Eur J Cancer Clin Onol, Vol. 17, No. 12, pp. 1287–1293, 1981.
Printed in Great Britain.

0277-5379/81/121287-07$02.00/0

An Experimental Approach to Chemotherapy for Occult Metastases: a Quantitative Model

H. SPEAKMAN*† and B. DIXON‡
University Department of Radiotherapy and ‡Radiobiology Department, Cookridge Hospital, Leeds LS16 6QB, U.K.

Abstract—*The population doubling time (PDT) of LMC_1 tumour cells in axillary and inguinal lymph nodes of tumour-bearing rats was estimated during the occult development of secondary tumours. Values increased with time and* 13 days *after implantation of the primary the PDT and the mean cell cycle time were the same, suggesting all tumour cells were in cycle. This information was used to predict the optimum time for administration of cyclophosphamide (CP) and to design rational treatments combining surgery with chemotherapy.* 150 mg/kg *CP, given* 13 ± 1 days *after implantation of the primary, reduced the incidence of metastases from* 50% *(in the surgery alone group) to zero. If the primary was left* in situ *reseeding was rapid and the incidence of metastases increased with time from drug administration. Within* 13 days 50% *of rats had metastases. However, a second dose of* 150 mg/kg *CP given at this time again reduced the incidence rate from* 50% *to zero. Lymph node metastases in rats given surgery alone reached* 8–10 mm *mean diameter* (T_{8-10}) 47.0 ± 3.0 days *after implantation of the primary. The equivalent* T_{8-10} *values for metastases reseeded from once- and twice-treated primary tumours should be about* 60 (47 + 13) *and* 73 (47 + 26) days *respectively. The observed values were* 57.4 ± 6.0 *and* 57.6 ± 2.6 days. *The reduced value for twice-treated animals suggest a greater number of cells present than expected, and this may be due to either an increase in 'seeding' efficiency or in the number of clonogenic cells shed from the primary tumour after the second dose of CP.*

INTRODUCTION

MOST solid tumours that have not metastasised at the time of clinical detection are curable by surgery or radiotherapy [1]. However, a critical reappraisal of clinical data and extensive animal studies have shown that cancer is often a systemic disease at the time of diagnosis [2, 3]. Relapse from metastases is thus a major cause of treatment failure, e.g., in breast cancer, irrespective of the method of local therapy[4]. Surgery in combination with chemotherapy has proved successful for the treatment of metastases in animal models [1], yielding cures when the residual burden of tumour cells with a high growth fraction and short generation time is smaller than the tumour cell kill potential of sublethal doses.

However, practical examination of the feasibility of designing rational approaches for the use of adjuvant chemotherapy requires the use of experimental animal models: firstly for defining the sites and cellular pathways of metastasis, and secondly for estimating the residual burden for clonogenic tumour cells, e.g., after excision of the primary tumour. Only then may the effect of adjuvant treatment be studied, e.g., in relation to the number and population kinetics of occult disseminated tumour cells.

The Leeds Mammary Carcinoma (LMC_1) is an isogenic, transplantable tumour which arose spontaneously in a breeding female John's strain Wistar rat. The tumour metastasises rapidly and reproducibly, and if the primary is either excised or its growth retarded by local radiotherapy and/or systemic chemotherapy, secondary deposits become manifest in the regional and distant lymph nodes [5, 6]. The incidence of visible metastases following excision of the primary tumour increases with the time it has remained *in situ*, and although metastatic foci may not be palpable at the time of surgery, after 10 days cells have 'seeded' in the ipsilateral inguinal and axillary nodes [5].

As reported in full in this paper and in preliminary form elsewhere[7], transplantation of (a) intact lymph nodes from tumour bearing hosts and (b) injection of isolated LMC_1 cells

Accepted 17 July 1981.
†To whom correspondence should be addressed.

into fresh isologous hosts may be used to estimate (i) the numbers of occult metastatic cells present within "tumorous" axillary and inguinal nodes, and (ii) the population doubling time (PDT) of these cells from their initial seeding from the primary tumour to the advent of palpable metastasis. We have now used this system to predict the optimum time for administration of a proliferation-dependent cytotoxic agent, cyclophosphamide [8], and have observed the control of metastatic disease *in situ* in animals whose primary tumours were excised following administration of adjuvant chemotherapy.

MATERIALS AND METHODS

Rats

John's strain Wistar rats were bred in our laboratory; 3–6-month-old, 100–150 g virgin females were used in all experiments. They were maintained in a 12 hr light/dark regime and allowed food and water *ad libitum*.

The cell assay

Tumour tissue for the preparation of monodisperse suspensions of LMC_1 cells was derived from primary tumours grown subcutaneously. At post-mortem, tissue from the margins of the tumour was dissected free and minced with scissors in a cavity block. The resulting tumour mince was transferred to a round-bottomed test tube containing 0.25% w/v trypsin in Hank's Balanced Salt Solution (HBSS, Flow Laboratories, Scotland) and agitated mechanically at 37°C for 20 min. Fragments of tumour mince were allowed to settle for 5 min and the cell suspension was transferred to another test tube with a Pasteur pipette and centrifuged at 180 g for 5 min. The resulting supernatant was discarded and the cell pellet was dispersed in fresh HBSS using a Pasteur pipette, centrifuged and resuspended in a small volume of HBSS. Fresh trypsin solution was added to the tumour mince and the above procedure was repeated, usually 4 times. Harvested cells were kept in HBSS on ice until the last incubation, when all cell suspensions were pooled, counted and examined microscopically to ensure that monodisperse cell preparations had been obtained.

A well-shaken sample of the cell suspension was counted in an improved Neubauer haemocytometer and viability, estimated by Trypan Blue exclusion, was $90 \pm 5\%$.

Serial dilutions were prepared in HBSS and, after shaking to ensure uniform dispersion, inocula containing from 3.3×10^{-2} to 4.6×10^{6} LMC_1 cells in 0.2 ml HBSS were injected subcutaneously at 4 inoculation sites per rat. The total number of inoculation sites used for inocula containing less than 1 cell exceeded the minimum required by calculation, e.g., a dilution of 1×10^{-2} would require 100 inoculation sites to be certain of injecting 1 cell. Total sites ranged from 80 (3.3×10^{-2} cells) to 8 (4.6×10^{6} cells).

The number of sites which subsequently developed tumours from each dilution of inoculated cells were recorded. Each tumour was measured daily across 3 mutually perpendicular axes and the time taken for each to reach 8–10 mm mean diameter was noted. A mean and standard error was calculated for each dilution of cells.

The lymph node assay

LMC_1 primary tumours were implanted subcutaneously in the form of 'sausages' at the right posterior abdominal flank. Tumour 'sausages' were prepared by the method of Thomlinson[9]. Briefly, tumour mince was introduced aseptically into the lumen of everted small intestine of a 50 g isologous rat. A length of gut and contents was tied at intervals along its length with surgical silk to form 'sausages' of 2–3 mm diameter. These were separated, washed twice in saline and once in distilled water to lyse any superficial tumour cells.

Groups of 10 rats were killed 1, 3, 6, 10, 12, 15, 20, 24 or 28 days after implantation. Their right axillary and inguinal lymph nodes were removed aseptically and implanted subcutaneously into fresh hosts at 2 abdominal sites per rat. 'Positive' nodes, i.e., those which gave rise to tumours, were scored and measured daily from first palpation. The time taken for each positive node to produce an 8–10 mm tumour was noted and a mean and standard error was calculated for positive nodes for each group of rats.

Adjuvant chemotherapy experiments

Other animals were assigned randomly to control and treatment groups on the day of implantation of the primary tumour. In each group there were 12 animals, and there were 4 groups of controls: untreated, surgery alone, single- and double-dose chemotherapy.

Animals randomised for surgery alone had their primary tumours excised 1, 3, 6, 12, 16, 20 or 28 days after implantation. Chemotherapy-treated animals were given 150 mg/kg (20 mg/ml) cyclophosphamide (CP) (Endoxana; Ward Blenkinsop, Wembley) intraperitoneally (i.p.) 13 days after implantation of their primary tumours. Rats randomised for two doses were

given a second dose of 150 mg/kg CP 13 days after the first, i.e., 26 days after implantation of the primary tumour. Primary tumours in untreated and chemotherapy-treated rats were measured daily across 3 mutually perpendicular axes from palpation to death of the host. These data were analysed by computer program [10]. For each day's measurements the program calculated the geometric mean of the diameters pooled from all rats within an experimental group. The mean radius ± S.E. was determined and the tumour volume and error were calculated using the formula $4/3\pi r^3$.

All animals randomised for CP in combination with surgery were given 150 mg/kg CP i.p. 13 days after implantation of the primary. Those scheduled for a single dose had their primary tumours excised 1, 3, 6, 12 or 20 days later. Rats randomised for 2 doses were given a further 150 mg/kg CP 13 days after the first and their primary tumours were excised 1, 3, 6, 12 or 20 days after the second dose.

All animals were examined to detect the development of metastases in the right axillary and inguinal nodes *in situ*. When palpated these were measured daily and the time taken for each to reach 8–10 mm mean diameter from implantation of the primary tumour was recorded. A mean value and standard error was calculated for positive nodes in each treatment group. Rats with local recurrence after surgery were excluded from the investigation.

The number of days that elapsed between implantation of the primary tumour and the killing of animals with metastases was used to calculate the mean survival time (MST) for each group of animals. Those animals which did not develop metastases were killed and examined 100 days after implantation of the primary.

RESULTS

LMC_1 tumours arising from either inoculation of single cells or from transplantation of lymph nodes exhibited the same growth curve (unpublished observations) from 9 ± 1 mm to 25 ± 1 mm as untreated primary tumours implanted by the method of Thomlinson [9].

The cell assay

The incidence of tumours arising from inoculation with isolated LMC_1 cells increased with the number injected (Fig. 1a) and the TD_{50} is thus 12.5 ± 1.7 cells [11]. The mean time taken for resulting tumours to reach 8–10 mm was plotted as a function of the number of cells

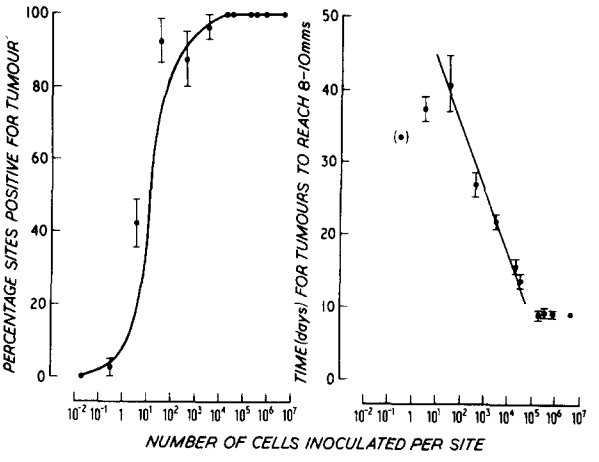

Fig. 1. (a) The incidence of tumours arising from subcutaneous inoculation with LMC_1 cell suspensions. Each point represents the mean (± 1S.D.) of 8 (10^6 cells/inoculum) to 80 (10^{-2} cells/inoculum) total sites. (b) Standard curve for subcutaneous inoculation with LMC_1 cell suspensions. Line derived by least squares fit (r = −0.98) through data points (± 2S.E.) resulting in more than 50% (Fig. 1a) and up to 100% (first incidence) takes. (●) represents one observation only.

inoculated for each dilution as a standard curve (Fig. 1b). From 35 to 3.2×10^5 cells per inocula there was a direct relationship between the logarithm of the number of cells inoculated and the mean time taken for tumours to reach 8–10 mm mean diameter.

The lymph node assay

Lymph nodes transplanted before 10 days after implantation of the primary tumour

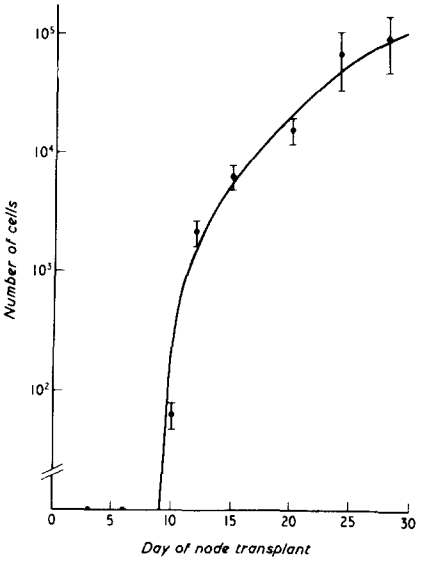

Fig. 2. The estimated number of clonogenic LMC_1 cells in axillary and inguinal lymph nodes at the time of their removal from the original tumour-bearing host and transplantation into the fresh host. Mean (± 1 S.D.) calculated for each group by interpolation on the linear relationship between T_{8-10} and the number of cells injected (Fig. 1b).

sausage did not produce tumours. Thereafter, the percentage of positive nodes increased with time of removal after implantation of the primary in the original host [7].

There was no significant difference either in tumour production rates or in the time for tumours to reach 8–10 mm mean diameter between axillary and inguinal lymph nodes in the same transplantation group, thus values were pooled.

The mean time taken for positive nodes in each group to produce 8–10 mm tumours was used to determine the number of cells in nodes at the time of transplantation into fresh hosts. This was achieved by interpolation on the linear relationship established between the time to reach 8–10 mm and the number of cells injected (Fig. 1b). A mean value and associated error (±2 S.E.) was calculated for each group of tumorous nodes and plotted as a function of the time of their removal and transplantation into fresh hosts (Fig. 2). The slope of the curve decreased with increasing cell number. From this curve the time taken for a given population of LMC_1 cells to double in number, i.e. the Population Doubling Time (PDT), at various stages in the evolution of a metastasis was estimated. Values increased with time after implanation of the primary tumour from 10–90 hr. Around 13 days the PDT was about 18 hr, which corresponds to the mean cell cycle time for LMC_1 cells *in situ* [12].

Adjuvant chemotherapy experiments

Primary tumours *in situ* did not regress after treatment with CP (Fig. 3). After a single dose their growth rate was considerably reduced but then returned to normal such that the overall delay in growth at 25 mm mean diameter (about 8000 mm^3) was 13.7 ± 1.9 days. With 2 doses of CP the corresponding net delay in growth was 27.0 ± 1.6 days. The mean time from implantation of the primary for positive nodes to reach 8–10 mm mean diameter *in situ* was 43.2 ± 1.2 days for animals given one dose and 54.0 ± 1.9 for those given two (Table 1). There was no significant difference between values for axillary and inguinal nodes and so the data were pooled.

Table 1. Mean survival time (MST) (days ± S.E.) and time for axillary and inguinal nodes to reach 8–10 mm mean diameter (T_{8-10})(days ± S.E.)

Treatment	MST	T_{8-10}
No treatment	29.3 ± 1.3	29.2 ± 1.3
1 × CP	43.0 ± 1.4	43.2 ± 1.2
2 × CP	55.7 ± 3.2	54.0 ± 1.9
Surgery alone	58.2 ± 1.7	46.9 ± 3.0
CP + Surgery	72.6 ± 4.5	57.4 ± 6.0
2 × CP + Surgery	68.2 ± 1.4	57.6 ± 2.6

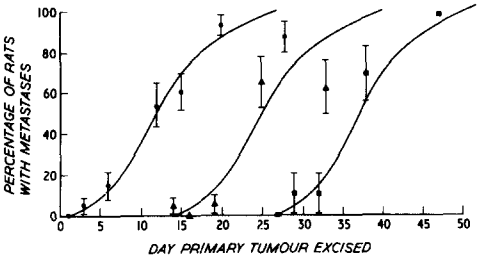

Fig. 4. The incidence of axillary and inguinal node metastases in situ. The percentage of rats (Mean of 12 observations ±1 S.D.) with metastases is expressed as a function of the time of excision of the primary tumour after implantation. ● Excision only, ▲ 1 × 150 mg/kg CP (13 days after implantation of the primary) and excision, ■ 2 × 150 mg/kg CP (13 and 26 days after implantation of the primary) and excision.

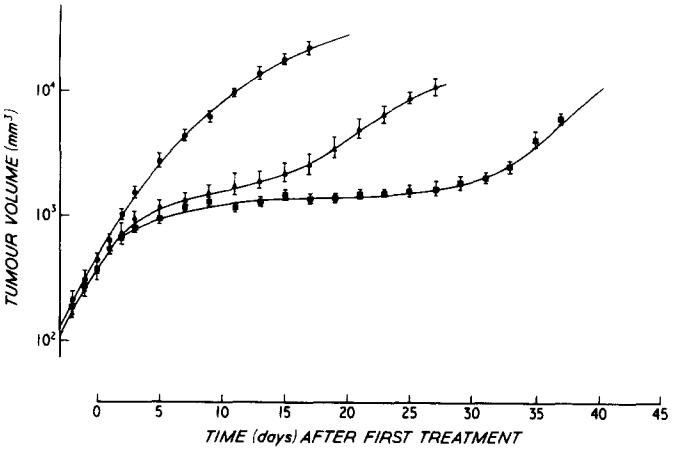

Fig. 3. Primary tumour response to CP (Mean ±1 S.E.). ● Untreated, ▲ 1 × 150 mg/kg CP administered at T = 0, ■ 2 × 150 mg/kg CP administered at T = 0 and T = 13. T = 0 when the mean diameter of the primary is 9 ± 1 mm (13 days after implantation).

The percentage of rats that developed metastases after surgery alone, or CP and surgery, increased with time from implantation of the primary tumour or final dose of drug to excision of the primary tumour (Fig. 4). Although 150 mg/kg CP given 13 ± 1 days after implantation reduced the percentage of rats with metastases from 50% to around zero, if the primary was left *in situ* after chemotherapy the incidence of metastases again increased with time. However, a second dose, given 13 days after the first, again reduced the incidence from 50% to zero (Fig. 4). For each treatment group the incidence increased from zero to 80% or more, and within experimental error the same curve translated along the time axis could be fitted for each set of data (Fig. 4).

The mean times for 'positive' nodes to reach 8–10 mm mean diameter in animals treated by surgery alone were independent of time from implantation to excision of the primary tumour. Equivalent values for double-dose combination therapy were also independent of the time interval from the final dose of drug to excision, and there was no definite trend for single-dose combination therapy. When the values were combined within a treatment group, mean times for both groups given CP in combination with surgery were significantly higher than those for surgery alone ($P < 0.01$ and $P < 0.001$). There was no significant difference between single and double doses of adjuvant therapy (Table 1). All treatment groups survived longer than the untreated group and MST's closely correlated with the time for nodes to reach 8–10 mm for each group.

DISCUSSION

In this series of experiments we have used a system which can be assayed macroscopically to study microscopic events from the dissemination of malignant cells from the primary tumour, through their development as micrometastases to their ultimate manifestation as palpable secondary tumours.

The linear relationship between the logarithm of the number of tumour cells inoculated and the time taken to produce tumours of a given size provides a standard reference curve which has been used in combination with transplantation to estimate survival of clonogenic cells in treated primary tumours [13]. Since, in this study, the growth rate of tumours arising from isolated cells was independent of the size of the initial inoculum and was the same as that of tumour cells in an implanted node, this method may be extended to calculate relative numbers of clonogenic cells present in tumorous nodes at the time of transplantation.

For LMC_1, linearity applies only between 5 and 5×10^5 cells. Results for less than 50 cells are stochastic and availability of diffusible nutrients prior to vascularization may be the limiting factor for inocula greater than 5×10^5 cells.

By estimating the relative numbers of LMC_1 cells in nodes at various times after implantation of the primary tumour, and attributing this increase largely to cell proliferation within the nodes [7], we have essentially constructed a growth curve for tumour cells in nodes (Fig. 2). Around 13 days after implantation of the primary, the PDT was equal to the mean cell cycle time for LMC_1 cells *in situ* [12], suggesting all tumour cells in inguinal and axillary nodes were in cycle and therefore susceptible to the proliferation dependent agent, CP. Beyond 13 days, the PDT increased progressively with time (Fig. 2) to 4–5 times the mean cell cycle time, suggesting a progressive increase in the fraction of non-cycling cells. This will presumably reduce the effectiveness of agents such as CP which are active mainly on proliferating cells [8].

As predicted, 150 mg/kg CP given 13 days after implantation of the primary reduced the incidence of metastases from about 50% to almost zero, and if the primary was removed within 5 days of CP this low incidence was maintained (Fig. 4). However, if the primary was left *in situ* for longer than 5 days, the incidence of metastases increased with time from drug administration and followed the same curve as that for surgery alone. Similarly, a second isodose of CP, given 13 days after the first, reduced the incidence of metastases from 50% to zero and again, if the primary was removed within 5 days, low incidence levels were maintained. If the primary was left *in situ* beyond 5 days the incidence increased sharply with time from the second dose of drug and followed the same curve as the other treatments. This suggests that the rate of reseeding of clonogenic cells from the treated primary tumour is unchanged.

As angiogenesis [14] is not *de novo*, and since there is a significant increase in the size of the primary (Fig. 3) and, therefore, theoretically an increase in the number of cells shed [15], one might expect reseeding from a treated tumour to be quicker than the initial seeding from the implanted tumour pellet. However, results suggest that either the number of cells shed are the same for both groups or, if there is an increase in the number of cells shed from a

well-established primary tumour, many of them are sterile due to the effect of CP, and the absolute number of clonogenic cells remains the same.

The mean time to reach 8–10 mm for positive nodes *in situ* in the surgery alone group was 46.9 ± 3.0 days. If the increase in incidence of metastases is due to reseeding, and reseeding rates are the same for each group, then equivalent values for 150 mg/kg CP + surgery and 2 × 150 mg/kg CP + surgery should be about 60 (i.e., 47 + 13) and 73 (i.e., 47 + 26) days respectively. Actual values were 57.4 ± 6.0 and 57.6 ± 2.6 days.

The reduced value for twice-treated animals suggest that a greater number of clonogenic cells are present than expected. This may be explained in several ways: if the number of cells seeded in nodes is proportional to the size of the primary tumour [15, 16], the number of tumour cells in nodes at 26 days will be greater than that at 13 days (Fig. 3). This may lead to a population of non-cycling cells at 26 days and increase the possibility of surviving cells after the second dose of CP. With other tumours the ratio of larger to smaller blood vessels increases with their growth, which permits dissemination of clumps of more than 6–7 tumour cells to leave the tumour [14]. Metastatic spread after the second dose may occur predominantly from dissemination of such clumps, against which mechanisms of non-specific immunity are relatively ineffective [17]. As the incidence of metastases is reduced to zero when the primary tumour is excised immediately after the second dose (Fig. 4), reseeding must be either instantaneous and by cell clumps and/or by a resistant population of clonogenic cells from a twice-treated primary tumour. These cells may have increased propensity to metastasise, seed and escape immunosurveillance, and may have different growth characteristics from their untreated counterparts.

Acknowledgements—We are grateful to Professor C.A.F. Joslin for his continued support and encouragement, Dr. J. P. Dickinson for helpful discussion and criticism, the Yorkshire Cancer Research Campaign for the support of Mr. S. A. J. Scott who bred and cared for our experimental animals, Mrs J. Thorogood of the Yorkshire Regional Cancer Organisation for the probit analysis and Mrs. D. Broadhead for typing the manuscript.

REFERENCES

1. SCHABEL FM. Surgical adjuvant chemotherapy of metastatic murine tumours. *Cancer* 1977; **40:** 558–568.
2. ZUBROD CG. Historic milestones in curative chemotherapy. *Semin Oncol* 1979; **6:** 490–505.
3. VALAGUSSA P, BONADONNA G, VERONESI U. Patterns of relapse and survival in operable breast carcinoma with positive and negative axillary nodes. *Tumori* 1978; **64:** 241–257.
4. BONADONNA G. Adjuvant chemotherapy of breast cancer. *Br J Hosp Med* 1980; **23:** 40–53.
5. DIXON B, SPEAKMAN H. Local recurrence and metastasis of excised breast carcinoma in the rat. *J R Soc Med* 1979; **72:** 572–577.
6. MOORE JV, DIXON B. Metastasis of a transplantable mammary tumour in rats treated with cyclophosphamide and/or irradiation. *Br J Cancer* 1977; **36:** 221–226.
7. SPEAKMAN H, DIXON B. Quantitation and cellular growth of occult metastases of a rat mammary carcinoma. In: HELLMANN K, HILGARD P, ECCLES S, eds. *Metastasis: Clinical and Experimental Aspects.* The Hague, Martinus Nijhoff, 1980; 55–59.
8. CALABRESI P, PARKS RE, Jr. Alkylating agents, antimetabolites, hormones and other anti-proliferative agents. In: GOODMAN LS, GILMAN A, eds. *The Pharmacological Basis of Therapeutics.* New York, Macmillan, 1975; 1254–1307.
9. THOMLINSON RH. An experimental method for comparing treatments of intact malignant tumours in animals and its application to the use of oxygen in radiotherapy. *Br J Cancer* 1960; **14:** 555–576.
10. MOORE JV. The response of a rat mammary tumour to cyclophosphamide and to subsequent irradiation. Ph.D Thesis, Univ. of Leeds 1976.
11. FINNEY DJ. *Probit Analysis.* Cambridge, 1971.
12. MOORE JV, DIXON B. Serial transplantation, histology and cellular kinetics of a rat adenocarcinoma. *Cell Tissue Kinet* 1977; **10:** 583–590.
13. ALFIERI AA, HAHN EW. An "In situ" method for estimating cell survival in a solid tumour. *Cancer Res* 1978; **38:** 3006–3011.
14. FOLKMAN J, TYLER K. Tumour angiogenesis: its possible role in metastasis and invasion. In: DAY SB, LAIRD MYERS WP, STANSLEY P, GARATTINI S, LEWIS MG,

eds. *Cancer Invasion and Metastasis: Biologic Mechanisms and Therapy.* New York, Raven Press, 1977; 95–103.

15. CARR J, CARR I, DREHER B, FRANKS CR. Lymphatic metastasis of tumour; persistent transport of cells. *Experientia* 1979; **35:** 825–826.
16. FISHER B, FISHER ER. Barrier function of lymph nodes to tumour cells and erythrocytes. I. Normal nodes. *Cancer* 1967; **20:** 1907–1913.
17. ALEXANDER P. Innate host resistance to malignant cells not involving specific immunity. In: DAY SB, LAIRD MYERS WP, STANSLEY P, GARATTINI S, LEWIS MG, eds. *Cancer Invasion and Metastasis: Biologic Mechanisms and Therapy.* New York, Raven Press, 1977; 259–275.