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The lymphnodal clonogenicity and kinetics of metastatic cells disseminated by a transplanted rat carcinoma

B. Dixon, D.A. Bagnall & H. Speakman

Radiobiology Department, Regional Radiotherapy Centre, Cookridge Hospital, Leeds LS16 6QB, UK.

Summary We report data on the transplantation of primary tumour cells and of lymph nodes containing metastatic cells disseminated by a mammary carcinoma (LMC_1) implanted s.c. in the Johns' Strain Wistar rat. A new method is described for deriving the TD_{50} of metastatic cells and for comparing their lymphnodal clonogenicity in the transplanted and the original, i.e. 'primary' tumour host. The TD_{50} for transplanted primary LMC_1 cells was ~12 (fiducial limits 8–20 cells), and the latency of the 8–10 mm tumours formed (T_{8-10}) after inocula of 10^2 to 10^5 cells decreased linearly with the logarithmic increase in the number of cells injected. From the T_{8-10} and tumour incidence data for transplanted inguinal, axillary and para-aortic nodes, the TD_{50} for metastatic cells was calculated to be 1120 cells (fiducial limits 790–1603 cells) indicating that the clonogenicity of naturally disseminated metastatic cells was about a 100 fold lower than that determined for transplanted primary tumour cells. The incidence and T_{8-10} data for axillary, inguinal and para-aortic lymph node metastases in primary-tumour-excised hosts suggests that, although metastatic cells may continue translymphnodal dissemination *in situ*, their TD_{50} is still consistent with that determined by node transplantation.

In contrast to the wealth of data on the cellular transplantation of experimental tumours there are few concerning the further seeding of cells that may be released once tumour growth is established *in situ*. In particular, the lack of an *in vivo* clonogenic assay for spontaneous metastatic cells has limited therapeutic studies of this aspect of the cancer problem to the experimental use of clinical end-points, e.g. prolonged survival or increased survival rates (Van de Velde *et al.*, 1977; De Ruiter *et al.*, 1982; Wondergem *et al.*, 1985). In the 1950s methods for assaying the clonogenicity and transplantation kinetics of primary tumour cells were developed (Hewitt, 1958; Berry & Andrews, 1961) and this produced rapid progress in quantifying the principal factors governing the clinical outcome of primary tumour therapy (Steel, 1977). A similar development in experimental tumour metastasis could be just as important, e.g. in defining the basic principles of adjuvant treatment of patients at significant risk from occult metastases (Salmon, 1977).

As indicated by Porter *et al.* (1973), a limiting dilution (TD_{50}) assay would be helpful in the study of tumour metastasis but, presumably due to the previous lack of a suitable animal model (Carr & Carr, 1981; Fidler & Hart, 1982; Kim, 1984; Vandenris *et al.*, 1985) such an assay for metastatic cells naturally disseminated by a transplanted tumour has not been reported. However, we have been able to assess the numbers of metastatic cells present in the inguinal and axillary lymph nodes of rats after implantation of an isologous mammary carcinoma LMC_1 (Speakman & Dixon, 1980; 1981). More importantly the method adopted was similar to a dilution assay but to avoid the need to isolate metastatic cells from a node, their numbers were assayed by determining the latency of the tumour it formed after transplantation to a fresh host. From the latency and incidence of tumour positive nodes, it is also possible to derive a TD_{50} curve for metastatic cells and thus provide the basis of an *in situ* cell survival assay after therapy (Speakman, 1986). In this paper we present and discuss our data on the lymphnodal clonogenicity of untreated metastatic cells.

All the data were derived from only a few LMC_1 generations using liquid-N_2 stored material and no changes in tumour transplantability or growth characteristics were observed. Three types of experiment were performed. The first to determine the TD_{50} of LMC_1 cells derived directly from the primary tumour. The second involved the transfer of axillary, inguinal and para-aortic lymph nodes from tumour-bearing to fresh hosts to determine from the incidence and latency of tumours formed the TD_{50} of transplanted metastatic cells. In the third, the subcutaneous primary tumour was excised at various times after its implantation, leaving the nodes to form overt metastases and thus determine their incidence and latency in the primary host.

Correspondence: B. Dixon.
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Materials and methods

Animals

Virgin female 180–200 g Johns' Strain Wistar rats, produced by strict inbreeding within the laboratory were used. They were allowed free access to food and water, maintained in a 12 h alternating light/dark regime and kept in groups of five or six throughout. Only aseptic surgical procedures were used.

Tumour

LMC_1 arose spontaneously in 1972 in an ex-breeding female and was maintained initially by serial s.c. transplantation until storage in liquid N_2 at the 35th generation. By this stage the histological differentiation of the tumour had been lost, but its growth rate, and the median cycle time of its clonogenic cells remained unchanged (Moore & Dixon, 1977*a*). Prior i.p. injection of 200 Gy irradiated minced tumour tissue, or s.c. inoculation with 10^6 irradiated tumour cells, does not influence the cellular transplantability of the s.c. tumour (Table I). In addition we have shown that axillary, inguinal and para-aortic nodes metastases have the same cellular kinetic and macroscopic growth rates as the untreated primary tumour (Carter *et al.*, 1980; Dixon & Bagnall, 1985). It was not established if the original or 1st to 35th generation tumour metastasized, but this property was present from 37th generation (Moore & Dixon, 1977*b*). In this study only the 37–45th tumour generations were used.

Cell transplantation assay

Suspensions of single tumour cells were prepared by the sedimentation of minced primary tumour tissue in PBS and syringing of the supernatant (Speakman, 1986). These were sampled to count microscopically intact trypan blue excluding cells and then diluted to contain the required number per 0.1 ml of inoculum. Four widely spaced abdominal sites per rat were used, each receiving the same s.c. inoculum. All sites, 8–80, depending upon the number of cells per inoculum, were scored for up to one hundred days to determine the percentage developing tumours. These were measured daily to determine when they attained a mean diameter of 8–10 mm (T_{8-10}) and that they subsequently exhibited the growth rate of the LMC_1. In some groups (Dixon & Bagnall, 1985; Figures 2 & 3) where less than 10^3 cells were given per site, rats developing either one, or two (bilateral) tumours, had these excised at T_{8-10} to allow for the possibility of tumours developing in the remaining sites.

Lymph node transplanatation assay

Animals were implanted s.c. with a primary tumour in their right posterior abdominal flank (Moore & Dixon, 1977*a*), and randomized for killing 3 to 30 days later. At post-mortem, the ipse-lateral inguinal, axillary and para-aortic nodes were transplanted immediately to a fresh host, using three of the sites used for single cell assay. All rats were then scored for up to 100 days to determine the incidence, T_{8-10} and growth rate of the tumours formed. In some groups where there was only a low take-rate, individual tumours were excised at T_{8-10} to show that there was no inhibition of negative sites. Also for other groups only the para-aortic node was transplanted to check that a simultaneous three node (i.e. inguinal, axillary and para-aortic) as opposed to a single node transplant, was without effect.

Lymph nodes in situ

Rats were implanted, randomized, and their growing primary tumours excised (Dixon & Speakman, 1979) up to 26 days later. After surgery, all animals were scored for up to 100 days to record the incidence of ipse-lateral inguinal, axillary and para-aortic lymph node metastases. Although metastases may also occur at other sites (Moore & Dixon, 1979*b*; Dixon & Speakman, 1979), positive inguinal, axillary and para-aortic nodes, develop earlier and adrenal and iliac lymph node metastases do not occur without seeding of the para-aortic node (Dixon & Bagnall, 1986).

For rats excised at 13–26 days, all axillary and inguinal lymph node metastases detected were measured to determine their T_{8-10} relative to the day the primary tumour was excised and to confirm their growth was characteristic of LMC_1. Para-aortic metastases could also be detected by palpation and their growth measured, although imprecisely, through the abdominal wall. However, at post-mortem all animals were examined for para-aortic metastases. These were removed to obtain their mean diameter and the day on which they had reached 8–10 mm diameter was determined from the growth curve for LMC_1 (Dixon & Bagnall, 1985, 1986).

Results

Primary tumour cell assay

No tumours formed in any of 80 sites inoculated with 3.3×10^{-2} cells, and all developed tumours if given 10^4 cells or more. Between these limits, the percentage of sites with tumours increased from 2.5 to 100% (Figure 1) and all data could be readily

Table I Transplantability of primary tumour cells in naive and 'immunized' hosts

	Number of cells injected	*Number of sites*	*Percent positive sites (±s.e.)*	*Latency of tumours (days ± s.e.)*
Naive	0.2	24	12.5±6.7	46
	20	22	50.0±10.6	46±8
	200	24	91.7±5.9	31±3
	200,000	39	100	9±1
'Immunized'	0.2	24	16.7±7.6	44
	20	47	40.4±7.2	42±4
	200	52	86.5±4.7	30±4
	200,000	25	100	9±3

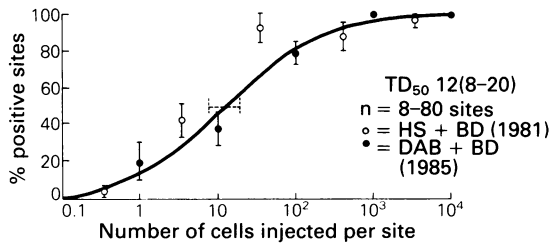

Figure 1 Transplantation of primary LMC_1 cells. All data; mean ±1 s.d.; one zero and six 100% datum points outside the limits shown not plotted; curve fitted as described in text; |----| computed TD_{50} and fiducial limits.

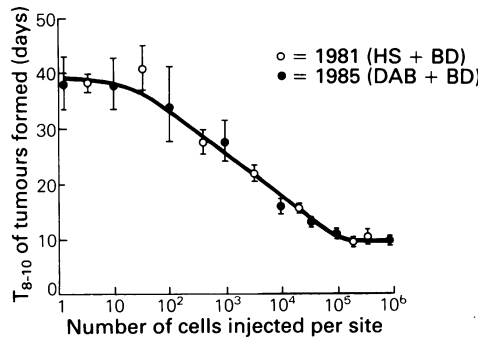

Figure 2 The latency of LMC_1 tumours formed by s.c. transplantation of primary tumour cells. All data, mean ±1 s.d.: n=6–38, depending upon the percentage takes.

fitted by a single probit curve (Speakman, 1986). The computed TD_{50}, i.e. the 'cell dose' that produced tumours in 50% of sites, was 12 (fiducial limits 8–20 cells), and for all data the computed chi-square was 11.96 with 13 degrees of freedom (df).

The T_{8-10} of tumours formed from inocula of 3×10^1 to 10^5 cells decreased linearly with the logarithmic increase in the number of cells injected (Figure 2). No further changes in T_{8-10} were obtained for inocula containing less than 3×10^1 or more than 10^5 cells. Analysis of all data for the tumours produced (not shown) confirmed that their growth was characteristic of LMC_1 and it was not affected either by the presence of two or more tumours in the same animal, or by the removal of all but one tumour at T_{8-10}.

Lymph node transplantation assay

The percentage of transplanted inguinal nodes producing tumours increased directly with the time they were left in the primary host (Figure 3a). In contrast, axillary nodes, from the same host, only produced tumours if they remained *in situ* for two weeks. Thereafter they produced tumours in fresh hosts at the rate observed for inguinal nodes. Consistent results were obtained for para-aortic nodes irrespective of whether they were transplanted singly or together with inguinal and axillary nodes (Table II). These data were pooled and showed that para-aortic nodes only produced tumours if they were left in the primary host for 10 days or more. Between 10 and 18 days about 35% of nodes were then found to be positive, but thereafter the percentage of tumourous nodes increased as for inguinal and axillary node transplants (Figure 3a).

In general, the T_{8-10} of tumours formed by transplanted nodes decreased the longer they were left in the primary host (Table III), and thus their latency was inversely correlated with the percentage of tumours formed. This relationship would be expected from the data for transplanted primary cells (Figures 1 and 2), and detailed analysis showed that it was the same relationship for inguinal,

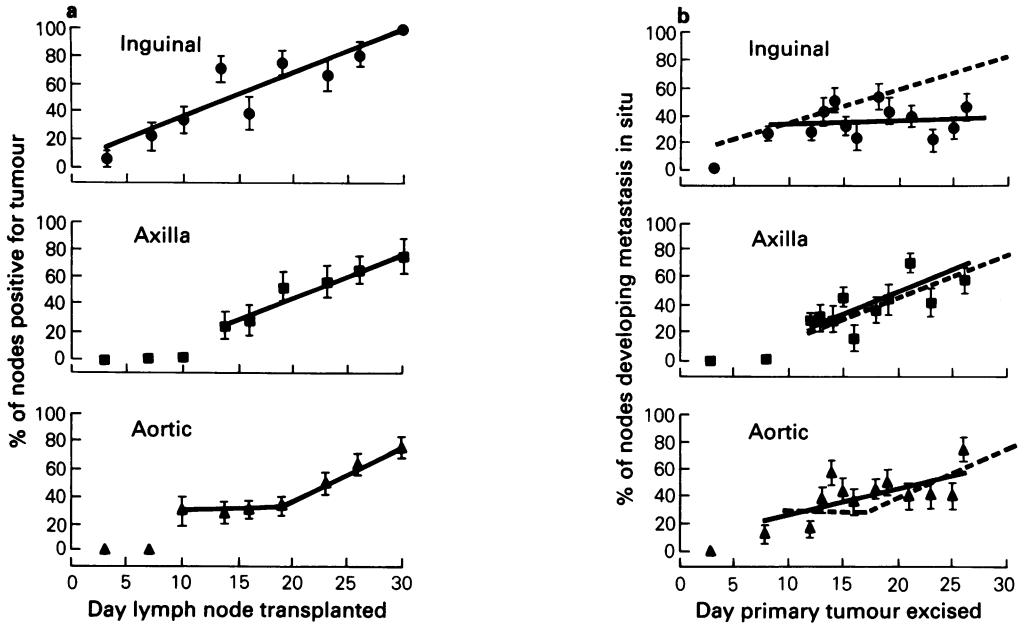

Figure 3 The percentage of nodes forming tumours in fresh and primary tumour-excised hosts.
(a) Transplanted nodes: ● Inguinal, mean ±1 s.d., $n=18$ all data fitted by least squares; ■ Axilla, mean ±1 s.d., $n=18$, 14–30 days fitted by least squares; ▲ Aortic, combined data from single and three node assay, mean ±2 s.e., $n=36$, 10–16 days free-hand fit, 19–30 days, least squares fit.
(b) *In situ* nodes: ● Inguinal, ■ Axilla, ▲ Aortic, all data means ±1 s.d., $n=24$–28. Solid lines, least squares fit of data, dashed lines, curves from corresponding transplanted nodes.

Table II Incidence of tumours formed by transplantation of para-aortic nodes

	Frequency of takes	
Day transplanted	*Pa alone*	*Pa with Ax+Ing*
10	[a]	5/18
14	2/24	14/36
16	6/23	6/18
19	9/24	6/21
23	12/23	8/18
26	8/12	14/23
30	14/20	10/12
34	12/16	[a]

[a]Not assayed.

Table III Latencies of tumours formed by transplantation of positive lymph nodes

	T_{8-10} (*days ± s.e.*)		
Day transplanted	*Inguinal*	*Axilla*	*Para-aortic*
3	27 (1)	[a]	[a]
7	35 (4)	[a]	[a]
10	30±5 (6)	[a]	30±7 (5)
14	23±6 (26)	32±9 (9)	31±10 (16)
16	25±8 (7)	38±7 (6)	31±7 (11)
19	17±11 (16)	26±5 (11)	27±8 (15)
23	18±6 (10)	19±6 (10)	23±7 (20)
26	16±7 (18)	23±8 (15)	22±7 (22)
30	16±7 (10)	13±4 (8)	18±7 (17)
34	[b]	[b]	17±7 (12)

[a]No positive nodes detected; [b]Not assayed.
Numbers in parentheses show number of measurable positive nodes.

axillary and para-aortic nodes. The T_{8-10} data (Table III) were therefore used to derive (from the data shown in Figure 2) the mean number of tumour cells present in positive nodes when removed from the primary host, and then plotted as a function either, of their time in the primary host (Figure 4a), or the incidence of tumours formed by transplantation of all (i.e. negative and positive) nodes to fresh hosts (Figure 5).

Between 5 and 14 days *in situ*, the mean number of metastatic cells in inguinal nodes increased from about 10^2 to 2×10^3, but between 14 and 19 days *decreased* to about 8×10^2 (Figure 4a). This apparent loss of metastatic cells coincided with the initial detection of about 10^2, and 2×10^2 tumour cells in the axillary and para-aortic lymph nodes respectively. After 19 days the mean number of metastatic cells in the inguinal node again increased, to about 2×10^4, but by then similar numbers were present in the other nodes. For the axillary node however, a transient loss of about 4×10^3 cells, may also have occurred between 23 and 26 days.

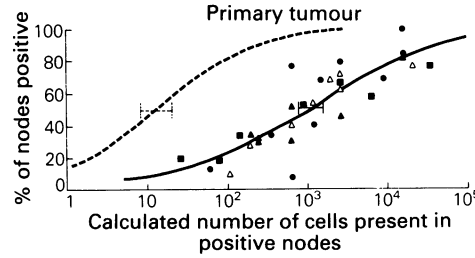

Figure 5 Data for metastatic cells spontaneously disseminated to and then transplanted within intact nodes removed from the primary tumour host. ● Inguinal, ■ Axillary, △ Aortic (single node), ▲ Aortic (with axillary and inguinal node), $n=18$–23 nodes transplanted to obtain each datum point. Zero take points not shown or used in computation of the fitted curve (——), the TD_{50} and its fiducial limits (|——|). The TD_{50} curve (----) established for transplanted primary tumour cells (**Figure 1**) is shown for comparison.

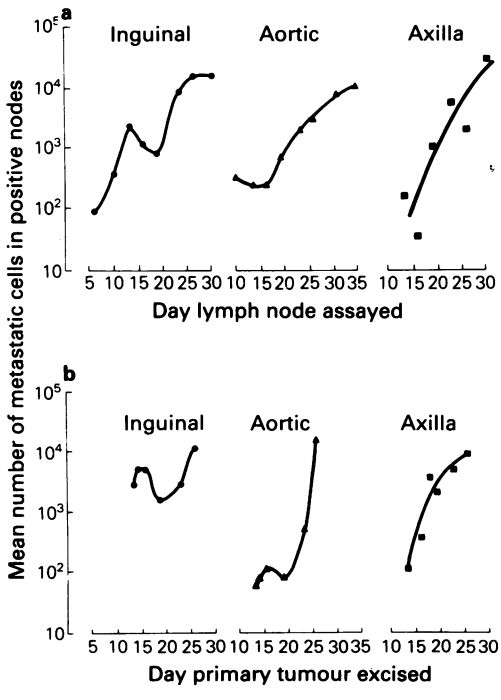

Figure 4 The mean numbers of metastatic LMC_1 cells in positive lymph nodes.

(a) Assayed by node transplantation to fresh host at times shown after primary tumour implantation. All curves are a free-hand fit, means calculated from data (**Table III**).

(b) Assayed in the primary tumour-excised host. All curves are a free-hand fit, means calculated from data (**Table V**).

Despite differences in the time of first detection and subsequent changes (Figure 3a), when the number of malignant cells present (Figure 4a) was plotted against the percentage of all nodes that produced tumours on transplantation (Figure 3a) all the data could be fitted ($\chi^2=17.73$, 17 df, $P=0.406$) with a common curve (Figure 5). Omitting zero takes, for which no estimate of cell number may be made, the computed TD_{50} was 1120 cells (fiducial limits 791–1603 cells). Thus this analysis indicates that metastatic cells in transplanted nodes have a clonogenicity ~100-fold lower than for primary tumour cells transplanted to the same sites. This conclusion applies for each type of node assayed, i.e. a single TD_{50} curve could be readily fitted to the data for each node and the $\sum\chi^2$ for all nodes was not significantly less than that for the pooled data (Table IV).

In situ *lymph node assay*

Few rats developed nodal metastases within 3–12 days after excision of their primary tumours (Figure 3b). Also there was no significant ($P>0.10$) increase in the incidence of positive inguinal nodes after 8–26 day excisions, and overall only $37\pm2.6\%$ (s.e.) developed metastases at this site. In contrast, axillary metastases after 12–26 day excisions increased at almost the same rate as tumours were formed by transplanted nodes. The data for metastasis to the para-aortic node were equivocal. Although their incidence was dependent on the time of surgery ($0.02<P<0.05$), the data were also

Table IV Computed parameters for transplanted lymph nodes

Lymph node	TD_{50}	*95% Fiducial limits*	χ^2	*Degrees of freedom*	*Goodness of fit (P)*
Inguinal	890	449–1553	6.25	6	0.396
Axillary	1155	367–4928	3.10	5	0.684
Para-aortic[a]	1170	678–2183	6.84	5	0.233
Para-aortic[b]	1541	772–4974	0.95	3	0.813

[a]Para-aortic node only transplanted; [b]Para-aortic node transplanted together with inguinal and axillary node; χ^2 pooled $-\sum\chi^2 = 0.59$, with 3 df ($P = 0.90$).

Datum points for lymph nodes calculated to contain the same number of cells but giving different percentage takes on transplantation were combined for computer probit analysis. Thus the degrees of freedom were based on $n-2$ where n is the reduced number of datum points used to derive each TD_{50} and its fiducial limits.

consistent (chi-square = 0.885 with 11 df) with the results for transplanted nodes.

The latencies of metastases were only determined during the later stages of the work (Dixon & Bagnall, 1985; 1986) and by day 26 the size of the primary tumour prevents its complete excision. However there were sufficient data (Table V) to compare with those obtained by node transplantation (Figures 4a and 5). The 'growth curves' for the metastatic cells *in situ* were similar to those determined by transplantation. The mean number of metastatic cells present in inguinal and axillary nodes between 13 and 19 days was however about double that estimated by transplantation. Conversely, over the same period fewer were present after *in situ* assay of the para-aortic nodes (Figure 4). Because of the greater errors in determining the mean latency of metastases *in situ* (Table V), these differences may not be significant.

Because metastases developed only in 20–60% of animals (Figure 3b), and there was a limited range of latency data (Table V), no independent TD_{50} analysis was attempted. However comparison of the data with the TD_{50} curves for transplanted primary and metastatic cells was possible (Figure 6). The *in situ* data were not compatible with the TD_{50} curve for primary tumour cells, although widely scattered they were better represented by the TD_{50} curve for transplanted metastatic cells, suggesting that in the primary host the TD_{50} for lymphnodal metastasis may also be ~1000 cells.

Table V Latency of metastases *in situ* after primary tumour excision

Day 1° excised	T_{8-10} (*days* ±*1 s.d.*) *Inguinal*	*Axillary*	*Para-aortic*
13	22±6 (11)	33±12 (9)	35±15 (10)
14	20±6 (14)	29±6 (8)	34±8 (16)
16	20±5 (6)	21±7 (4)	33±10 (9)
19	24±10 (10)	23±8 (11)	34±9 (12)
23	22±9 (5)	20±8 (10)	28±7 (8)
26	17±6 (11)	18±8 (14)	16±7 (18)

24–28 animals assayed on each of the days shown. Figures in parentheses show number of rats with measurable metastases at stated site.

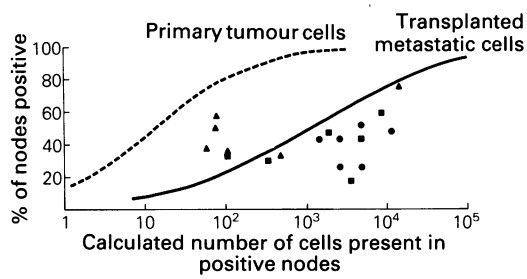

Figure 6 *In situ* TD_{50} data for metastatic cells in the lymph nodes in primary tumour-excised rats. ● Inguinal, ■ Axilla, ▲ Aortic nodes. The number of animals excised, and the incidence and latency of metastases formed is given in **Table V**; —— TD_{50} curve for transplanted lymph nodes, ---- TD_{50} curve for primary tumour cells.

Discussion

Lymphatic metastasis does not exclude haematological dissemination (Weiss, 1985), but the latter plays no significant role in inguinal, axillary and para-aortic lymph node metastasis of LMC_1 and is not considered further. For convenience of

presentation we discuss separately: the transplantation of primary tumour cells, the nodal transplantation of metastatic cells, and because they are clearly interdependent, the *in situ* seeding and translymphnodal passage of metastatic cells. Despite their potential significance, these aspects of spontaneous metastasis, aside from the work of Hewitt and Blake (1975; 1977), have not been studied previously using non-immunogenic tumours.

The transplantation of primary cells

With a TD_{50} of between 8 and 20, relatively few primary tumour cells were required for the direct transplantation of LMC_1 subcutaneously and nearly all sites developed tumours if inoculated with 35 cells or more (Figure 1). For those given less than 35 cells, the mean latency of the tumours formed remained constant at about 37 days (Figure 2) and this presumably represents the average time needed for sites seeded by one clonogenic cell to produce a T_{8-10} tumour. For inocula containing from 10^2 to 10^5 cells the data indicate (Bruce *et al.*, 1967) that cells in occult subcutaneous LMC_1 tumours have a population doubling time of about 2.5 days. This compares with a doubling time of 2 days measured (Moore & Dixon, 1977*a*) for T_{8-10} tumours formed by the implantation of a pellet of minced LMC_1 tissue containing about 10^4 viable cells (Speakman, 1986). This suggests that the growth curve of LMC_1 may be comparable before and after its subcutaneous detection.

According to Figure 2, 1.4×10^6 cells should have produced an 8–10 mm diameter tumours immediately. However the minimum T_{8-10}, achieved with 10^5 cells or more, was about 10 days (Figure 2). This presumably reflects, firstly the limits of subcutaneous and abdominal wall microvasculature in meeting the initial requirements of more than a few thousand cells and secondly, the time required to meet this demand through neovascularization (Folkman & Tyler, 1977) and the provision of other stromal elements required for sustained tumour growth (Falk, 1980). This minimal latency corresponds, to within a day or so, to the time required for implanted LMC_1 tumour tissue to vascularize (Speakman, 1986), initiate sustained growth and produce a significant incidence of occult metastases (Speakman & Dixon, 1981; Dixon & Bagnall, 1985, 1986; and Figure 3b).

The transplantation of metastatic cells

Even when transferred within lymph nodes, metastatic LMC_1 cells still exhibited single cell transplantation kinetics (Figure 5). Their reduced clonogenicity may not, however, be attributed simply to lymphnodal transplantation since the *in situ* data were comparable with those for transplanted metastatic cells (Figure 6). Also for LMC_1 tumour-specific immunity does not occur. The tumour is isogeneic and pre-immunisation either with viable tumour tissue which is then later completely excised (Dixon & Bagnall, unpublished), or with radiation sterilized LMC_1 cells or tissue, does not change its transplantability (Speakman, 1986, and Table I). Although non-immune host response factors produced by a macroscopic primary tumour may lead to 'metastatic inefficiency' (Weiss, 1985), with LMC_1 this should exert no effect once nodes had been transplanted to fresh hosts. Nevertheless this will need to be investigated experimentally, e.g. by the disaggregation of positive nodes and subcutaneous transplantation of the metastatic cells released, and by the determination of the latency of transplanted normal nodes after their injection with known numbers of tumour cells. However, with the CBA Carcinoma NT, which has a relatively high TD_{50} of about 4000 cells, the release of nodal metastatic cells *reduced* their tumour forming capacity and conversely the incorporation of tumour cells within normal nodes, increased their transplantability (Hewitt & Blake, 1977). Thus, if such effects occur with LMC_1 the transplantation of naturally disseminated metastatic cells within intact nodes should have reduced rather than increased their TD_{50}. There are, however, two other mechanisms that could have lead to the differences observed, *viz*, tumour cell heterogeneity (Fidler & Poste, 1982) or a Révész Effect (Révész, 1956).

Malignant cells within a tumour are known to be heterogeneous in many respects (Owens *et al.*, 1982) and it may be argued that whereas experimental disaggregation of a primary tumour selects for highly clonogenic cells, the metastatic dissemination of cells does not. With other tumours when highly metastatic cell populations have been isolated, this usually involves their *in vitro* passage (Weiss, 1985). For LMC_1 this does not provide unequivocal evidence that spontaneous metastases must arise from subpopulations of either high or low clonogenicity. If natural selection for metastatic properties does take place, for LMC_1 this could have already been completed during the sequential transplantation history of the tumour. With LMC_1, metastases from the 37th and subsequent tumour generations have the same microscopic DNA content, cell-cycle and macroscopic growth characteristics as primary tumour cells (Carter *et al.*, 1980; Dixon & Bagnall, 1985). Moreover, subcutaneous tumours produced by the transfer of LMC_1 positive lymph nodes, metastasize no more frequently than tumours produced by the direct passage of the primary tumour. In addition the

sequential passage of LMC_1 by the transplantation of tumour positive lymph nodes still does not enhance metastasis (Dixon & Bagnall, unpublished).

The TD_{50} of many tumours may be reduced by a factor of 10^2 or more by adding an excess of radiation sterilized cells to the viable inoculum to stimulate the formation of fibrin at the site of assay (Révész, 1956, 1958; Peters & Hewitt, 1974; Steel, 1977). However, for LMC_1 a Révész Effect could only reduce the TD_{50} of primary tumour cells 8- to 20-fold and their clonogenicity may already be fully enhanced by the non-clonogens and angiogenesis factors present within inocula prepared directly from the macroscopic tumour (Folkman & Tyler, 1977). In contrast, the natural dissemination, trapping and nodal transplantation of occult metastatic LMC_1 cells requires no direct, i.e. cellular manipulation, and insufficient to have stimulated angiogenesis (Figure 4) within the node were involved. Also lymphoid cells are unable to produce a Révész Effect (Hewitt *et al.*, 1973; Steel, 1977) and metastatic cells contained within the node may not be exposed to fibrin stimulated to form by node transplantation. Although also requiring further investigation, these contrasting circumstances could readily account for the 100-fold difference in the TD_{50} of transplanted primary tumour and metastatic LMC_1 cells. Irrespective of the mechanism, however, a clonogenic assay is only valid if the method used has no influence on the clonogenicity of tumour cells and this is not true of LMC_1. On this basis therapeutic studies of tumour 'metastases' initiated by the intravenous or intra-lymphatic injection of primary tumour cells may over-estimate their clonogenicity and the therapy required to ablate spontaneous metastatic foci.

The seeding and lymphnodal kinetics of metastatic cells

Although the *in situ* data conform approximately to the TD_{50} curve derived for transplanted metastatic cells, their poor fit (Figure 6) indicates that factors additional to clonogenicity may be involved in nodal metastasis in the primary host. About one third of cells spontaneously disseminated by a transplanted tumour may be micro-emboli of 2–8 cells or more (Liotta *et al.*, 1976). However, emboli of 2, 3, or 4 clonogenic cells etc., would have progressively smaller TD_{50}s and steeper transplantation curves than single cells. Alternatively, if each clonogenic cell was surrounded by non-clonogens, this may change the TD_{50} but not the steepness of the curve. Neither of these mechanisms therefore provides a ready explanation of the *in situ* data which although widely scattered suggests a clonogenicity comparable with transplanted metastatic cells.

Variability in the TD_{50} data would occur if nodes differed in their ability to trap and initiate the growth of metastatic cells or if they continued to spread through the lymphatics after excision of the primary. No significant variability was observed for each of the nodes when assayed by transplantation and the TD_{50} for each was about the same (Table IV). However, whilst transplantation 'traps' metastatic cells within nodes they may continue dissemination, although possibly at a modified rate in the excised host. If this is the factor modifying the *in situ* data it is amenable to assay, e.g. by the use of the lymph node transplants at various times *after* primary tumour excision. However we have already shown that whereas inguinal and axillary nodes are seeded by the primary tumour, para-aortic nodes are seeded largely by cells from already positive nodes (Dixon & Bagnall, 1986). It is presumably this same mechanism that delays the increased frequency of tumour formation by transplanted para-aortic nodes (Figure 3a), and is expressed in cellular terms when latency is used to derive the numbers of metastatic cells they contain (Figure 4).

For LMC_1, about 100 cells are required to produce positive inguinal nodes (Figure 4a). When these are left *in situ* for a further 7 days their metastatic cell content is increased with an effective population doubling time of about 2 days, i.e. similar to that determined for subcutaneously injected single cells. When the metastatic cell content in the inguinal node reaches about 2×10^3 cells, i.e. a tumour cell population at least a 100-fold smaller than that required to demonstrate physiological inhibition in the development of tumours produced by single cell inocula (Figure 2), there is a transient loss of cells. Since no comparable loss is indicated for axillary and para-aortic nodes transplanted with this number of cells (Figure 4a), and it also occurs for inguinal nodes left in the primary host (Figure 4b) it is most unlikely to be an artefact. Qualitatively similar changes in the number of metastatic cells passing through the prepopliteal nodes draining the footpad of rats given 2×10^7 Walker carcinoma cells 6 days previously has also been reported (Carr & Carr, 1981).

Hewitt and Blake (1975) have also shown, using the isogeneic WHT carcinoma, that the translymph-nodal passage of spontaneously disseminated cells may occur. With their intradermal tumour, about 40% of axillary nodes were positive on assay in fresh hosts, irrespective of their time of removal from the primary host. However, only about 4% of primary tumour-excised hosts developed axillary metastases and they concluded that disseminated tumour cells mostly only pass through the node to the blood, to be destroyed. Later Hewitt and Blake

(1977) supported this conclusion with data for four other tumours but in each case only did one comparative transplantation/*in situ* assay. Our data for LMC_1 shows, however, that their conclusion may not be applied indiscriminately to all tumours and indeed to all lymphatic nodes draining the same tumour.

For LMC_1 no significant loss of metastatic cells occurs from the axillary node, i.e. the incidence of positive nodes was the same *in situ* and after transplantation, and once initiated the incidence of metastases was directly dependent on the duration of lymphatic drainage of the primary tumour (Figures 3a, b). In contrast, comparison of the two sets of data for the inguinal node clearly indicate an early and later a substantial loss of metastatic cells from the node when left *in situ* after excision of the primary tumour. Although equivocal, comparison of the *in situ* and transplant data for the para-aortic node (Figures 3c, 4), indicates that after its inital seeding and the passage through it of inguinal node-disseminated tumour cells, no further significant loss of metastatic cells may occur. From these data it may also be concluded that, unlike the WHT carcinoma, the LMC_1 tumour has a high overall rate of initiating lymphatic metastases *in situ*, and that the translymphnodal passage of tumour cells when it occurs has a high probability of seeding metastases elsewhere, e.g. in the mediastinum (Dixon & Bagnall, 1985, 1986).

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