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Ploidy Distribution of Tumour Cells Derived from Induced and Spontaneously Arising Metastases of a Murine Radiation-induced Sarcoma, RIF-1

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Abstract—*Flow cytometric analysis of the X-radiation-induced sarcoma RIF-1 has shown that the parent tumour is composed of diploid and tetraploid subpopulations of cells, each capable of independent proliferation. We have now examined artificially induced and spontaneously arising metastases of the RIF-1 tumour and have found in both cases that, unlike the parent tumour from which they are derived, they exhibit a single level of ploidy which is stable throughout successive* in vivo *and* in vitro *passaging. This finding suggests that at least in this tumour system metastasis is a clonal event.*

INTRODUCTION

THERE is now considerable evidence to show that tumours can contain subpopulations of cells with differing metastatic abilities [1–4]. Furthermore, the process of metastasis has been shown to be highly selective, with only a few cells within a primary neoplasm being able to complete all the stages involved in the production of overt metastases [5–9]. Whether or not a metastasis develops from one cell or many is a question of fundamental and clinical importance. Results obtained using the B16 melanoma [10] indicate that cells populating lung colonies, which arise after intravenous inoculation of parent tumour cells, are more homogeneous with respect to metastatic capability than cells derived from the parent tumour. Thus, clones derived from cells isolated from a single pulmonary metastasis were of uniform metastatic potential, as determined by the lung colony assay [1], whereas clones derived from cells isolated from the parent tumour differed markedly in metastatic ability using the same assay. These data suggest that, at least in these tumour systems, metastases probably arise from one or a few cells.

We have investigated the cellular origin of metastases using the X-radiation-induced murine sarcoma RIF-1, recently described by Twentyman *et al.* [11]. Two characteristics of the RIF-1 tumour make it a particularly useful model system for the study of metastasis: firstly, it is non- or weakly immunogenic in its syngeneic host, having a transplantation TD_{50} (median tumour dose) of between ten and twenty cells in both unimmunized and pre-immunized animals [11]; and secondly, flow cytometric analysis of the RIF-1 tumour has revealed that it is comprised of both diploid and tetraploid subpopulations of cells, each being capable of independent proliferation [11]. The existence of these two subpopulations of differing ploidy allows an assessment of their relative distribution in artificially induced and spontaneously arising RIF-1 metastases.

MATERIALS AND METHODS

C3H/Km mice bred in this unit were used in most experiments. The breeding colony was established using mice from the colony at Stanford University, Stanford, CA in which the RIF-1 tumour was induced. In some of the earlier experiments, however, C3H/He-mg mice (obtained from OLAC 1976, Oxford) were used and these experiments are specified in the text.

RIF-1 tumours were established from the frozen stock previously described in the protocol for maintenance of the RIF-1 tumour [11] and were grown either intradermally or intramuscularly [11]. Thus tumours were derived

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from cells which were no more than three animal passages away from the primary tumour. The medium used throughout was Eagle's Minimal Essential Medium with Earle's salts supplemented with 20% new-born calf serum (both Gibco Biocult Ltd.) and antibiotics.

Production of artificial metastases

Intradermal RIF-1 tumours, when they reached an approximate volume of 200 mm^3, were aseptically excised. They were minced finely with scissors in a plastic Petri dish containing 1 ml of medium and bacterial neutral protease (Sigma London Chemical Co. Ltd.) at a concentration of 1 mg/ml [12]. The fragments were then transferred to a glass universal container and 14 ml of medium and neutral protease (1 mg/ml) were added, together with a 2-cm long, sterile, Teflon-coated, magnetic bead. The container was placed on a magnetic stirrer at room temperature for 30 min, following which the contents were filtered through a cotton gauze. An equal volume of medium was added to the cell suspension and the suspension was centrifuged at 200 ***g*** for 5 min. The pellet was resuspended in medium and the single-cell suspension was counted with the use of a haemocytometer. Cells (10^5) in 0.25 ml of Hanks' Balanced Salt Solution (HBSS) were then inoculated into the tail veins of C3H/He-mg mice. Animals were killed 18 days later and all tissues carefully examined. The artificial metastases from various body organs were excised and disaggregated as described above. Samples of the resulting cell suspensions were removed and prepared for flow cytometry (see below).

Artificial metastases were maintained *in vivo* and *in vitro* as follows: 10^6 cells from each cell suspension were seeded into 75-cm^2 tissue culture flasks (Sterilin Ltd.) containing medium and maintained at 37°C in an atmosphere of 5% CO_2–95% air. At confluence cells were removed from the flask by the use of a double rinse with medium containing bacterial neutral protease (1 mg/ml) followed by incubation at 37°C for 15 min. Cells were then harvested in medium and counted. For *in vivo* transplantation, 10^5 cells in 0.1 ml of medium were inoculated intradermally in the flanks of C3H/He-mg mice. When tumours reached an approximate volume of 200 mm^3, a single-cell suspension was prepared and 10^6 cells were seeded into tissue culture flasks as described above and a second cycle was begun. For routine *in vitro* passage, 10^6 cells from the original tumour suspension were seeded into 75-cm^2 tissue culture flasks containing medium. After 3 days of growth the medium was changed daily and when at confluence the cells were removed from the flasks as described above. Subsequent *in vitro* passages were established from 10^5 cells.

Production of spontaneously arising metastases

Animals bearing large intramuscular leg tumours were anaesthetized with ether. The skin around the base of the leg was aseptically incised and folded back to reveal the underlying tumour and musculature. The femoral artery was then ligated and the leg carefully amputated above the knee. Animals were examined regularly and were killed upon appearance of overt metastases. Tumours were excised, disaggregated and the cells stained for flow cytometry as described below.

Flow cytometry

In vivo or *in vitro*-derived cells (5×10^5) from suspensions of parent RIF-1 tumour, artificially induced metastases or spontaneously arising metastases were centrifuged at 200 ***g*** for 5 min at room temperature. The supernatant was removed and cells were stained with ethidium bromide solution using a rapid technique [13]. Cell suspensions were then vortexed for 1 min, left at 4°C for 30 min and analysed for DNA content per cell by means of the Cambridge dual laser, multiparameter flow cytometer. The ratio of the peak channel numbers for the G1 phase of the tumour cells to that of a diploid standard (normal mouse bone marrow) was calculated and used for ploidy identification.

RESULTS

Upon death, 90% of animals receiving 10^5 RIF-1 cells intravenously were found to bear artifical 'metastases'. Flow cytometric analysis of RIF-1 sublines derived from such artificial metastases revealed that all were of a single level of ploidy after one *in vitro* passage to eliminate host cell infiltrates. This was in contrast to the parent RIF-1 tumour which was comprised of both diploid and tetraploid clonogenic tumour cells. Thus, flow cytometric analysis of the parent tumour yielded a DNA distribution comprised of two prominent peaks in channels equivalent to one and two times the DNA content of G1 phase normal mouse bone marrow, with S phase diploid cells lying between the two main peaks and S and G2 phase tetraploid cells lying to the right of the second main peak (Fig. 1a, b). On the basis of DNA distribution, metastases, however, could be divided into two types. For one, the DNA dis-

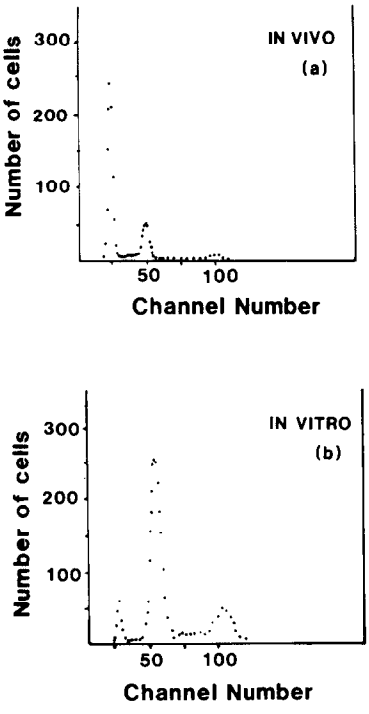

Fig. 1. Distribution of DNA content for cells in suspension taken either from (a) solid flank tumour or from (b) exponential phase culture of RIF-1 cells. For (a) and (b) the left-hand peak is in the same channel as the G1 peak for a suspension of normal mouse bone marrow.

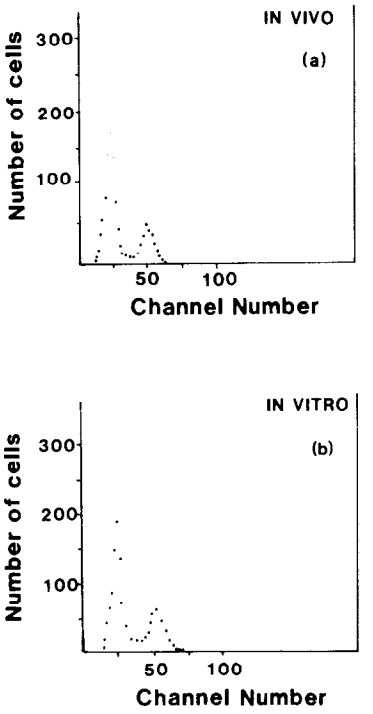

Fig. 2. Typical DNA distribution for cells taken either from (a) solid tumour or from (b) exponential phase culture of diploid RIF-1 artificial or spontaneous metastases.

tributions of both *in vivo* and *in vitro*-derived cells were similar (Fig. 2a, b), being comprised of a single main peak in channel 25, corresponding to the DNA content of the G1 phase of normal mouse bone marrow cells, a smaller peak in channel 50, corresponding to the DNA content of G2 phase cells, and S phase cells between the two peaks. Such metastases were therefore judged to be comprised of a single population of diploid tumour cells. For the second type of metastasis, all stained cells derived from *in vivo* tumours showed a small peak in channel 25 followed by a larger peak in channel 50, corresponding to the DNA content of G1 phase tetraploid cells, with the S and G2 phases of the tetraploid cells lying to the right of the larger peak (Fig. 3a). When cells from such metastases were stained after one *in vitro* passage, the small peak in channel 25 was no longer present (Fig. 3b), indicating that this peak corresponded to the DNA content of non-clonogenic diploid host cells. [The RIF-1 tumour is known to be comprised of approximately 30% infiltrating host cells (D. W. Siemann, personal communication).] Table 1 shows the site and ploidy distribution of such artificially induced metastases. No metastases having ploidy levels intermediate between diploid and tetraploid values were observed.

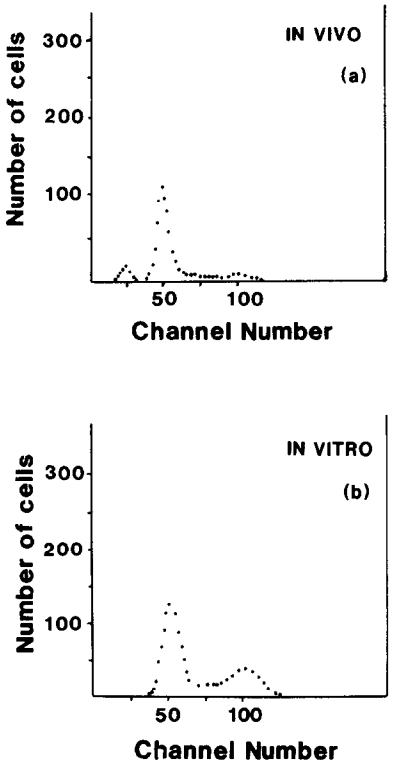

Fig. 3. As for Fig. 2 except for tetraploid RIF-1 artificial or spontaneous metastases. For (a) the left-hand peak corresponds to the G1 phase peak of infiltrating host cells. For (a) and (b) the main peak in channel 50 corresponds to the G1 phase peak of tetraploid tumour cells.

Table 1. *Site and ploidy of RIF-1 artificial metastases*

Metastasis	Site	Ploidy*
1	Chest wall	D†
2	Chest wall	T‡
3	Axilla	D
4	Chest skin	T
5	Axilla	D
6	Chest wall	D
7	Behind eye	T
8	Ovary	T
9	Rear ribs	D
10	Rear ribs	T
11	Heart	D
12	Lung	T
13	Lung	T
20	Ovary	D
21	Behind eye	D
22	Abdominal wall	T
23	Axilla	D
24	Leg	D
25	Chest wall	T
26	Back	D
27	Brain	T
28	Lungs	T
29	Lungs	T
30	Body wall	D
31	Chest wall	D

*Ploidy of *in vitro*-derived cells.
†D = Diploid, peak channel ratios of G1 phase diploid tumour cells to G1 phase normal mouse bone marrow cells being in the range of 0.9–1.1:1.
‡T = Tetraploid, peak channel ratios of G1 phase tetraploid tumour cells to G1 phase normal mouse bone marrow cells being in the range of 1.8–2.2:1.

Flow cytometric analysis of the metastatic sublines after as many as 10 *in vivo* and 6 *in vitro* passages revealed that each line was stable as evidenced by similar flow cytometric distributions at early and late stages of maintenance and growth.

Spontaneously arising RIF-1 metastases

Of the 65 animals which had undergone removal of large intramuscular tumours by leg amputation, only 6 exhibited detectable spontaneous metastases. These appeared between 13 and 168 days after leg amputation, and in 3 animals were present in the lungs only, whilst the remaining 3 animals exhibited metastases in the shoulder, diaphragm and neck.

Flow cytometric analysis of 7 spontaneous metastases so far isolated revealed that all exhibited a single level of ploidy after one *in vitro* passage to eliminate host cells (Table 2). All 4 lung metastases examined and two extrapulmonary metastases (to shoulder and diaphragm) were judged to be tetraploid after one *in vitro* passage to eliminate infiltrating host cells, producing a DNA distribution similar to that of tetraploid RIF-1 artificial 'metastases' (Fig. 3b). The remaining spontaneous metastasis, isolated from the neck, was comprised of a single population of diploid cells producing a DNA distribution similar to that of diploid RIF-1 artificial 'metastases' (Fig. 2a, b).

DISCUSSION

The ploidy distribution of the parent RIF-1 tumour is not random but is grouped into diploid and tetraploid values [11]. The line is very stable, giving similar flow cytometric distributions at early and late protocol stages of maintenance and growth [11]. In this study flow cytometric DNA analysis has been used to yield information on the ploidy of artificially induced and spontaneously arising RIF-1 metastases with a view to assessing their clonal heterogeneity.

Our data clearly show that RIF-1 metastases, whether artificially induced or spontaneously arising, exhibit a single level of ploidy, unlike the parent tumour from which they are derived, and that this is stable throughout successive *in vivo* and *in vitro* passaging. This finding suggests that, at least in this tumour system, spontaneous metastasis is likely to be a clonal event, for if metastases arose from emboli of the parent tumour, they too would exhibit a mixed ploidy distribution. The validity of this interpretation is clearly dependent

Table 2. *Site and ploidy distribution of RIF-1 spontaneous metastases*

Site	Ploidy*
Lung	T†
Lung	T
Lung	T
Lung	T
Shoulder	T
Neck	D‡

*Ploidy of *in vitro*-derived cells.
†T = Tetraploid, peak channel ratios of G1 phase tetraploid tumour cells to G1 phase normal mouse bone marrow being in the range of 1.8–2.2:1.
‡D = Diploid, peak channel ratios of G1 phase diploid tumour cells to G1 phase normal mouse bone marrow being in the range of 0.9–1.1:1.

upon the sensitivity of the flow cytometric technique in detecting mixed populations of diploid and tetraploid cells. Data from studies in which RIF-1 diploid and tetraploid cells were mixed in various proportions show that the histograms produced by the Cambridge dual laser, multiparameter flow cytometer are capable of detecting a minority subpopulation that comprises less than 5% of the total tumour cell population. Furthermore, in studies of *in vitro* isolated RIF-1 clones, we have observed no significant difference in the growth rates of diploid and tetraploid cells either *in vitro* or in the intramuscular site *in vivo* (Reeve and Twentyman, unpublished). Therefore it is unlikely that RIF-1 metastases arise from mixed ploidy emboli and that differential growth rates of diploid and tetraploid cells, leading to overgrowth of one population by another, is the basis of the single ploidy level seen in overt metastases. It is possible, however, that cells of the same ploidy are grouped together within the RIF-1 tumour; if so, it is feasible that small emboli of the parent tumour could also exhibit a single level of ploidy. Experiments are now in progress to elucidate this point.

The conclusion that RIF-1 spontaneous metastases are clonal in origin further depends on lack of interconversion between diploid and tetraploid cells. We have examined this problem at some length by studying the stability of *in vitro* isolated RIF-1 diploid and tetraploid clones when successively passaged both *in vivo* (in the lung and intramuscular site) and *in vitro*; in no case has interconversion been observed (Reeve and Twentyman, unpublished).

An alternative explanation for our findings is that the apparent single level of ploidy expressed by RIF-1 metastases results from a site-specific, ploidy-dependent selection event. However, this is unlikely to be a valid interpretation since metastases of differing ploidy have been isolated from a common organ, and in one animal a metastasis to the left adrenal gland was diploid and a second metastasis, to the right adrenal gland, was tetraploid.

The artifical introduction of cells into the circulation bypasses the first two steps of the metastatic process, namely invasion of vessels and detachment from the primary tumour mass. However, the subsequent steps of the process, which are transport and survival in the circulation, arrest, extravasation and growth, must all occur for overt metastases to develop. That single levels of ploidy were observed for both artificially induced and spontaneously arising metastases suggests that artificial metastasis well approximates the process of spontaneous metastasis.

The use of flow cytometric analysis to obtain information on ploidy and the proliferation characteristics of tumour cell populations has been used to assess the clonal heterogeneity of small-cell anaplastic carcinoma in man [14]. Although some metastases to the lungs were comprised of two tumour cell clones with different ploidy, the majority were found to consist of cells exhibiting a single level of ploidy. These findings are in good agreement with those of the present study and illustrate the usefulness of the RIF-1 tumour as a model for the study of metastasis.

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