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Studies of Three Canine Mammary Cell Lines—II. *In Vivo* Properties*

MARY NORVAL,†‡ JEAN MAINGAY† and RODERICK W. ELSE§

†*Department of Bacteriology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, U.K. and* §*Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies, Summerhall, Edinburgh EH9 1QH, U.K.*

Abstract—*Three cell lines, REM* 134, 111 *and* 367, *derived from canine mammary carcinomas have been used to induce tumours in athymic nude mice after subcutaneous injection. The histopathology of the tumours was compared and each was found to resemble closely the original tumour. This did not change after serial* in vivo *passage. Metastasis never occurred. Injection of REM* 134 *cells intracranially resulted in a fast-growing tumour which also did not metastasize; injection intrapleurally resulted in growths most commonly on the mediastinum with confinement to the chest cavity. Fibronectin was present in the subcutaneous tumours. Two of the cell lines were cloned in semi-solid agar. When tested, these clones induced tumours identical histologically to the uncloned ones. Finally, male and female mice were injected subcutaneously with the same number of cells from each of the three lines but the rate of tumour growth did not differ significantly between the two sexes.*

INTRODUCTION

THE ORIGIN of three epithelial cell lines, REM 134, 111 and 367, derived from canine mammary carcinomas and their properties *in vitro* have been described in a previous paper [1]. This communication deals with their tumorigenicity in athymic nude mice. REM 134 cells have already been reported to induce tumours in nude mice and these could be serially transplanted [2]. There was the suggestion of a difference in the rate of tumour growth between male and female mice and this has been further investigated. Recently a study was made of four canine mammary tumours in which fragments of tissue implanted in nude mice induced formation of tumours which could then be serially transplanted [3]. The oestrogen receptors of the induced tumours were assayed [4].

MATERIALS AND METHODS

Cell lines

The origin, growth and maintenance of REM 134, 367 and 111 cell lines have already been described [1]. For inoculation into mice the cells were removed from the surface of the culture vessel with trypsin–versene, washed twice in medium, counted and re-suspended at the required concentration in 0.1 ml saline.

Mice

CBA athymic mice, 4–6 weeks old, were purchased from the Clinical Research Centre, Harrow, U.K. They were kept in an isolator with autoclaved bedding, filtered air, sterile food and acidified water (pH 2.8). Injections of cells were carried out subcutaneously, intracranially and intrapleurally. Tumour diameters in two directions (and height in some instances) were measured using Vernier callipers, and the mice were weighed at weekly intervals. When the tumours reached around 2 cm in diameter or became necrotic the mice were killed by cervical dislocation. The tumours were excised using an aseptic technique and, in some instances, disaggregated with a mixture of collagenase/dispase (Boehringer) before re-injection into another mouse as cell suspensions or before culturing *in vitro*. Part of each was fixed for histopathology in 10% buffered formalin, sectioned after embedding in paraffin wax and stained with haematoxylin and eosin. Various organs were also prepared in the same way. Pieces of tumour were prepared for electron microscopy

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‡To whom requests for reprint should be addressed.

by fixing in 2.5% glutaraldehyde and embedding in 'Transmit' resin (Taab Ltd).

RESULTS

Initially various numbers of cells from REM 134, 367 and 111 were inoculated subcutaneously into female athymic nude mice. REM 134 cells were found to be highly tumorigenic, an inoculation of 10^7 cells producing a solid tumour easily visible by eye within 5 days which continued to grow steadily until approximately 2 cm in diameter at 21 days. If 10^5 cells were used, 3/4 mice developed tumours. Inoculation of less than 10^5 cells produced no tumour. With regard to the other two cell lines, 1.5×10^6 cells of 367 produced visible tumours in 3 weeks and less than 1.5×10^5 cells produced no tumour, while 10^6 111 cells produced visible tumours in 2–3 weeks. Metastases were never seen macroscopically. In addition to the three cell lines, various epithelial cell strains derived from canine mammary carcinomas were also tested in nude mice, plus a fibroblastic strain. None induced tumours when injected subcutaneously at 10^7 cells per mouse. Hamsters, five male and six female, injected subcutaneously on the day of birth with 2×10^6 REM 134 cells did not develop tumours.

Subcutaneous inoculations of REM 134 cells in nude mice produced solid-type carcinoma formations as sub-epidermal or dermal nodules. There was extensive cellular pleomorphism in tumours with varying cell size and indistinct cell outlines. Some cells stained more deeply than others and some pyknotic cells were seen. Nuclei were often variable in size and bizarre shapes were seen; mitoses were commonly observed. A striking feature was the presence of squamous metaplasia in many parts of the tumour nodules (Fig. 1). Central areas of larger tumours were sometimes necrotic, with pyknotic cells sandwiched between necrotic and viable zones. The borders of neoplasms were irregular but well-demarcated; however, there was no well-recognisable complete compression capsule. In some sites the tumour cells infiltrated into surrounding muscle or connective tissue. Part of this effect may have been related to the relative depth of initial subcutaneous inoculation of cells. In some tumours there was evidence of satellite tumour nodules in connective or muscle tissue and, not uncommonly, tumour cells impinged on capillaries at the periphery of tumour foci. The cells were equally undifferentiated in both female and male nude mice.

In some mice inoculated with REM 134 cells the tumours invaded into the lumbar musculature and at autopsy were found to have tumour masses encroaching on the peritoneal cavity. In none of these mice, howver, was there evidence of spread of tumour to abdominal or other body organs, either continuously or by metastasis.

Tumours induced by 111 cells were solid aggregations of epithelial cells (Fig. 2). Although tumour foci were well-demarcated there was only thin and incomplete compression capsule formation. The tumour cells did not appear to infiltrate the surrounding tissues but the peripheral connective tissue was well-vascularised. In contrast to REM 134, there was no evidence of squamous metaplasia, although areas of central necrosis were a regular feature with the same pyknotic cell interfaces as described above. Cellular pleomorphism was observed but, compared with REM 134-induced tumours, cells were more uniform. Typically, they had indistinct cell borders with large pale, but regularly ovoid, nuclei. Mitotic figures were common. Male and female tumours were similar.

Murine tumours produced by inoculation of 367 cells contrasted markedly with REM 134 and 111 tumours (Fig. 3a). Tumour architecture was typically well-organised tubular or papillary adenocarcinoma (Fig. 3b). This appearance, as in the case of REM 134 and 111 tumours, resembled closely the original canine tumours. Larger tumours exhibited central foci of necrosis and aggregations of pyknotic cells were present at the interface between necrotic and viable tumour zones. Sometimes macrophage-like cells were seen. Tumour borders were fairly well circumscribed by compression capsules but the tumours induced in male mice had less prominent encapsulation than in females. Tumour cells had large pale-staining ovoid nuclei with sparse cytoplasm and indistinct cell membranes.

Mitoses were less commonly observed than in REM 134 and 111 tumours. An interesting feature was the fact that 367 tumours in male mice were apparantly more differentiated and organised as tubular elements than in female mice.

Frozen sections of tumours induced by REM 134, 367 and 111 were shown to contain fibronectin as measured by indirect immunofluorescence (Table 1), despite the cells not synthesising it in detectable amounts *in vitro* [1]. Fibronectin was also present in three canine mammary carcinomas tested. Serial tumour passage from one mouse to another was carried out three times using tumours induced by REM 134, 367 and 111 cells which were excised, disaggregated with collagenase/dispase and reinjected subcutaneously. In each case the resulting tumours were the same as regards gross morphology and histopathology as the originals. Cell cultures derived from the disaggregated

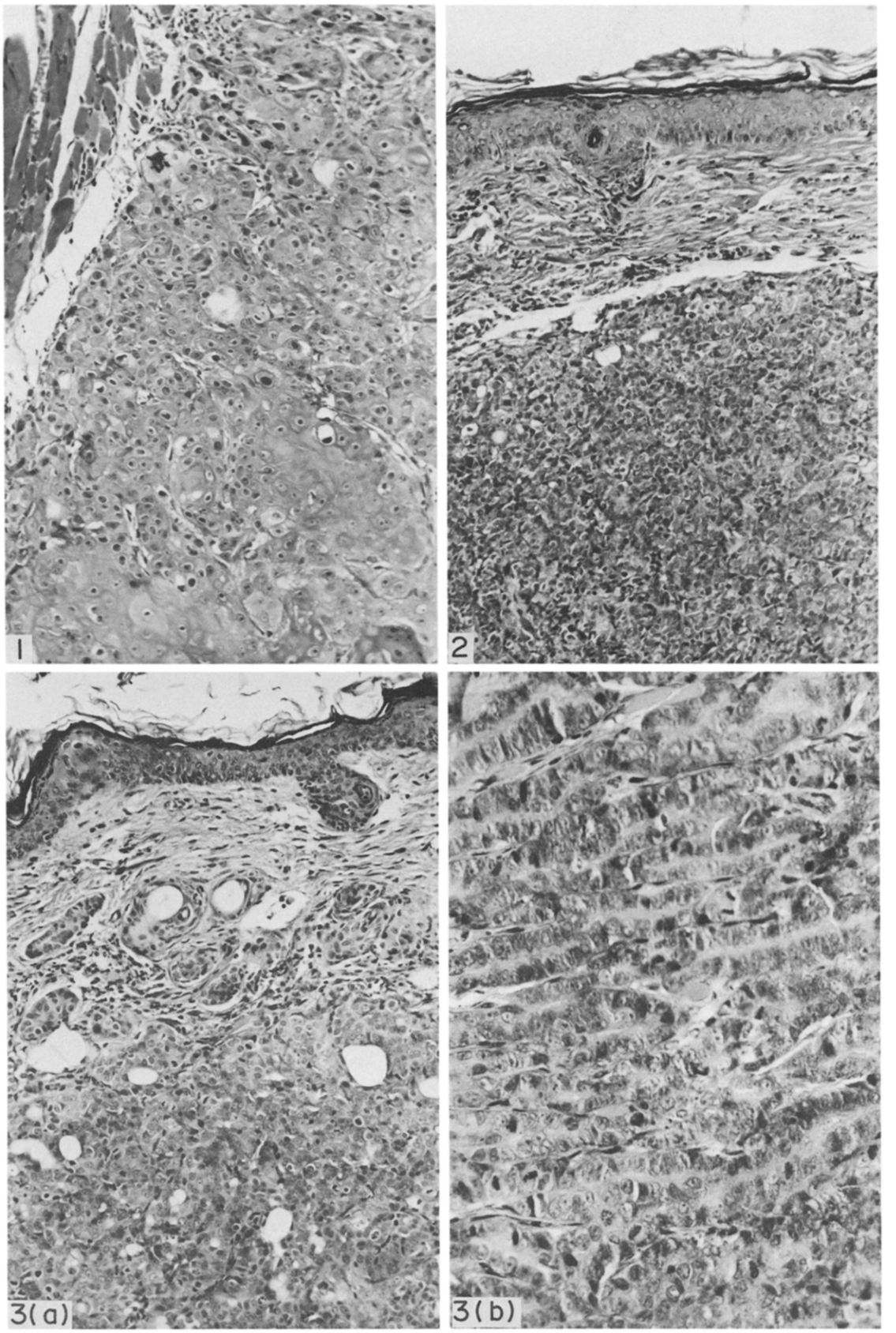

Fig. 1. *Histopathology of tumour induced by REM* 134 *cells in athymic nude mouse* (×160; *H and E*).
Fig. 2. *Histopathology of tumour induced by* 111 *cells in athymic nude mouse* (×160; *H and E*).
Fig. 3. *Histopathology of tumour induced by* 367 *cells in athymic nude mouse* (*a*; ×160; *H and E*), *showing* (*b*) *the well-organised tubular adenocarcinoma structure* (×320).

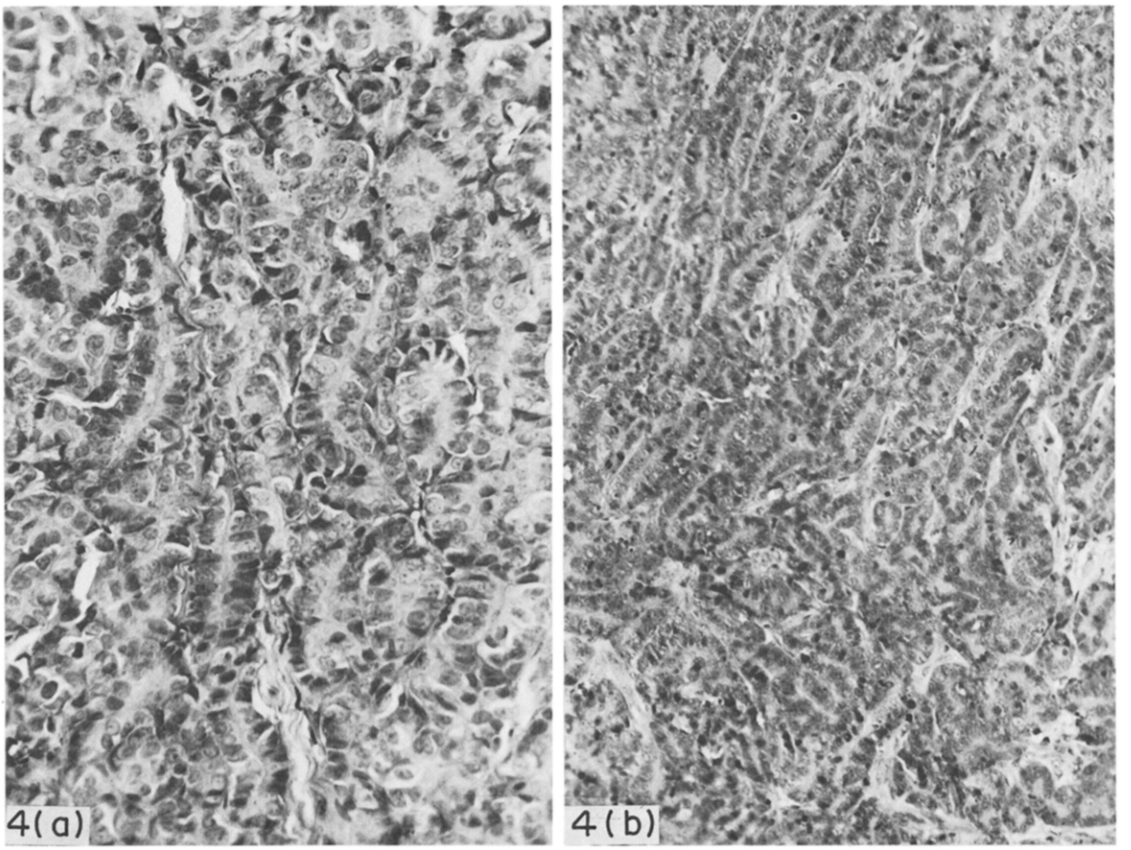

Fig. 4. *Histopathology of tumours induced by two clones of* 367 *cells in athymic nude mice* (*a*, ×320; *b*, ×160; *both H and E*).

Table 1. *Presence of fibronectin as measured by indirect immunofluorescence on sections of tumours*

	Dilutions of antiserum 1/20	1/100	1/250
Mouse tumour induced by REM 134 cells	+++	++	-
Mouse tumour induced by 367 cells	++	-	-
Mouse tumour induced by 111 cells	+++	++	-
Mammary carcinoma A	+++	++	+
Mammary carcinoma B	++	++	+
Mammary carcinoma C	++	+	-

+++, ++, + = degrees of immunofluorescence; - = no immunofluorescence.

tumours yielded monolayers with the same properties as the parent cells.

It has been reported that injection of MCF-7 mammary carcinoma cells in different sites in athymic mice may lead to development of tumours with different rates of proliferation and invasiveness [5]. Thus 1.5×10^6 REM 134 cells were injected intracranially and 5×10^5 intrapleurally. A tumour was visible in the first case on the head of the mouse within 4 days. It was killed after 11 days, when the tumour measured 8 mm. Histological examination showed extensive invasion of tumour cells around and below the meninges into the brain and cranially into the ethmoid and frontal regions of the head. The neoplasia was anaplastic carcinoma with squamous metaplastic foci, as observed in the original canine tumour and the subcutaneous murine lesions described above. Although there was intra-cerebral growth of tumour cells, there was no evidence of metastases elsewhere in the mouse.

After intrapleural injection the mice were kept for 1 month and then killed. In the case of REM 134 there was copious pleural fluid when the mouse was opened. This was cultured *in vitro* and within 48 hr there was a good growth of cells with typical REM 134 morphology. Macroscopically, mice inoculated intrapleurally showed friable white nodular growths on the mediastinum and parietal pleura, and frequently on the diaphragm. The neoplasia appeared to originate commonly on the mediastinum. Gross sectioning of lung tissue showed no obvious tumour involvement of the parenchyma or pleural surfaces.

Histologically, tumour tissue showed typical REM 134 morphology with a marked degree of pleomorphism, high mitotic index and bizarre nuclei, together with early squamous metaplasia. Where parietal pleural nidation was observed there was neoplastic infiltration of adjacent musculature. Tumour growth was confined to the chest cavity macroscopically, and histological examination of other selected organs showed no tumour metastases.

Intrapleural inoculation of 367 and 111 cells resulted in similar localised tumour growth patterns but no evidence of metastatic disease was observed. In each case the tumours induced were characteristic of the original subcutaneous lesions.

REM 134 and 367 cells were cultured in semi-solid agar and several clones grown up individually [1]. Four REM 134 clones (2×10^5 cells of each) and three 367 (2×10^6 cells of each) were then tested for their tumorigenicity in nude mice by subcutaneous injection. In all cases tumours were induced but their rate of growth varied, a property which correlated with their *in vitro* rate of growth. Histologically, the tumours induced in mice by different clones of REM 134 were identical and similar in type to the initial subcutaneous uncloned mouse tumours; for example, the histopathology of two tumours induced by two such clones is depicted in Fig. 4(a and b).

There was some preliminary evidence to suggest that REM 134 cells induced a slower rate of tumour growth in male mice than in females [2]. In addition, a response to oestrogen had been noted with these cells *in vitro*, a property not shared by the 367 and 111 cell lines [1]. Thus three male and three female mice of the same age were injected subcutaneously with 5×10^5 REM 134 and 367 and 10^6 111 cells. The mice were weighed weekly and the dimensions of any tumour formed measured.

The area of each tumour was calculated by multiplying the diameter measurements together. Height was not included as it was only possible to measure this dimension when the tumour was large. Figure 5(a, b and c) depicts the results obtained for male and female mice injected with REM 134, 367 and 111 respectively. Regression coeffieients were calculated using the area of the tumour as the dependent variable and days post-injection as the independent. All regressions were significant. Weighted T tests were performed with these coefficients as independent variables weighted by the reciprocal of the estimated variance of each coefficient. The weighted means for the male mice were tested against those of the female for each of the three cell lines, giving the results of $T_4 = 1.6$ for REM 134, $T_4 = 0.33$ for 111 and $T_4 = 0.54$ for 367. None of these was significant.

The regressions were repeated using various log transformations (log area vs day, log area vs log day, area vs log day) which produced improved fits for some mice, notably two females injected with REM 134, although for most mice the

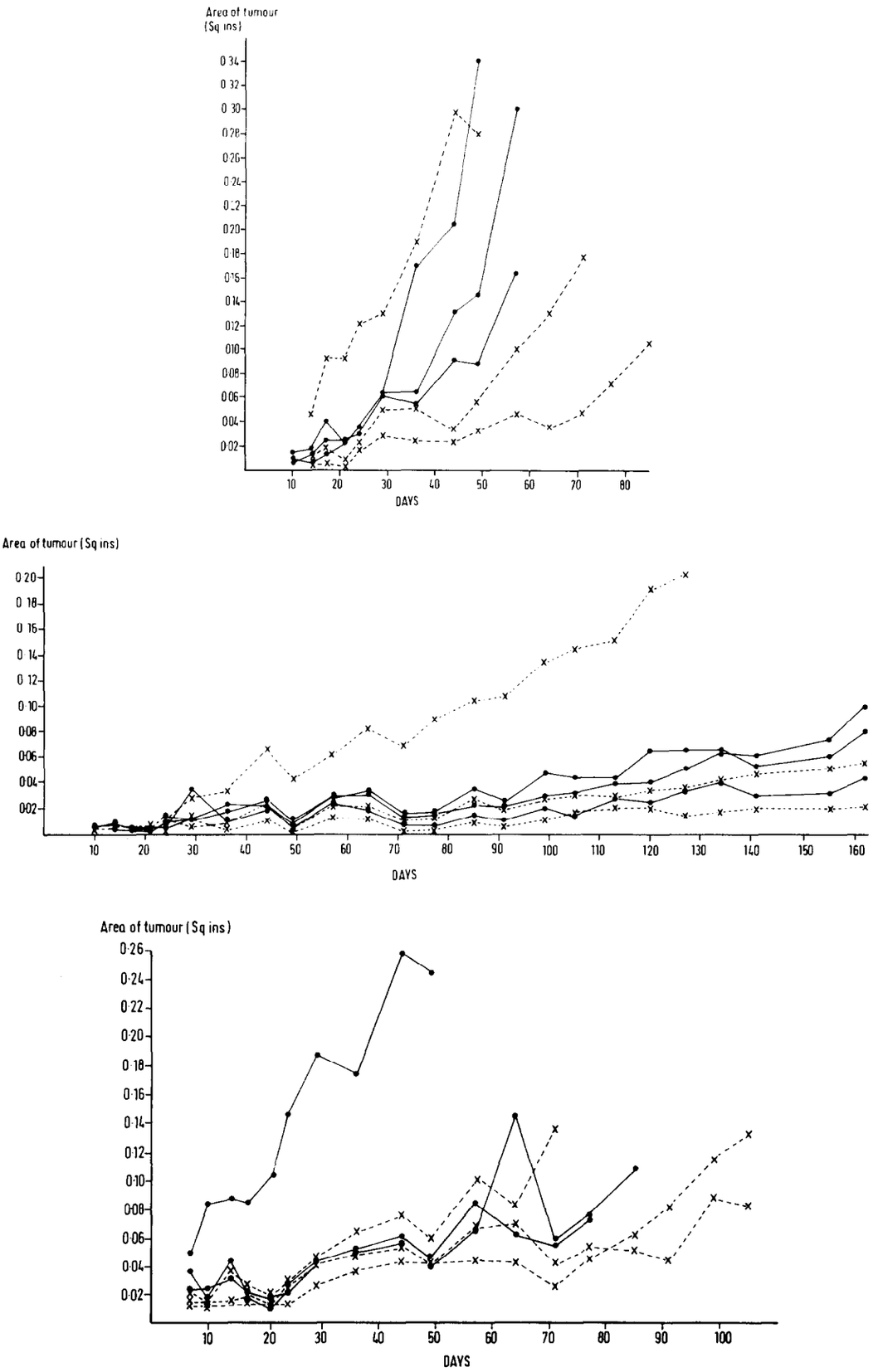

Fig. 5. Rate of growth of tumours in male (×-----×) and female (●——●) athymic nude mice induced by (a) REM 134, (b) 367 and (c) 111 cells.

original regression gave the best fit. Thus the weighted T test was calculated on the transformed (log area) data for REM 134, giving $T_4 = 2.05$, $0.1 < P < 0.2$. This was considered of marginal significance only.

Calculation of the regression coefficients from an analysis of weight of mice on days post-infection did not suggest any relationship with the growth rate of tumours (data not shown).

DISCUSSION

Cell suspensions from three canine mammary carcinoma cell lines were found to induce tumours when inoculated subcutaneously in athymic nude mice. These tumours were similar in general histological type to their canine originals, and could be passaged as cell suspensions from one mouse to another directly or as *in vitro* tissue cultures.

An interesting feature of the lines was that 367 appeared to be less aggressive, with a greater number of cells required to induce tumour growth subcutaneously. Morphologically, 367 tumours were better differentiated tubular or papillary carcinomas, as opposed to the anaplastic epithelial neoplasms induced by REM 134 and 111 cells. In all the mice inoculated, however, there was no gross or microscopic evidence of metastatic disease with any of the tumour types. Some tumours induced by REM 134 showed early infiltration of adjacent musculature but, in common with all the subcutaneous neoplasms, the cells were well-circumscribed by a compression pseudo-capsule. In some cases with 134 and 111 cells there was evidence of 'pushing' expansion of tumours but capillaries at the edge of tumours showed no convincing evidence of tumour cells within their lumens, as might have been expected in early metastasis. One possible explanation for apparent tumour-to-muscle infiltration may be related to site of original cell inoculation such as inadvertently seeding cells into muscular tissue rather than subdermal areolar connective tissue. The lack of metastasis agrees with most other studies [3, 6, 7], although Ozzello and Sordat report that some human mammary cell lines do metastasize at variable frequency [8]. It might be worthwhile allowing tumours to remain *'in situ'* for longer periods or to 'trigger' any metastatic potential by use of agents such as collagenase.

Attempts were made to induce metastatic disease by inoculating cells intracranially in the case of REM 134, and intrapleurally for all three lines. Florrid tumour growth occurred but there was still no induction of metastatic disease.

Examination of clones of 134 and 367 prepared by growth of colonies in semi-solid agar showed differences in rates of growth in mice but histologically the tumours induced had no well-defined differences from the originals. Ultra-structural examinations have so far yielded no further explanation for the different growth rates.

Initial studies [1] showed that the cell lines exhibit no hormone receptors when subjected to standard receptor assay; this is in contrast to the findings of Thomas *et al.* [4], who reported the presence of oestrogen receptors in four solid cell lines which grew in nude mice. *In vitro* studies for REM 134 cells indicated an increased growth rate in the presence of oestrogen and luteotropic hormone [1], and some preliminary work showed that there may be a difference in growth rate of tumours induced by REM 134 cells in male and female mice [2]. However, the present results, using larger numbers of mice and statistical analysis, showed that there was no significant difference in growth rate between males and females. It is possible that any hormone receptors present on the original canine tumours from which the cell lines were derived may have been masked or lost during passage, and the apparent response of REM 134 cells *in vitro* due to some other factor. To clarify this matter, we are currently testing the effect of tamoxifen, an anti-oestrogenic agent, on growth rate of REM 134 cells *in vitro* and tumour induction in male and female nude mice.

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