Local recurrence and metastasis of excised breast carcinoma in the rat[1]

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Introduction

The radical treatment of breast cancer by surgery alone or combined with radiotherapy is often precluded by regional or distant metastases (Nichini & Tsien 1971). In these cases and in those developing local recurrence after treatment, chemotherapy may be used palliatively. In others, where the risk of metastases is high, prophylactic chemotherapy may be given (Carter 1976). However, in patients treated by surgery and irradiation, chemotherapy may potentiate the formation of metastases (Finney 1971), as with some animal tumours (Steel & Adams 1977, Brown & Marsa 1978). This suggests that adjuvant chemotherapy requires a detailed knowledge of the biological pattern of tumour spread both in patients and in animal tumour models, particularly if the latter are to be used in developing rational general principles of combined therapy.

We have already reported the effect of chemotherapy with cyclophosphamide, and/or local irradiation on the growth of the transplantable breast tumour LMC_1 in rats (Moore & Dixon 1978, Dixon *et al.* 1978). We have also shown that the incidence of metastases from this tumour is higher if systemic cyclophosphamide is given 4 to 10 days before local irradiation (Moore & Dixon 1977*a*).

We now present data from a preliminary study of the local recurrence, spread and growth of metastases of LMC_1 following surgical excision of the primary tumour at various times after implantation in host animals. The objective of these experiments was to obtain data to enable the spread of the tumour to be quantified, and to define accurately the problems that need to be overcome when giving chemotherapy for metastases before or after treating the primary tumour by other forms of therapy.

Material and methods

The LMC_1 tumour arose spontaneously at the level of the third and fourth mammary gland in a breeding female Wistar rat of John's strain. The tumour at post-mortem was unattached to the overlying skin and the underlying abdominal wall, and on histology proved to be a well differentiated adenocarcinoma of the breast (Moore & Dixon 1977*b*). No metastases were detected and the tumour has since been maintained by serial transplantation in isologous virgin female and, from the 37th transplantation generation, stored in liquid nitrogen. As is common with most serially transplanted tumours (Heiman 1934), the histological architecture of the spontaneous neoplasm has been lost but its original macroscopic pattern of growth and the cycle time of its cells have been maintained (Moore & Dixon 1977*b*). The tumour is not responsive to hormonal manipulation of the host (Jordan *et al.* 1979).

Experimental tumours were prepared by a method modified from that of Thomlinson (1960). Tumour from a donor animal was removed aseptically, minced finely and transferred into the lumen of a 15–20 cm length of inverted small bowel, prepared from a 50–70 g rat of the same strain. The gut was then tied at intervals with surgical silk to form 3–5 mm diameter

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spherical 'sausages' which after separation were washed twice by stirring vigorously for five minutes in normal saline. They were then subjected to a similar treatment in sterile distilled water to lyse any tumour cells adherent to the external surface of the spheres. After a further wash in saline, each of these 'sausages' was implanted subcutaneously into the posterior abdominal flank of an anaesthetized virgin female rat. This technique produces well-encapsulated tumours that grow, usually spherical and unattached to skin or body-wall, and may be excised easily or subjected to daily caliper measurement across three mutually perpendicular axes.

After implantation, rats were randomized into groups as controls or for surgical excision of their tumours at 1, 3, 6, 8, 12, 16, 20 or 28 days after implantation. Excision usually took 5–10 minutes in the anaesthetized rat, and was carried out with a minimum of manipulation by removing the tumour and the overlying elipse of skin. After excision the animals were examined daily to detect either locally recurrent tumour or the development of metastases in the axillary and inguinal nodes. These were measured daily across at least two, and usually three, diameters until the rat developed cachexia, and had to be killed. The number of days that elapsed between implantation of the tumour and killing of the animal was used to calculate the mean survival time for each group of animals.

At post-mortem each rat was dissected to expose metastases at other sites, e.g. in the abdomen and thorax, and when found these were removed and measured across three diameters. All animals excised and surviving apparently tumour-free at 100 days were killed and a full post-mortem carried out. With this tumour model, surgically treated animals remaining tumour-free for 100 days do not subsequently develop local recurrence or metastases (Dixon & Moore, unpublished).

Results

For 28 control rats implanted with LMC_1 the mean survival time was 39 ± 2 days and the growth curve established for their tumours (Figure 1A) was the same as that determined previously (Moore & Dixon 1977*b*). Of these animals, 46% had small but definite metastases which were only detected at post-mortem.

Of 145 rats in which the primary was excised, 52% subsequently developed further tumours: 9% with local recurrence, 12% with local recurrence and metastases, and 31% with metastases only (Table 1). The mean survival time for each of these groups was 50 ± 4, 54 ± 3 and 63 ± 2

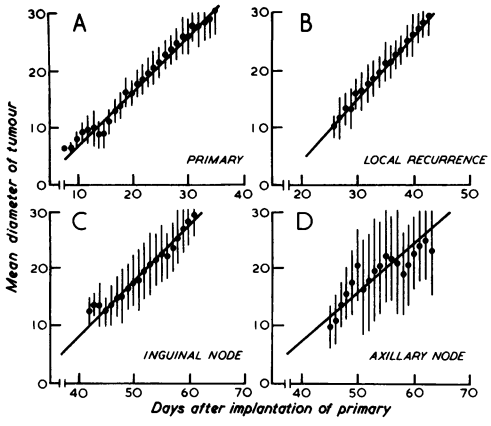

Figure 1. Growth curves for tumour LMC_1 measured *in situ* at each of the sites shown. All tumours were measured daily and the mean value (± 2 s.e.m.) calculated for all tumours present at that site on each day. Growth curves fitted by method of least squares

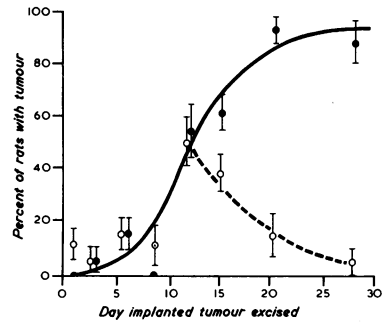

Figure 2. Incidence of local recurrence and metastases of LMC_1 in surgically excised rats. Number of rats excised at each time-interval is given in Table 1. ○, local recurrence; ●, metastases. Vertical bars represent ± 2 s.e.m.

Table 1. Follow-up data for rats after excision of primary tumour

Day of excision	Diameter of tumour (mm)	Number of rats	Local recurrence only	Recurrence and metastasis	Metastasis only	Tumour free
1	1.2●	17	2	0	0	15
3	1.8●	18	0	1	0	17
6	2.9●	20	0	3	0	17
8	4.2●	8	1	0	0	7
12	8.0±1.0	22	7	4	8	3
15	9.2±1.8	26	3	6	10	7
20	15.9±1.4	17	0	3	13	2
28	15.9±2.4	17	0	1	14	2

● Values derived from growth curves obtained previously for LMC_1 measured *in situ* (Moore & Dixon 1977*b*)

days respectively. Thus only 48% of all treated rats remained tumour-free for 100 days after implantation, and none of these had overt metastases at post-mortem.

Local recurrence developed within the muscle wall or occasionally in the skin at the site of the excision. Recurrences were detected readily within 10–20 days of excision, as a firm 2–3 mm diameter nodule that developed quickly into a spherical tumour with growth characteristics indistinguishable from those of primary tumours (Figure 1B). The incidence of local recurrence depended, however, upon the time and therefore the diameter (Table 1, second column) at which the primary was excised. The greatest number of recurrences occurred after the excision of 8 mm diameter tumours at twelve days from implantation (Table 1, Figure 2). After excision of 16 mm tumours at 20 and 28 days local recurrence rates were reduced to the same levels as for rats from which 1–4 mm tumours were removed within eight days of implantation (Figure 2).

Metastases following surgery were detected at one or more of six sites: commonly in ipsilateral inguinal, axillary and para-aortic lymph nodes and, less frequently (usually at post-mortem), in lungs, thymus, and at the adrenal glands (Figure 3). In the lungs and at the adrenals, metastases were nearly always bilateral. Although inguinal and axillary metastases only became palpable about 20 days later than locally-recurrent tumours, their growth rates thereafter were similar and thus also characteristic of LMC_1 (Figure 1 C, D). However, analysis of the post-mortem data of the mean diameter of the metastases found within the para-aortic nodes, lungs and at the adrenals (Figure 4) indicated that either these arose later than in the inguinum and axilla or that they grew more slowly, particularly in the lungs and adrenal.

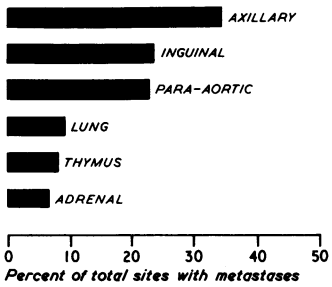

Figure 3. Frequency distribution of metastases in rats positive for tumour at post-mortem

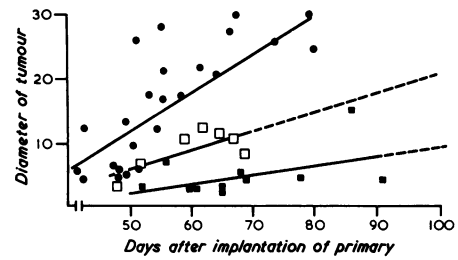

Figure 4. Growth curves for metastases of LMC_1 in para-aortic nodes, adrenal and lungs. Each data point represents the mean diameter of an individual metastasis measured at post-mortem on day shown. O, para-aortic node; □, adrenal; ■, lung. Growth curves fitted by method of least squares

In contrast to local recurrence, the number of animals that developed metastases increased progressively with delay in excision of the primary tumour and was maintained in animals subjected to surgery at 20 and 28 days (Figure 2). However, in affected rats the number of 'available' sites with metastases was independent of the time of surgery (Table 2).

Discussion

With LMC_1 tumours excised at or before 8 days from implantation, distant metastases never occurred without an animal first developing a local recurrence (Table 1), suggesting that in these groups the few metastases detected originated from the recurrent tumour. In all experiment groups, local recurrence could be attributed either to cells disseminated by surgery, or to their local infiltration beforehand. If due to surgery the incidence of recurrence should increase with the size and hence the time elapsed before the primary was removed (Ketcham & Sugarbaker 1977). However, for LMC_1 excised after 12 days the reverse occurred (Figure 2). Moreover, nearly all local recurrences clearly originated within the muscle of the abdominal wall rather than in the overlying fascia and skin. Local cellular infiltration before surgery is thus the more likely mechanism of local recurrence and, if so, this happened mainly between 8 and 12 days and decreased rapidly thereafter.

For other tumours, neovascularization of implanted tissue is necessary for distant metastases to occur (Butler & Gullino 1975, Folkman & Tyler 1977, Kleinerman & Liotta 1977, Warren *et al.* 1978). For LMC_1 significant numbers of distant metastases occurred only when the established tumour was excised at 12 days or more after implantation (Table 1, Figure 2). A latent period is also required for the development of metastases from other histologically different transplantable tumours (Greene & Harvey 1960, Paslin & Triglia 1977, Liotta *et al.* 1974) and in the MTW9 mammary carcinoma of the rat (Butler & Gullino 1975) and the T241 fibrosarcoma of C57B1 mice (Kleinerman & Liotta 1977) precedes the shedding of 10^5 and 10^6 cells per gram of tumour tissue per day into the efferent blood supply. Also, although a demonstrable lymphatic network may be absent from the tumour, 'convective currents' within the extracellular elements represent a major factor in the release of cells into the lymphatic system of the host (Gullino 1977). If these processes occur in LMC_1 with its cell loss factor of about 30% (Moore & Dixon 1977*b*) more than 10^5 cells would be shed from the primary tumour between 8 and 20 days from implantation. Since about only 10^2 cells are required to transplant LMC_1 subcutaneously with 100% success (Speakman, unpublished) the 80% rise in the incidence of metastases appearing in rats after excision of the primary between these times is readily accounted for.

In spite of the propensity of LMC_1 to spread to a number of anatomical sites (Figure 3), the mean number of sites positive for metastases in affected animals did not increase with progressive delay in excision of the implant (Table 2). There may be therefore a limit to the number of sites able to support the growth of LMC_1 at any one time, even though all potential

Table 2. Number of sites with metastases. No animal with an LMC_1 tumour excised at 1 or 8 days developed metastases, and only 1 at 3 days (4 sites). Standard errors of the mean are calculated on the binomial probability of metastases growing at each of six sites (Figure 2) per affected animal

Day primary excised	Number of rats with metastases	Number of sites with metastases	Mean number of positive sites per rat
6	3	7	2.3 ± 0.4
12	12	31	2.6 ± 0.2
15	16	37	2.3 ± 0.1
20	16	37	2.3 ± 0.1
28	15	37	2.5 ± 0.1

sites may be seeded with viable cells. This in turn may represent simply a limit on the total body burden of tumour tolerated by the host, or an active suppression of newly seeded cells by actively growing metastases. Others have postulated that primary implanted tumours may inhibit the growth of distant metastases (Schatten 1958, Ketcham *et al.* 1961, Van den Brenk & Sharpington 1971, Sheldon & Fowler 1973, Gorelik *et al.* 1978). In LMC_1 after excisions at 12 days or more, as the overall incidence of local recurrence decreased, overall incidence for distant metastases increased (Figure 2). In those animals with local recurrence the incidence of distant metastases is low compared with those who remained tumour free locally (Table 1), and the difference in mean survival time between these two groups was not sufficient to account for this. Metastases in para-aortic and adrenal nodes, thymus and lungs appeared to develop later and/or grow more slowly than those in axillary and inguinal nodes (cf. Figure 1B, C, Figure 4).

In general, the pattern of spread and overall incidence of metastases observed for LMC_1 after excision was similar to the data obtained when the primary tumour was left *in situ* and irradiated locally with ^{60}Co gamma-rays and/or the host was pretreated with cyclophosphamide (Moore & Dixon 1977*a*). In that study, treatment with drug appeared to enhance the incidence of metastases and facilitate their more widespread distribution, compared to the effect of irradiation alone. This may be explained now by the low incidence of occult metastatic foci present at the time of using either irradiation or cyclophosphamide 10–12 days after implantation of the tumour, and the additional 4–10 days delay before irradiation when both forms of treatment were combined. These particular time intervals would be sufficient to permit the release of surviving clonogenic cells from the relatively drug-resistant primary tumour (Dixon *et al.* 1978) to reseed potential sites following the systemic but transient effect of the cyclophosphamide. Similar effects may be expected with other forms of combined treatment, involving surgery, irradiation and/or chemotherapy, for experimental tumours and should also be considered as a possibility in the design of clinical regimes of cancer therapy.

Summary

The incidence and growth is reported of local recurrence and metastases – to axillary, inguinal, para-aortic nodes, lungs, thymus and adrenals – from a spontaneous breast carcinoma transplanted subcutaneously into 172 isologous 150–200 g virgin female Wistar rats.

Of 28 controls, 45% developed metastases. In 145, after excision of the tumour at 1 to 28 days, 9% developed recurrence, 12% recurrence and metastases, 31% metastases alone, and 48% remained tumour-free. After excision at 1–8 days, local recurrence and metastases was 10%, but increased to 50% at 12 days. After surgery at 15 to 28 days, local recurrence was reduced to 10%, but metastases occurred in about 90% of animals.

The average number of sites with metastases was 2.5 per affected animal, and was independent of the time of surgery. Local recurrent tumours and metastases in axilla and inguina grew at the same rate as the primary in control animals, but metastases at other sites either grew more slowly or arose later.

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