the HI MXT tumors at the 24th and 36th h after its administration to the recipient mice (Fig. 4).

All the control values recorded in the tumors borne by the castrated OVX-II mice (HI tumors) were significantly higher (P < 0.01) when compared with their corresponding values in the castrated OVX-I HS tumor-bearing animals.

Table 2 illustrates the data obtained with 17β-estradiol on MXT tumors grafted onto animals bearing pituitary grafts, i.e., the MXT T-16 IPI-bearing mice, or on the tumors from the mice previously given daily injections of prolactin, i.e., the MXT T-35-bearing animals. The MXT T-16 tumors corresponded to HI tumors already grafted onto IPI animals for 4 passages whereas MXT T-35 corresponded to HI tumors already grafted for 4 passages (T-31–T-34) onto intact animals treated daily with 10 μg of PRL and for 1 passage onto non-treated intact animals. The MXT T-16 neoplasms recovered most of the hormone-sensitive properties (see “Growth of the Tumors” and “Histology and Steroid Hormone Receptor Contents”) whereas MXT T-35 neoplasms only partly recovered these properties and indeed did not recover significant amounts of ER and PgR (see “Growth of the Tumors” and “Histology and Steroid Hormone Receptor Contents”). However, estradiol induced a significant and transient TLI increase over the control values in both the MXT T-16 and the T-35 strains.
Fig. 4. Late 17β-estradiol-, Pg-, or PRL-induced responses (within hours) in MXT HI or HS tumors, as assessed by TLI. The animals were treated [castration, hormone, i.e. 17β-estradiol (EB); Pg (I); PRL (III); or placebo, i.e., saline (C), injections] as described in the legend to Fig. 3. In the present case, they were sacrificed 12 to 48 h after receiving the injection. One h prior to sacrifice each mouse received 1 µCi [3H]dThd/g body weight, after which the tumors were removed, fixed for histology, sliced, and submitted to autoradiography. The mean TLI value ± SEM of 17β-estradiol-, Pg-, or PRL-treated groups were statistically (Fisher F test) compared to the corresponding control value (*, P < 0.05; ***, P < 0.001). The "HI" tumors were devoid of ER and PgR, but they responded to 17β-estradiol or PRL.

Table 2. Influence of 17β-estradiol in the TLI of MXT tumors grafted onto IPI mice (T-16) and of MXT ones grafted onto animals treated daily with prolactin (T-35)

<table>
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<th>24 h</th>
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<th>48 h</th>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.0 ± 1.2</td>
<td>6.2 ± 1.6</td>
<td>5.9 ± 1.0</td>
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<td>Estradiol</td>
<td>11.5 ± 0.9</td>
<td>14.4 ± 0.7</td>
<td>7.8 ± 0.8</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>12 h</th>
<th>24 h</th>
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<tr>
<td><strong>MXT T-35</strong></td>
<td></td>
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<tr>
<td>Control</td>
<td>5.1 ± 0.8</td>
<td>6.3 ± 1.4</td>
<td>6.1 ± 0.8</td>
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<td>Estradiol</td>
<td>9.5 ± 0.7</td>
<td>15.2 ± 1.1</td>
<td>8.7 ± 1.4</td>
<td>6.2 ± 0.9</td>
</tr>
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</table>

* Animals received a bilateral tumor graft arising from the T-16 (IPI animals) or the T-35 ("mice treated daily with PRL") strains. These animals were ovariectomized 6 days prior to sacrifice in order to suppress the endogenous influence of gonadal steroids and were given i.p. injections of 0.1 ml saline (control) or 0.25 µg 17β-estradiol/animal, respectively, 12, 24, 36 or 48 h prior to sacrifice. One h before killing, all the mice were given i.p. injections of 0.1 ml of a saline solution containing 1 µCi [3H]dThd/g body weight. After removal, the MXT tumors were fixed and processed for traditional autoradiography. The mean TLI values (± SEM) recorded in 17β-estradiol-treated MXT tumors were compared (Fisher F test) with the control value assessed at the same time. */< 0.001. NS, not significant (P > 0.05).

** DISCUSSION **

Many investigators have shown on the basis of biochemical studies that breast cancers can be classified as hormone dependent or hormone independent according only to their steroid hormone receptor status (21, 22). In the present work, the presence of ER and PgR was correlated to in vivo findings on tumor growth. From this, it became clear that tumors with detectable ER and PgR did not regress after ovariectomy but that their growth was slowed down. We therefore prefer to refer to them as HS. Under the conditions of this work, HS and HI only concern sensitivity to ovarian hormones. Moreover, it is generally accepted that mammary tumors in humans and animals contain subpopulations of neoplastic cells capable of reacting differently to the same hormonal stimulus (5, 6, 9, 23).

Our results show that MXT HS tumors may be defined as well-differentiated steroid hormone receptor-positive adenocarcinoma the growth of which is significantly slowed down by ovariectomy whereas HI ones are defined as poorly differentiated steroid hormone receptor-negative adenocarcinoma the growth of which is not influenced by castration. The first MXT passage used in the present work was HS as defined above, and this model progressively evolved to autonomy in such a way that the 12th passage was HI, a feature already reported earlier in the MXT model (5) and in other ones (3, 8-10). In order to characterize the cell biology of such different HS and HI cells an attempt was made to reverse the HI stage of the MXT tumor toward a HS stage to see if the HI stage of a mammary tumor (in the present case the MXT model) was irreversible or if HS properties would appear again. For this purpose, we grafted a pituitary gland under the renal capsule of the tumor-bearing animals 1 week prior to each monthly transplantation procedure in order to obtain an endogenous and continuous secretion of prolactin that would no longer be under the negative influence of the hypothalamus; animals operated in this way were defined as IPI animals. We chose prolactin since it was previously shown that this hormone exerted a stimulating influence on MXT tumor growth (15). As a consequence, we also studied the influence of a daily injection of prolactin on the behavior of HI MXT tumors over a 4-month period (four MXT passages). The main result of this work is that in both the animals bearing pituitary isografts and in those treated daily with PRL, the transplanted HI tumors recovered most of the criteria of hormone sensitivity, i.e., the slowing down of growth by ovariectomy and the presence of ER (but not PgR) along with one complete histological differentiation. Within the MXT tumors borne by animals treated daily with prolactin, we did not observe any significant amounts of ER or PgR, but estradiol was able to provide significant stimulation for the thymidine labeling index of such tumors; this is discussed below.

Ovarian grafts were also tried in preliminary experiments but without satisfactory results since the success of an ovarian isograft on a mouse is more aleatory and prolonged estrogenic stimulation leads to a refractory effect or an increase in cell loss in target organs such as the uterus (25) or MXT neoplasia (11, 12, 14).

With regard to steroid hormone receptor data, the hormone-
sensitive MXT T-1 tumors grafted onto OVX-II mice, i.e., those ovariec-tomized prior to tumor transplantation, seemed to have completely lost their ER and PgR. This result is apparently unacceptable if one refers to the experimental OVX-II condition that would correspond to the postmenopausal state; in this case available sites for estradiol should remain the same or possibly increase in number in comparison with the intact animals. We thus think that the experimental OVX-II schedule performed prior to the transplantation procedure might favor the initial proliferation of HI cells, with the result of such a selection leading to the appearance of HI tumors. This finding has already been observed in the MXT model (11).

As further proof of the sensitivity of tumor cells to ovarian hormones and prolactin, we tried the "actinomycin D staining method" of Brachet and Hulin (26) followed by autoradiography and the thymidine labeling index (27). With respect to the actinomycin D staining method, it should be pointed out that since the slides were fixed before being exposed to [3H]AMD, this substance was not used as an antimetabolite but as a "staining" reagent. The amount of intercalating dye such as acridine orange or labeled actinomycin bound to DNA has been shown to be directly proportional to the degree of chromatin decondensation and would partly reflect genomic derepression (28, 29). In this work, the binding of [3H]AMD resulting in a significant increase of the SGN over basal values was observed in the HS but not in the HI MXT tumors 5 to 40 min after 17β-estradiol, Pg, or PRL stimulation. A similar observation was described previously in the uterus, where specific mRNA synthesis was induced by 17β-estradiol as little as 2 min after its administration to the animals (30, 31).

The proliferative influence of 17β-estradiol, Pg, or PRL on the MXT tumors was approached by means of a [3H]Tdt labeling technique which, developed by Hughes et al. (27), represents the percentage of cells engaged in the DNA synthesis phase and remains a first class technique enabling in vivo cells to be evaluated in the reproductive cycle. Although this parameter does not measure all the cells engaged in the S phase (32) and is not even always the sign of a true mitogenic effect (33, 34), we have shown previously that TLI represents a good indicator of cell proliferation in the MXT model stimulated by estradiol (7, 11, 12), progesterone (13, 14), or prolactin (15). The same features were also observed here since the three hormones, i.e., 17β-estradiol, Pg, and PRL, induced a transient and significant mitogenic effect on HS tumor cell proliferation. This effect lasted from the 12th to the 36th or 48th h after administration to the tumor-bearing animals.

Rather unexpectedly, a significant 17β-estradiol-induced increase in TLI was also observed in "HI" MXT tumors, where ER and PgR were undetectable. An explanation of this finding might be found in the work of Ikeda et al. (35). These authors are in favor of the possible role of mediator growth factors and mitilate along the others (36–38) against the hypothesis that estrogens are directly mitogenic. Their working hypothesis is that estrogens promote the growth of normal target tissue cells and hormone-responsive tumors via at least three levels of regulation. They suggest that one of these levels may be that estrogens induce the production of endocrine or of circulating-type growth factors which have their primary action on distinct target tissues (a traditional endocrine mechanism) such as the uterus, the kidney, and the pituitary gland (35). A second type of control may be that estrogens (and progesterone) act locally on target tissue cells to induce biosynthesis and/or the secretion of 17β-estradiol-dependent growth factors with primary sites of action that are either adjacent cells (paracrine mode of action) or a direct return to the tumor cell of origin (autocrine mode of action) (36, 37). A third type of estradiol-mediated effect would be that 17β-estradiol acts on target cells and via the ER mechanism induces the synthesis of a substance which abolishes the inhibitory activity present in serum (38). Thus, the early "genomic derepression" in HS tumors might agree with the transcription of genes involved in growth factor synthesis (39).

In HI MXT tumors, the situation is somewhat different. The high basal "genomic derepression" observed might be in accordance with an elevated basal hormone-independent growth factor synthesis. Such a hormone-independent synthesis of growth factors might be regulated by oncogenes (40–42) and growth factors have recently been divided into positive autocrine and negative paracrine ones (42). It has thus been suggested that malignant cell transformation might arise not only from the excessive production, expression, and action of positive autocrine growth factors but also from the failure of cells to synthesize, express, or respond to the specific negative growth factors which they normally release to control their own growth (39, 42). The acquisition in HI cells of some of these properties would explain that "HI phenotype" presents a selective advantage over the "HS phenotype," thus leading to autonomous HS-HI tumor evolution.

Our unexpected finding that "HI" MXT tumors devoid of detectable ER and PgR nevertheless react to 17β-estradiol by increased TLI is consistent with other works (43–45). Indeed, Dao et al. (43) have shown that physiological doses of estrogen and progesterone administered for 3 consecutive days to women with breast cancer with undetectable ER could induce a significant TLI increase in these tumors. Conte et al. (44) reported that diethylstilbestrol, administered in vivo to women with breast cancer, induced a significant increase in TLI (evaluated in vitro), independently of their estrogen receptor content. Hug et al. (45) have reported that, at pharmacological doses, the in vitro growth-stimulatory and -inhibitory effects of estrogens and antiestrogens are not necessarily mediated by hormone-specific receptors. However, as already mentioned by Dao et al. (43), since the hormone responsiveness of mammary cancer was claimed to be dependent on the presence of specific hormone receptors (21, 22), the enhanced replicative cell activity following the administration of estradiol in cases of HI tumors is a phenomenon contrary to the concept of hormone receptors. It is conceivable that the mitogenic effects of 17β-estradiol on MXT HI tumors point to an indirect stimulation mechanism, e.g., through the production of some autocrine and paracrine growth factors (as mentioned above), that amplify the 17β-estradiol-induced stimulatory influence, with the ER being in such a small quantity that it would not be possible to detect them by the conventional technique of dosage. The PRL-induced response in HI tumors can appear less conflictual than the 17β-estradiol-induced one if one refers to the fact that the term "hormone independence" is related only to steroid hormone receptor status. Thus, the possibility remains that PRL receptors exist in HS as well as in HI tumors. We are presently conducting experiments to evaluate both the PRL receptor amounts within HS and HI tumors and the PRL-induced effects on ER and PgR concentrations within HI tumors borne by intact animals receiving continuous prolactin injections.

In conclusion, we have shown that it is possible to reverse autonomous hormone sensitivity in the direction of hormone independence in MXT mouse mammary tumors either by grafting a putitary gland under the renal capsule of the tumor-bearing mice (IPI animals) or by daily giving intact mice injections of prolactin. Most of the characteristics, i.e., the presence of significant amounts of ER (in IPI recipient mice but not in PRL-treated ones), well-differentiated histological patterns, sig-
significant sensitivity to ovariectomy, and the slow proliferation rate of HS tumors were recovered after 6 months of serially transplanting MXT HI tumors on IPI animals or 3 months of treating intact animals daily with ovine prolactin.

Furthermore, from our experiments on actinomycin D binding and TLI it would appear that the difference between HS and HI MXT tumors is quantitative rather than qualitative. HI tumors more readily bind AMD and dThd, even in the absence of ovarian hormones or prolactin. 17β-Estradiol or PRL increased AMD and dThd binding by HS tumors and further increased dThd binding by so-called HI tumors. When dThd binding was tried in HI tumors transplanted either in animals bearing pituitary isografts or in ones that had been treated with PRL, the tumors returned to HS TLI values in good agreement with the conclusions reached in the first part of this work. Thus, all the present findings strongly suggest that prolactin might be the major hormone involved in the recovery of HS properties in HI tumors.

The precise biochemical mechanisms underlying the modulating actions of ovarian steroids and prolactin on MXT growth deserve further investigation, and we are now involved in this.

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Influence of Pituitary Grafts or Prolactin Administrations on the Hormone Sensitivity of Ovarian Hormone-independent Mouse Mammary MXT Tumors

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