Characterization of a Slow-growing, Transplantable Rat Mammary Tumor (MCR-83): A Model for Endocrine-related Cell Kinetic Studies

Jan Hein van Dierendonck, Cornelis J. Cornelisse, Peter Willem G. van der Linden, Luke M. van Putten, and Cornelis J. H. van de Velde


ABSTRACT

Out of 24 primary mammary tumors, arising in rats of the WAG/Rij Wistar strain after low dose irradiation, with or without prolonged treatment with estrogen, a slow-growing, well differentiated adenocarcinoma (MCR-83) was selected. This tumor, induced by radiotherapy alone, is independent of estrogen pellets for growth after transplantation into adult female rats, but nontransplantable into males or ovariectomized females. Measurements of tumor growth and contents of both estrogens and progesterone receptors on three successive passages are not indicative of a rapid progression in growth rate or to hormone independency. Ovariectomy and treatment with tamoxifen give a pronounced inhibition of tumor growth, whereas neither methotrexate nor cyclophosphamide is effective. Growth rate is significantly increased when rats are given 17β-estradiol. Flow cytomteric DNA analysis as well as in situ S-phase cell detection with anti-bromodeoxyuridine antibodies show a 3-fold increase in S-phase fraction cells within 4 days after the onset of estrogen treatment.

No spontaneous metastases have been found so far, but lung nodules develop after i.v. inoculation of tumor cells. From one of these nodules a fast-growing, hormone independent subline (MCR-86) has been derived, showing both lymphatic and hematogenous dissemination upon s.c. transplantation.

By showing several features of hormone responsive human disease in its early stage of progression the MCR-83 tumor system may be a clinically relevant model for studies on endocrine regulation of tumor growth and its therapeutic manipulation.

INTRODUCTION

Tumors with large growth fractions are most susceptible to the cytotoxic effects of many chemotherapeutic agents (1–4). A major reason for the poor drug activity correlation between human and experimental cancers might be that transplantable rodent tumor models generally are less differentiated and their growth rate is accelerated (5, 6). This lack of suitable models, relevant for the phase of slow growth, is especially striking in the case of hormone responsive breast cancer. Whereas an increasing number of clinical studies are testing combinations of hormonal manipulation and chemotherapy, the feasibility and optimal methodology of these strategies is still not firmly established in animal models resembling early human disease.

Mammary carcinomas which are chemically induced in normal young female rats most often have an inherent sensitivity to hormonal manipulation but show a rapid increase in growth rate and acquisition of hormone independency after one or two transplantations (7). Furthermore there is a tendency of these tumors (possibly as a result of the mutagenic effects of the carcinogenic agents involved) to express immunogenicity (8), whereas in human tumors there is no unequivocal evidence for a role of host immunity (9).

The objective of our study was therefore to evaluate whether mammary tumors, which arose in the WAG/Rij Wistar rat strain after low dose irradiation and/or prolonged treatment with 17β-estradiol, could provide transplantable, low antigenic, hormone responsive tumor models.

This article describes the characteristics of a selected slow-growing adenocarcinoma (MCR-83), which originated in an irradiated female. We compared the effects of MTX, CY, TAM, OVX, and estradiol on tumor growth and used flow cytometric DNA analysis as well as immunohistochemical detection of incorporated BrdUrd to monitor effects on cell cycle parameters.

The results indicate that the MCR-83 mammary carcinoma shares several features of hormone responsive human breast cancer in its early stage of progression and is fairly stable upon transplantation.

This model might be useful in investigating to what extent the low proliferative state of many human breast tumors (10, 11) relates to treatment failures and whether hormonal stimulation of tumor growth might augment the chemotherapeutic efficacy in these tumors (12).

MATERIALS AND METHODS

Animals and Tumors. The Wistar-derived WAG/Rij albino rat, originally obtained from the Glaxo Laboratories (Greenford, Middlesex, England), is inbred by brother-sister mating since 1953 in a pathogen-free breeding colony of the REP Institutes TNO (Rijswijk, The Netherlands). Using ionizing radiation and/or s.c. implanted estrogen pellets, a wide range of benign and malignant mammary tumors develop in these rats (13).

From adenocarcinomas, histopathologically classified by Dr. Matthew J. Van Zwieten (14), a series of 24 was investigated with respect to transplantability to syngeneic females, growth behavior, estrogen requirement, and ER content. One of these tumors, MCR-83, was selected for further characterization and model development.

Tumor Transplantation. Tissue blocks (1–3 mm³) of MCR-83 were implanted s.c. into the flanks. In some experiments a single cell suspension was made by incubating finely minced tissue for 4 h in RPMI 1640 tissue culture medium containing 1 mg/ml collagenase III (Worthington; Freehold, NJ) at 37°C under constant agitation. Cells were suspended in PBS and injected into the caudal vein or s.c.

Tissue blocks or cell suspensions were frozen in RPMI 1640 containing 10% rat serum and 10% dimethyl sulfoxide (Baker Chemicals, Deventer, Holland) and stored in liquid nitrogen. Caliper measurements of flank tumors were taken once or twice per week and the volume was calculated as

\[ \text{Volume} = \text{Length} \times \text{width} \times \text{height} \times \frac{\pi}{6} \]

Receptor Assay. Tumor samples for estradiol and progesterone receptor assay were frozen immediately after excision and stored at

1 The abbreviations used are: MTX, methotrexate; CY, cyclophosphamide; TAM, tamoxifen; OVX, ovariectomy; EP, estrogen pellet; ER, estrogen receptor; PgR, progesterone receptor; BrdUrd, bromodeoxyuridine; PBS, phosphate buffered saline; TD, tumor doubling time.
was found between tumors from estrogenized and untreated could be transplanted successfully to untreated females, but in rats. of whole body X-radiation or 6-100 rads of 0.5 MeV neutrons a generous gift from Dr. Frank Dolbeare, Livermore, CA) in the considerable period of time. Removal of the EPs invariably resulted growth, suggesting that these cells remain dormant for a con fraction. Bilateral ovariotomy was performed with a dorsal midline incision. Flow Cytometry. Samples for flow cytometry were obtained by sequential fine-needle aspiration or mincing small tissue blocks. Suspensions of single nuclei were prepared as described by Vindelev et al. (17) and stained with propidium iodide (Sigma). The samples were measured on an ICP 22 flow cytometer (Ortho, Westwood, MA). The percentages of cells in the cycle phases were estimated in a graphical method as described by Ritch et al. (18).

Immunohistochemical Detection of Incorporated BrdUrd. BrdUrd (Sigma), dissolved in 0.9% NaCl solution, was administered as a single injection (50 mg/kg) i.p. After 60 min tumor-bearing rats were sacrificed, and tumor tissue was fixed in 3.7% buffered formalin and embedded in paraffin. After deparaffinization of tissue sections, DNA was denatured by immersing the slides for 5 min in 0.07 M NaOH containing 70% ethanol, followed by dehydration in a graded series of ethanol and incubation with 0.1 mg/ml proteinase K (Boehringer, Mannheim, Federal Republic of Germany) in 10 mM Tris-HCl-2 mM CaCl, pH 7.0, for 10 min at room temperature. After another dehydration in ethanol, sections were preincubated in PBS containing 0.5% bovine serum albumin and 0.1% Tween 20 for 30 min, incubated with a 1:3000 dilution of a peroxidase-conjugated rabbit-anti-mouse IgG antibody (Dakopatts, Glostrup, Denmark) in a 20% dilution of normal human serum in PBS. The peroxidase location was developed with diamino- benzidine-H2O2. The sections were lightly counterstained with hematoxylin, and 2000 cells were counted at random to determine S-phase fractions.

RESULTS

Isolation of MCR-83 Mammary Adenocarcinoma. All 24 adenocarcinomas, which were tested with respect to transplantability in EP-bearing, ovariectomized, or intact females, originated from animals irradiated with a single dose of 100-100 rads of whole body X-radiation or 6-100 rads of 0.5 MeV neutrons at 8 weeks of age. A majority of this group (17 females) had received an EP 1 week prior to irradiation. The primary tumors had a mean ER content of 50.5 ± 12.8 (SD) fmol/mg protein (range, 30-74). No significant difference was found between tumors from estrogenized and untreated rats.

None of the tumors that were induced in estrogenized rats could be transplanted successfully to untreated females, but in 14 cases tumor growth could be sustained in EP-bearing rats. It appeared that implantation of an EP in females 4 months after tumor transplantation resulted in a rapid onset of tumor growth, suggesting that these cells remain dormant for a considerable period of time. Removal of the EPs invariably resulted in a rapid regression of the tumors.

Three of seven primary carcinomas, induced in rats that were not treated with an EP, gave rise to slowly growing flank tumors when transplanted to untreated females. One of these, adenocarcinoma MCR-83, which originated in a female irradiated with a dose of 6 rads of 0.5 MeV neutrons 21 months before transplantation, was chosen for further characterization. It showed a predictable growth pattern in normal, non-EP-bearing females, but did not grow in males or ovariectomized females.

Hormone Treatment. Tamoxifen (a gift from the Imperial Chemical Industries, Pharma, Macclesfield, England) and 17β-estradiol (Sigma, St. Louis, MO) were injected i.c. and i.p. respectively. None of the tumors that were induced in estrogenized rats were not treated with an EP, gave rise to slowly growing flank tumors when transplanted to untreated females. One of these, adenocarcinoma MCR-83, which originated in a female irradiated with a dose of 6 rads of 0.5 MeV neutrons 21 months before transplantation, was chosen for further characterization. It showed a predictable growth pattern in normal, non-EP-bearing females, but did not grow in males or ovariectomized females. Histology. A histological section of the MCR-83 tumor is shown in Fig. 1. This tumor is a mucous tubulopapillary carcinoma, composed of multiple branching papillae of fibrovascular tissue which are covered by one or more layers of cuboidal or columnar epithelial cells. The tumor is noninvasive and encapsulated and therefore can be easily removed without primary site regrowth.

Hormone Receptor Content. Table 1 shows TDs (as determined from the linear part of growth curves; approximately, between 0.1 and 1 cm3), as well as ER and PgR values of tumors from three successive passages. These data are not indicative of a rapid increase in growth rate or loss of receptor content after successive tumor transplantations. However, the variability in ER and PgR values was large (ranging from 20-130 and 61-638 fmol/mg protein, respectively). Measurements in five different parts of a single large tumor yielded values of 37.4 ± 4.3 and 471 ± 164 fmol/mg protein, respectively, whereas measurements of three tumor implants in a single rat yielded 27.0 ± 7.1 and 387.3 ± 137.3 fmol/mg protein, respectively.

Take Rate and Growth Behavior. Take rate of 1-mm3 tissue fragments or 0.1 ml 104-107 viable cells (two s.c. bilateral implants) was 96% in intact females (n = 117) and 0% in ovariectomized females (n = 8) or males (n = 10), as determined up to 8 months after transplantation. In the case of four s.c. implantations/animal take rate was 83% (n = 92), and tissue from a first passage tumor that was frozen and stored at -170°C had a take rate of 60% (n = 20).

The time required for flank tumors to grow to 500 mm3 from a 1-mm3 implant ranged from 70-150 days. This difference in lag period was even greater for multiple implantations within the same animal. Examples of this phenomenon are shown in Fig. 2. In the case of two bilateral tumor implants both tumors generally followed the same Gompertizian growth pattern (Fig. 2.4). In the instances of more than two implants, growth rates became more heterogeneous (Fig. 2, B and C). In 8 of 10 rats we observed an accelerated growth of smaller tumors after surgical removal of the largest tumor load (Fig. 2C). TDs were
Table 1  Tumor doubling times and estrogen and progesterone receptors of MCR-83 rat mammary tumors

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<th>TD (days)*</th>
<th>ER fmol/mg protein</th>
<th>ER $K_d$ ($\times 10^{-18}$ M)</th>
<th>PgR fmol/mg protein</th>
<th>PgR $K_d$ ($\times 10^{-18}$ M)</th>
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<tr>
<td>Primary</td>
<td></td>
<td></td>
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<td>First passage</td>
<td>26.0 ± 2.6$^e$ (3)'</td>
<td>48.0 ± 39.6</td>
<td>1.6 ± 0.8</td>
<td>207.0 ± 206.4</td>
<td>6.5 ± 3.8 (2)</td>
</tr>
<tr>
<td>Second passage$^d$</td>
<td>22.5 ± 3.6 (10)</td>
<td>65.1 ± 37.8</td>
<td>2.0 ± 1.4</td>
<td>350.8 ± 145.1</td>
<td>8.2 ± 6.8 (6)</td>
</tr>
<tr>
<td>Third passage$^d$</td>
<td>21.3 ± 3.3 (7)</td>
<td>62.4 ± 33.6</td>
<td>1.0 ± 0.3</td>
<td>284.7 ± 181.6</td>
<td>7.5 ± 4.5 (7)</td>
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* Tumor doubling time in exponential growth phase.
* Mean ± SD.
$^d$ Numbers in parenthesis, number of tumors assayed.
$^e$ Second passage tumors resulted from tissue transplantation of one first passage tumor.
$^f$ Third passage tumors were induced by pooling cell suspensions of 10 second passage tumors.

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Fig. 2. Growth behavior of tumors in three female WAG/Rij rats (A, B, and C, respectively) bearing multiple second passage transplants of mammary carcinoma MCR-83. j, surgical removal of a (large) flank tumor.

Fig. 3. Pulmonary metastasis from MCR-83 cells inoculated i.v. 6 months prior to sacrifice. H&E, × 200.

shortened to approximately 50% of control values when rats were bearing EPs (TD, 13.2 ± 2.3 days; n = 5).

Necrosis and hemorrhage were often observed in MCR-83 tumors exceeding 4–5 cm$^3$, but ulceration was a relatively infrequent phenomenon and flank tumors did not become life threatening.

Metastatic Behavior. In order to study possible metastatic behavior of MCR-83 tumors, in 10 rats bearing a 1-cm$^3$ outgrowth of a hindleg muscle inoculum the tumor-bearing leg was amputated and 5 rats were given an EP. Eight to 9 months later both groups still showed no evidence of metastatic disease in lymph nodes, lungs, or other tissues.

The effect of i.v. inoculation of dispersed tumor tissue was evaluated by injecting $10^6$–$10^7$ viable cells into the caudal vein of two females. Six months later grossly and microscopically apparent metastases had developed in peripheral as well as central parts of the lungs. As shown in Fig. 3, these nodules had a similar well-differentiated appearance as did the flank tumors, although the stromal component was less prominent.

From one of these experimentally induced metastases we isolated a relatively fast growing, hormone-independent subclone, designated MCR-86. S.c. transplants of this variant showed a TD of 4 days, and their growth did not seem to be affected by ovariectomy or treatment with TAM. Both ER and PgR were negative. In contrast to the parent line, s.c. implanted MCR-86 tumors readily metastasize to lymph nodes and lungs.

Effects of Chemotherapeutic and Hormonal Treatments on Tumor Growth. In a first experiment we studied the effect of 5 subsequent daily doses of 0.45 mg/kg MTX (i.p.), OVX, or 21 subsequent daily doses of 0.5 mg/kg TAM on relatively large second passage tumors. As shown in Fig. 4, MTX treatment in this dose gave a minimal growth delay, whereas both OVX and TAM therapy led to complete and partial remissions. The ultimate growth rate after OVX was less than a third of the initial growth rate. In the TAM treated group we observed a growth delay of 3–4 months.

In a second experiment on second passage tumors we evaluated the effects of two daily doses of 150 mg/kg CY, OVX, and 3 weeks of 1.0 mg/kg daily TAM on palpable tumors. Fig. 5A shows curves of the mean tumor load of all treatment groups. No difference was found between the CY and the control group, whereas both endocrine therapies effectively prevented tumor growth.

Furthermore we evaluated the effects of five daily doses of 0.75 mg/kg MTX, ovariectomy, three times/week doses of 1.0 mg/kg TAM during 10 weeks, and five daily doses of 1.0 mg/kg 17β-estradiol on palpable third passage tumors (Fig. 5B). The treatment results of MTX, TAM, and OVX were not different from the treatment results on second passage tumors, whereas estradiol caused an immediate increase in growth rate (TD, 6 days) that lasted for approximately 10 days, followed...
Fig. 5. Effects of chemotherapeutic and hormonal treatments on the growth of second (A) and third (B) generation MCR-83 tumors. Treatment schedules were as follows: A, daily 150 mg/kg body weight i.v. CY during 2 days, daily 1.0 mg/kg body weight s.c. TAM in arachis oil during 3 weeks, or arachis oil vehicle; B, daily 0.75 mg/kg body weight i.p. MTX during 5 days, three times a week 1.0 mg/kg body weight s.c. TAM during 10 weeks, daily 1 mg/kg body weight s.c. 17β-estradiol (E2) during 5 days, or arachis oil vehicle. Points, mean tumor volumes of 6, 8, or 10 different tumor bearing rats; for graphical clarity, the SE is not included but ranges from 12–20% of each value. \[ start of treatment.

by stasis and gradual regression to control growth rate (TD, 21 days).

Sham OVX control groups were omitted in these experiments, since surgical trauma had not been found to inhibit the growth of MCR-83 tumors (see also Fig. 2C).

Flow Cytometric Estimation of Hormone-induced Cell Cycle Changes. Cell cycle changes induced by endocrine manipulation of MCR-83 tumors were monitored by flow cytometric DNA analysis of tumor samples obtained by fine-needle aspirations of the tumors used in the experiment described in Fig. 5B. DNA histograms showed a narrow single G1,0 peak with a coefficient of variation varying between 3 and 4%. Percentages of cells in the cell cycle phases are plotted in Fig. 6 as a function of time after treatment. Estradiol induced a change in cycle distributions within 100 h after the onset of treatment, comprising a decrease in the fraction of G1,0 cells accompanied by increasing fractions of S-phase and G2,M cells.

In Situ S-Phase Cell Detection Using BrdUrd Labeling. Fig. 7 shows a section of a s.c. growing MCR-83 tumor in a rat which received 50 mg/kg BrdUrd i.p. and was sacrificed 1 h later. BrdUrd-labeled cells can be easily discriminated by the strong dark staining of their nuclei.

In Table 2 fractions of S-phase cells calculated from flow cytometric DNA histograms are compared with results obtained by BrdUrd labeling in MCR-83 tumors of both control rats and

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<th>S-phase fraction (%)</th>
<th>DNA-FCM</th>
<th>BrdUrd</th>
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<tr>
<td>CONTROL</td>
<td></td>
<td></td>
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<tr>
<td>Estragon</td>
<td>2.3 ± 0.9</td>
<td>2.7 ± 0.8</td>
</tr>
<tr>
<td>E2</td>
<td>8.0 ± 2.2</td>
<td>7.0 ± 1.3</td>
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* DNA flow cytometry of propidium iodide stained nuclei.

* Immunocytochemical detection of DNA-incorporated BrdUrd in tissue sections. Rats were given 50 mg/kg body weight i.p. BrdUrd and sacrificed 1 h later. Rats were treated daily with 1 mg/kg body weight estradiol during 3 days and sacrificed 72 h after starting the treatment.

4 Number of tumors assayed.

5 Mean ± SD. Student's t test: P < 0.05, control versus estrogen; flow cytometry versus BrdUrd, not significant.

Fig. 7. Tissue section of MCR-83 reacted with anti-BrdUrd monoclonal antibody followed by staining with indirect peroxidase method and lightly counterstained with hematoxylin. \( \times 500. \)
rats treated during 3 days with daily doses of 1.0 mg/kg estradiol. Results from BrdUrd labeling are not significantly different from the flow cytometric approach. Both methods reveal an approximately 3-fold increase in the S-phase fraction following the estrogen treatment.

**DISCUSSION**

The objective of our study was to evaluate whether mammary carcinomas, which arose in WAG/Rij Wistar rats treated with a single low dose of ionizing radiation or/and prolonged treatment with 17β-estradiol, could provide transplantable, hormone-dependent tumor models. These tumors have been shown not to be immunogenic (14). Although it has been demonstrated that sequences related to the genome of murine mammary tumor virus are present in normal rat DNA (19), Bentvelzen et al. (20) did not find evidence for the presence of murine mammary tumor virus-related antigens in these tumors.

Most primary mammary tumors we obtained were induced in EP-bearing animals. Our transplantation study of a variety of adenocarcinomas confirmed the findings of Noble et al. (21) and Noble and Hoover (22) that tumors arising in estrogenized rats continue to require hormones for growth after transplantation. Without such extraneous hormonal stimulation small clumps of nonpalpable epithelial cells remain viable and will respond at any time to become a growing tumor if properly stimulated.

Obviously, tumor models suitable for endocrine-related cell kinetics studies should respond in a way similar to that of hormonal manipulation in human disease. Therefore they should not depend on long-term estrogen supplementation for growth. A number of tumors originating in unestrogenized females fulfilled this requirement, but only one showed a predictable growth pattern after transplantation. Histology, hormone receptor contents, growth behavior and response to OVX and treatment with TAM of successive transplantations of this MCR-83 adenocarcinoma did not indicate rapid progression to autonomous tumor growth and dedifferentiation.

Because of this relative stability of MCR-83 tumors, the selection of fast-growing, autonomous and/or spontaneously metastasizing sublines by serial s.c. transplantations would be a long-lasting process. It was found, however, that i.v. inoculation of dispersed tumor tissue provides an opportunity for selecting metastatic clones in this tumor system. From metastatic lung noduli, developed 6 months after inoculation, we isolated a fast-growing, spontaneously metastasizing subline (MCR-86), showing similar histology and diploid DNA index. Preliminary results have shown that this variant does not respond to OVX or treatment with TAM after transplantation.

Ovary-independent tumor growth was also observed in rats that were ovariectomized when bearing a relatively large tumor, but growth rate of these tumors was considerably retarded (Fig. 4B). In three of six nearly stationary tumors in ovariectomized rats we observed a rapid growth stimulation following implantation of an EP, 1 year after OVX (data not shown).

All MCR-83 tumors growing in intact rats had a positive ER and PgR content, showing higher PgR than ER levels. However, the values of receptor concentrations varied considerably (Table 1).

In human breast cancer a wide range of receptor concentrations is a common finding. Biochemical as well as immunohistochemical techniques have identified mixed populations of receptor-positive and receptor-negative cells, but at present it is unclear whether these tumor cell differences are due to the presence of different clones, differences in receptor protein expression, or both. In MCR-83 tumors PgR values showed a more pronounced variability than did ER values. The finding that TAM treatment significantly reduced PgR levels (data not shown) indicates that the PgR levels are largely mediated by the action of estrogens in these tumors (23). The loss of PgRs was not observed upon OVX, since PgR measurement of a stationary tumor in an ovariectomized female showed a value of 770 fmol/mg protein.

Since MCR-83 tumors show relatively little necrosis (when smaller than 3 cm³), cell loss is probably a minor factor with respect to the growth kinetics of these slow-growing tumors. In accordance with this observation is the finding that the fraction of S-phase cells, as determined by flow cytometric DNA analysis, did not exceed 5%. This relatively low percentage puts some limits to the detection of cell kinetics changes induced by chemo- or hormonal therapy (Fig. 6). However, the cell kinetics response to estrogen is significant. The magnitude and temporal pattern of this response is in agreement with recent reports on the effect of hormones on cellular replication in a number of human breast tumors (24, 25). Using in vitro thymidine labeling of surgical biopsies these studies show a mean increase in labeling index of approximately 2.5 times control values 3 days after starting hormonal treatment. In the MCR-83 model we used both flow cytometric DNA analysis and the application of recently developed monoclonal antibodies against DNA-incorporated BrdUrd (26) on paraffin sections of tumors labeled in vivo. Both methods revealed an approximately 3-fold increase in S-phase cells 3 days after the onset of estrogen treatment (Table 2).

The use of the BrdUrd labeling technique has several advantages above classical [3H]thymidine labeling and autoradiography in being nonradioactive, rapid, less labor intensive, and more sensitive (27). The excellent preservation of morphology permits a good discrimination between neoplastic and nonneoplastic cells.

A remarkable observation was that a relatively large s.c. tumor mass in the host seemed to have a growth retarding effect on smaller tumor implants. This inhibition was released after surgical removal of a large tumor load (Fig. 2). Similar effects have been described for other experimental tumor models as well (28-31). It has to be emphasized, however, that further experiments are needed to confirm these findings with larger experimental groups.

A number of studies using human breast cancer cells in vitro (32, 33) or transplantable mouse mammary tumors (34, 35) are indicative of a synergistic action of hormonal stimulation and drug treatment. These experiments have initiated a number of clinical studies, not only testing the possibility of recruitment by low-dose estrogens (24), but even strategies aimed at the synchronization of cells with TAM and subsequent estrogenic stimulation (36). Present data suggest that the MCR-83 mammary carcinoma is a model for human breast cancer in an early stage of progression, particularly suitable for a preclinical evaluation of these endocrine-related treatment schedules. Furthermore it might be used to investigate mechanisms of growth regulation and tumor progression, possible interactions between tumor subpopulations, and metastatic behavior.

**ACKNOWLEDGMENTS**

The authors express their appreciation to Dr. M. J. Van Zwieten and coworkers of the Department of Gerontology, REPOO-TNO, Rijswijk, for providing the rat mammary tumor biopsies and discussing...
the histological classification. The authors thank Dr. F. Dolbeare, Livermore, CA, for kindly providing the IU-4 anti-BrdUrd antibodies and Dr. A. K. Raap, Department of Histochemistry and Cytochemistry, University of Leiden, and Dr. B. Schutte, Department of Internal Medicine, University of Limburg, Maastricht, for their advice on the application of BrdUrd in the MCR-83 model. Special thanks goes to Dr. J. A. Foekens and coworkers of the Department of Biochemistry, Radiotherapeutic Institute, Rotterdam, for performing and discussing the hormone receptor assays. The authors also wish to thank V. T. H. B. M. Smit, H. M. Ceha, H. R. De Koning, N. J. Dijkshoorn, and Dr. J. A. Bruijn, Department of Pathology, University of Leiden, for their excellent technical assistance and/or helpful advice.

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