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Studies on relationships between metastatic and non-metastatic tumor cell populations using lineages labeled with dominant selectable genetic markers

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ABSTRACT The relationships between metastatic and non-metastatic cell populations co-existing in composite neoplasms have been studied using cell lineages marked with a dominant selectable marker (neomycin resistance), by transfection. The experimental circumstances were arranged so that the lineages were known to be genotypically distinct (i.e. not merely phenotypic variants of the same lineage) and so that a single metastatic clone was each time combined with a mixed polyclonal non-metastatic population and *both* partners were distinctly and recognizably marked. This made it possible to ascertain the fates of clones with different metastatic capabilities during tumor progression and metastasis and evaluate their relative contributions to the clinical extent of disease. It was found that metastatic and non-metastatic cell lineages co-existed in most of the late-stage primary tumors examined and that a cell lineage that is invariably non-metastatic, when growing on its own, can with surprising frequency be found thriving in distant metastatic deposits, when it grows to form a primary tumor in combination with a metastatic partner. In fact, occasional metastases from such tumors contained no detectable cells of the metastatic lineage. The endowment of a tumor cell lineage with a new, clinically significant, capability which it convincingly and reproducibly did not manifest before, by another coexisting cell population raises several new questions about the contribution of such phenomena to the overall debilitating properties of the neoplasm and the geometric progression of its impact on the host.

KEY WORDS: *metastasis, clonal lineages, tumor composition, genetic tagging*

Introduction

The recent formulation of methods to reliably label chosen tumor cell lines and clones possessing interesting properties, with unique genetic markers, has made it possible to ask many searching questions about their behavior and fate *in vivo*. These include enquiries about the contribution of individual constituent cell lineages to the clinical impact of the neoplasm.

The insertion of dominant, selectable markers into mammalian cells became a practical reality when Mulligan and Berg (1980) and Southern and Berg (1982) succeeded in constructing vectors which could be used to introduce the bacterial *gpt* and neomycin resistance genes into cultured cells by DNA transfection. The latter gene encodes the enzyme aminoglycosyl transferase, conferring resistance to the aminoglycoside antibiotic neomycin, which is toxic to eukaryotic cells as well as to most prokaryotes. The purpose of designing these constructs was to provide a method for recognizing and selecting cells which had incorporated exogenous DNA, including the marker.

In essence, this work showed that a proportion of cells transfected with this gene integrate it into their genome and so become indelibly marked with a label that replicates with the cell. The presence of the marker can be detected in one of three ways; by survival of the cells in neomycin-containing culture medium, by PCR and by Southern blot hybridization. Further, as the site of integration is random in each transfected cell and stable over many cell generations *in vitro* and *in vivo*, its progeny can be recognized and distinguished from those of other neomycin-tagged cells by the possession of a characteristic pattern of bands in Southern blots of their digested DNA, when probed with the neomycin resistance gene.

In 1987, Talmadge and Zbar published two reports describing their application of this method to the study of tumor cell populations *in vivo*. They showed that it can be used to obtain information on many aspects of tumor growth and behavior as well as to speedily and simply provide individually marked cell lineages, from a chosen tumor line, for use in such investigations. These studies were rapidly followed by further work by Waghorne *et al.* (1988) and several others, including ourselves, which confirmed its value for such

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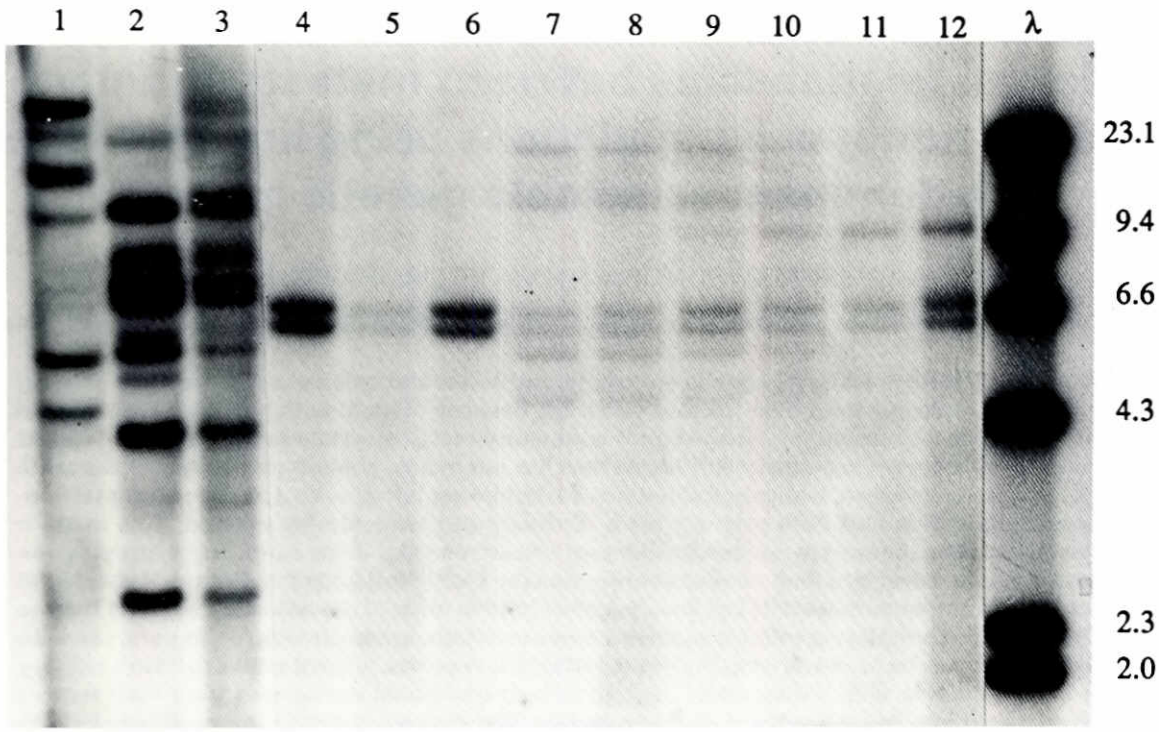

Fig. 1. Autoradiogram showing neo^R integration patterns of cell lines T6 (lane 1) and K1 (lane 4) and primary tumors produced by injection of T6 (lanes 2 and 3) alone, K1 (lanes 5 and 6) and the mixture of K1 and T6 (lanes 7-12). *Tumors of mixed lineage origin continue to contain bands representative of both lineages although there is reduction in the complexity of the banding patterns and hence of numbers of surviving clones. DNA digested with EcoR1.*

purposes. As a result, the technique is becoming recognized as a powerful new means of analyzing cell population changes during neoplasia and of tracking and identifying cell lineages during tumor dissemination and metastasis. Drug resistant mutant cell lines have been used very effectively in the study of tumor cell behavior for several years (e.g. see review by Miller and Heppner, 1990) and still make important contributions to understanding the metastatic process (Aslakson and Miller, 1992), but the present methods of obtaining lines marked with dominant selectable markers are more reliable and efficient and have the additional advantage of unequivocal identification of individual lineages by their neo-integration patterns.

In a recent study of the fates of clonal cell lineages in three separate tumor cell lines, during progression and metastasis, using these methods, we observed that the tumor cell populations composing primary and secondary tumors often remained heterogeneous into advanced stages of malignancy and that under-represented clones in the original inoculum or in the primary tumor could be found in its metastases (Moffett *et al.*, 1992). This suggested that metastatic cell populations within a tumor are not necessarily ones which can rapidly gain a numerical ascendancy in the site of primary growth and that the underlying mechanisms of invasion and metastasis can be separated from those which confer growth supremacy upon a clone of cells. However, it remained formally possible that, in a neoplasm comprised of many metastatic (M^+) cell lineages, all of them would be competitively superior to the non-metastatic (M^-) ones and clear dominance by a single M^+ clone would not occur, although the M^- ones had been overwhelmed and eliminated.

The present investigation was therefore undertaken to answer two specific questions, namely:

Does a single M^+ clone routinely over-run and eclipse several reliably M^- tumor cell lineages when they are mixed and co-injected? and

If metastases are formed by tumors initiated by mixed M^+ and M^- cell lineages, what is their cell composition? Do such deposits routinely only contain M^+ cells?

The findings presented below demonstrated that M^- and M^+ clones co-existed in most of the primary tumors formed by the cell mixtures, even at late stages of tumor progression. It was also observed that many metastases were formed by these tumors and that cells from the population which was unequivocally M^-, when injected on its own, were present and thriving in some of the secondary tumor deposits. If operative among tumor cell populations generally, such direct and indirect effects of genotypically and phenotypically heterogeneous clonal cell lineages upon each other could influence or determine the clinical behavior and therapeutic susceptibilities of naturally occurring neoplasms and therefore warrant further study.

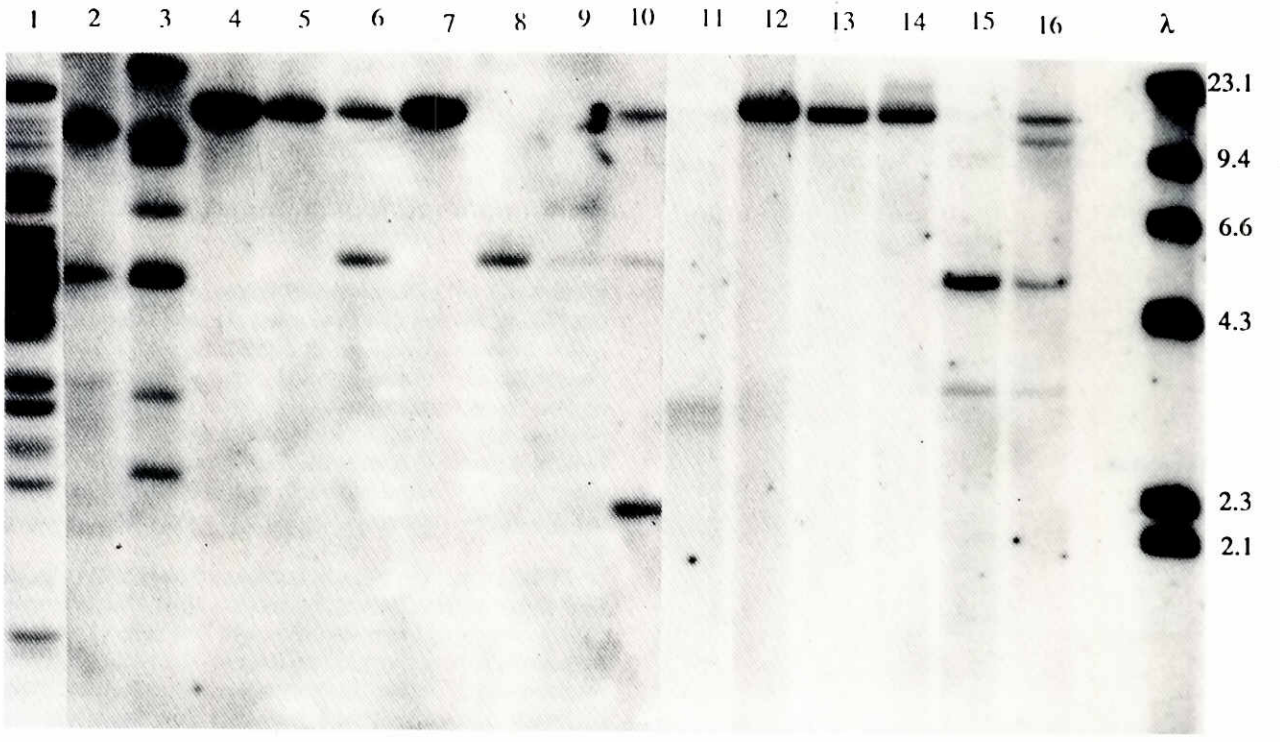

Fig. 2. Autoradiogram showing neo^R integration patterns of cell lines K5 (lane 4) and T3 (lane 1) and examples of primary tumors and metastases produced by injection of these lines separately and as mixtures. *Lane 5 contains DNA from a primary tumor produced by K5 and lanes 2 and 3 contain DNA from primaries produced by T3 alone. The latter show some reduction in bands relative to their parent cell line but differ from each other. Lanes 11-16 show the compositions of some tumors derived from mixed cell lineage inoculations. In these examples there is much more drastic reduction of clones present. The tumors in lanes 11 and 15 appear to be composed only of T3 cells, those in lanes 12 and 13 of only K5 and those in 14 and 16 are mixed. Lanes 6-10 show the composition of some metastases from tumors of mixed lineage origin. The lung metastases in lanes 6 and 10 contain bands from both cell populations, whereas that in lane 7 contains only K5. The sample in lane 8 is from a lymph node metastasis and that in lane 9 from a lung metastasis, both of which contain only T3. DNA digested with EcoR1.*

Cell lines

Experimental design

Two separate cell lines, both of mouse origin, were used in this study: TR4 Nu is a fibrosarcoma line derived, as described previously (Moffett *et al.*, 1992), from non-neoplastic Swiss NIH 3T3 cells. This cell line has been well characterized in many experiments in our laboratory and was chosen because it is reproducibly tumorigenic and locally invasive, but not metastatic in any animals injected subcutaneously (see Table 1). It has never shown any capability to form metastases when injected alone, in studies performed over many years in hundreds of animals. The median latent period of tumorigenicity is 24 days and growth is rapid, resulting in 2.5-3 cm diameter tumors by 40 days. The neo^R transfected TR4Nu polyclonal cell populations used in these experiments were confirmed to be completely non-metastatic (Table 1) by both macroscopic and histological observations.

The other cell line used, namely KHT, is also a fibrosarcoma line and is spontaneously metastatic from tumors formed at the site of inoculation. Polyclonal KHT tumor cells inoculated orthotopically in the skin form visible local tumors at the site of injection, after a median latent period of 10 days, which grow to reach a diameter of 2.5-3.0 cm in 35 days. Metastases are seen in the lungs, mediastinal lymph nodes and regional lymph nodes in more than 95% of animals surviving more than 35 days. The behavior of the individual neo^R transfected clones used in this experiment is given in Table 1.

This experiment was specifically designed to avoid the possibility that apparently M$^-$ clones from the M$^+$ cell line might reacquire metastatic capability at some stage during tumor growth and thus obviate the point of the experiment. Therefore, we chose to mix individual M$^+$ KHT clones with unequivocally M$^-$ polyclonal TR4Nu populations. This experimental design allowed us to clearly see M$^-$ cells participating in metastasis, an event which would, with a different choice of M$^+$ and M$^-$ partners, have been open to alternative interpretations.

Three metastatic KHT clones (designated K-1, -5 and -7) and three separate TR4Nu polyclonal populations (designated T-1,-3 and -6) were used in these experiments.

Mixtures prepared for inoculation contained a single M$^+$ (KHT) clone and one of the polyclonal M$^-$ (TR4Nu) population in ratios of 1:10 and

TABLE 1

METASTATIC CAPABILITIES OF CELL LINES

Cell line	Ratio of mixture	Animals with lung metastases (regional and mediastinal)	Animals with lymph node metastases
a) Pure cell lines			
TR4			
T-1		0/8	0/8
T-3		0/8	0/8
T-6		0/13	0/13
KHT			
K-1		13/13 (15±10)	8/13
K-5		10/15 (11±5)	5/15
K-7		2/9 (5±2)	1/9
b) Mixed cell lineages			
K1+T6	(1:10)	4/9	3/9
	(1:100)	7/13	2/13
K5+T3	(1:10)	5/10	2/10
	(1:100)	17/32	19/32
K7+T1	(1:10)	3/10	2/10
	(1:100)	4/10	6/10

1:100 respectively, so as not to give the former an undue starting advantage. The partners chosen to compose each mixture had distinct non-overlapping banding patterns of neo^R integration sites, visualized by Southern blot hybridization, so that the constituent cells in primary tumors and their metastases could be unequivocally identified. As both constituents of the mixture were marked it was possible to study the contribution of each of the interacting cell populations to tumor growth and spread. The combinations used were K-1 with T-6 (K1/T6), K-5 with T-3 (K5/T3) and K-7 with T-1 (K7/T1).

Results

Behavior of cell lines when injected separately (Table 1) and stability of neo integration banding patterns in vivo (Table 2)

All three KHT clones were metastatic (M^+) and the tumors and metastases that they formed were unanimously characterized by the unique clonal banding pattern of the progenitor cell line (representative examples are shown in Figs. 1 and 2). No extra bands were seen, nor were any detected in our previous studies with these and other KHT clones (Moffett *et al.*, 1992). Hence the specific markers of these clones, used for recognition of tumor cell composition in this work, were stable over many cell generations *in vivo* and *in vitro*. These findings are supported by similar previous observations by other investigators (Radinsky *et al.*, 1987; Talmadge and Zbar, 1987; Talmadge *et al.*, 1987; Waghorne *et al.*, 1988) on other cell lines.

The tumors formed by polyclonal TR4Nu populations were all consistently *completely* non-metastatic (M^-). They showed individual differences in the number of visible bands on the autoradiograms of the Southern blot hybridizations, reflecting differences in the proportions of various constituent clones surviving from each of the three original inoculated populations, but none acquired any bands which could be confused with KHT patterns. As can be seen from the representative samples illustrated in Figs. 1 and 2, some of the tumors formed by TR4 Nu populations showed marked reduction in the number of surviving marked clones, whereas others did not (see also Table 2 for further information).

The measurement of the growth rates of the K5 and T3 cell lines *in vivo* and *in vitro* (Figs. 3 and 4) showed that T3 grew faster *in vivo*, although it has a longer initial lag phase before tumors appear and that the two lines grew at almost the same rate *in vitro* with a doubling time of approximately 20 hours.

Metastatic behavior of mixed lineages (Table 1) and cell composition of tumors and their metastases (Table 2)

All three cell mixtures, in both 1:10 and 1:100 ratios, were metastatic to the lungs and to regional and mediastinal lymph nodes (Table 1). DNA analysis showed that both M^+ (KHT) and M^- (TR4Nu) cell populations were detectable in 20 out of 24 primary tumors examined (Table 2). Two further tumors contained no detectable KHT cells. Hence, in only 2 of the 24 tumors of mixed lineage origin analyzed did the M^+ cells become the sole constituent.

Of the 18 metastases which gave sufficient DNA for gel analysis, from these tumors, 6 contained cells with TR4Nu (M^-) markers (Fig. 2), either present in combination with KHT cells (3 metastases) or apparently on their own (3 metastases) [see Table 2 for details]. The remainder (12 metastases) contained no detectable TR4Nu cells and their banding patterns indicated that they were composed solely of KHT cells.

TABLE 2

COMPOSITION OF TUMORS BY DNA ANALYSIS

Cell line	Type of tumor	Number	Result
K5	primary	5	Identical to K5
K5	secondary	8	Identical to K5
T3	primary	5	T3 only (reduction in banding)
K5+T3	primary	2	T3 only
	primary	2	K5 only
	primary	8	K5+ T3
	lung secondary	**3**	**K5+T3**
	lung secondary	**1**	**T3 only**
	lymph node secondary	**1**	**T3 only**
	lung secondary	4	K5 only
	lymph node secondary	4	K5 only
	kidney secondary	1	K5 only
K7	primary	2	Identical to K7
T1	primary	3	T1 only (2 identical to inoculum, 1 reduced)
K7+T1	primary	5	K7+T1
	lymph node secondary	**1**	**T1 only**
	lung secondary	1	K7 only
K1	primary	2	Identical to K1
	lung secondary	2	Identical to K1
T6	primary	2	T6 (more bands than inoculum)
K1+T6	primary	7	K1+T6
	lung secondaries	2	K1 only

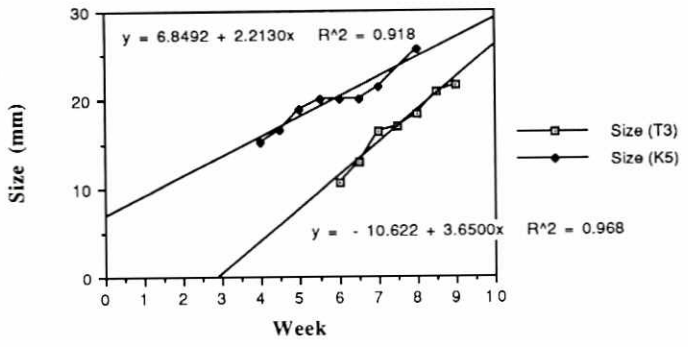

Fig. 3. Growth rates of cell lines separately *in vivo*.

Discussion

There are two main conclusions to be drawn from these data on interactions between $M^+$and M^- tumor cell lineages:

The first is that primary tumors formed by these cell lines can (and very often do) remain heterogeneous with respect to M^+ and M^- cell lineages, into advanced stages of malignancy, at which time metastases are present. There is often reduction in the number of surviving clones, judged by the number of bands, but the degree of this is quite variable.

Apparently random elimination of one or other of these lineages did occasionally occur in individual animals injected with aliquots of the same cell inoculum, but we detected no systematic process of selection favouring M^+ or M^- phenotypes. This result is consistent with our earlier work (Moffett *et al.*, 1992) demonstrating the persistence of heterogeneous cell lineages into advanced stages of tumor growth and extends it to specifically include the continuing coexistence, in such neoplasms, of lineages with differing metastatic behavior.

Some recent reports from other laboratories have presented data suggesting that, during tumor growth and progression, a single clone often becomes the dominant cell population (Miller *et al.*, 1988; Waghorne *et al.*, 1988; Enoki *et al.*, 1990; Price *et al.*, 1990; Radinsky and Culp, 1991) and that metastatic ones eliminate non-metastatic counterparts in this process (Waghorne *et al.*, 1988; Theodorescu *et al.*, 1991). Conversely, some other groups have described findings indicating persistence of heterogeneity in tumors and secondary deposits (Talmadge *et al.*, 1987) as well as lack of correlation between metastatic capability and capability for dominant growth (Samiei and Waghorne, 1991). In the tumor cell lines we have studied, dominance of the primary tumor by a single clone and the elimination of all non-metastatic lineages by a metastatic variant did sometimes occur, but they were not frequent or essential events in the metastatic process. The findings we have documented therefore demonstrate that, although there are often changes in clonal composition during tumor growth, manifested by changes in the relative proportions of the genetic markers visualized autoradiographically, M^+ clones do not inevitably outgrow and eliminate ones with M^- phenotype in the primary tumor, nor is this an essential prelude to metastasis. On the contrary, measurement of the growth rates of representative KHT and TR4Nu cell lines *in vivo* and *in vitro* indicated that the TR4Nu cells grew faster than their KHT partners.

The question of why there is such marked reduction in complexity of the banding pattern during tumor formation by some cell populations is interesting and deserves brief consideration. We believe this indicates that some populations contain only a few genetically distinct subpopulations and that, when the cells are initially labeled by transfection, many individual cells of the same subclone may each be labeled differently because of the random integration of the plasmid. Individual tumors and tumor cell lines vary in the proportion of stem cells (operationally defined as the clonogenic cells capable of propagating the tumor) that they contain, relative to amplification cells (which divide a few times and increase the population, but then senesce) [see Steel, 1977 for further discussion]. Some can reproducibly be transplanted by a single cell, demonstrating that they are composed solely of stem cells, while others will grow only if they are initiated with a large inoculum, indicating that the bulk of the population has limited life span and is being continually replaced. Loss of amplification cells, labeled at the time of transfection, could account for the simplification of banding pattern sometimes seen after cell inoculation. This process would then not strictly be one of dominance by a given clone, but rather one of fairly rapid loss of individuals with a limited life span and survival of one or a few marked, immortal progenitors. Conversely, the random integration of the marker gene could label several daughter stem cells of the *same* clone differently and give the impression that coexistent, genetically distinct clonal lineages from a given tumor cell population are being studied. These considerations do not affect the conclusions drawn from the data presented in this paper because the two populations selected for the work were known to be genetically distinct and the M^+ lineage chosen was also known to be of clonal origin.

The second main conclusion of this work is that cell populations which are definitely *incapable* of metastasis (i.e., M^-) on their own can, if combined with appropriate M^+ cells, participate in this process and contribute to the growth of the deposits. This deduction is reinforced by the absence of any detectable M^+ (KHT) cells in some of the metastatic deposits, which paradoxically, therefore, appeared to be composed solely of M^- cells. It seems likely that these metastases were originally composed of cells of both phenotypes and that the M^+ ones disappeared, but formal investigation is

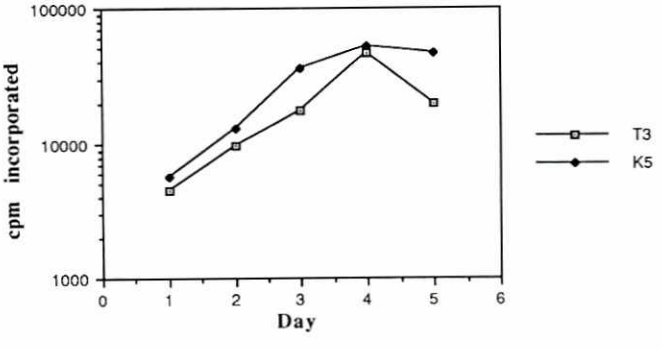

Fig. 4. Growth rates of cell lines separately *in vitro*.

required for confirmation because, if some of the originally M^- cells have become independently capable of metastasis on their own, this would have implications concerning the possible mechanisms involved and the clinical consequences. The observation that a minority component of the tumor cell population can, by as yet unknown means, induce others which are usually sedentary to join them in metastasis is clinically significant because it highlights a process which might, in appropriate combinations of tumor cell lineages, be capable of accelerating the geometric progression of the disease.

The strength of these conclusions rests on the use, in these experiments, of two separate cell lines, which are both mouse fibrosarcomas, but which differ completely with regard to the phenotype under study. Had we used M^- and M^+ clones from the same metastatic cell line the findings would have been more open to the interpretation that latent or dormant M^+ capability in the putative M^- clone had merely been released or re-awakened. In the framework of the present experiment it is clear that a new and different capability has been unmasked in the (previously) M^- cells which appeared in the deposits. It is only because *both* the M^+ and the M^- cells were labeled that the presence of M^- cells in the metastases could be definitively recognized. If the M^+ ones had been the only ones that were marked, the others would not have been identifiable with confidence and the relatively frequent occurrence of this phenomenon in these animals might have passed unnoticed.

There have been occasional previous reports suggesting presence of M^- cells in metastatic deposits: Miller (1983) cultured lung nodules obtained after i.v. or s.c. injection of mixtures of M^+ and M^- cells, and deduced the presence of M^- cells on the basis of their morphological characteristics. Also, Waghorne *et al.* (1988) described metastases, recovered from animals injected s.c.with a cell mixture composed of a neo^R labeled M^+ clone and a polyclonal unlabeled M^- population of the SP1 mammary carcinoma, which they inferred were composed of M^- cells because they did not see neo^R homologous bands on Southern blot analysis and failed to recover tumor cells that were resistant to G418 in culture.

The present work, by using distinguishable genetic markers on both populations, now provides direct proof that M^- cells can co-participate with *some* M^+ clones in metastasis. It now becomes an important priority to ascertain the mechanisms involved. Do the M^+ cells induce or recruit the M^- cells to become independently M^+ by transfer of some agent; or do they open a pathway which the M^- cells could not enter on their own, or are the M^- cells carried passively to their new residence because they are adherent to their more migratory partners? The techniques are now available to answer these questions.

Materials and Methods

Animals and inoculations

MF1 nude mice were obtained from the breeding facility at the John Radcliffe Hospital, Oxford University. Tumour cell inoculations were standardized to contain 10^6 cells in a volume of 100 µl injected subcutaneously into the right flank. Animals were observed daily and killed when the resulting tumor reached 3 cm diameter, or sooner if the tumor ulcerated or the animal appeared distressed or moribund.

Cell marking, mixing and reinoculation procedures

Genetic tagging of tumor cell populations with the neomycin resistance gene was performed as described previously (Moffett *et al.*, 1992). Briefly, cells were transfected with 25 µg plasmid pSV2 neo by sonication during log phase growth and those which had not incorporated exogenous DNA were eliminated by exposure to the neomycin analogue G418 (800 µg/ml). They were then either pooled or individually isolated using cloning rings and expanded for cryopreservation, DNA extraction and animal inoculation.

Confluent cultures of single transfected clones with unique banding patterns, or the entire polyclonal products of an individual transfection, following neomycin selection and growth to confluence, were stripped from the flasks by trypsin-EDTA treatment, suspended in culture medium and injected subcutaneously into nude mice to assess their metastatic capabilities and verify the stabilities of their neo^R integration patterns during tumor growth *in vivo*.

Autopsies and sample preparation

When the resulting tumors reached 2.5-3 cm diameter the animals were killed and autopsied and samples from the primary tumors and from metastases were taken for histological confirmation and DNA analysis. Pieces of tissue for histological examination were fixed in 10% formal-saline and processed for microscopy. Tumour samples for DNA analysis were placed in vials and frozen immediately in liquid nitrogen. DNA was later extracted and Southern blots of endonuclease digested (DNA) samples were probed to determine the neo integration patterns of the tumors (see below). These were then compared to the banding patterns of the inoculated cells and of the separate individual clones, where appropriate, to determine the clonal composition of the neoplastic tissue.

DNA analysis

High molecular weight DNA was isolated from cell lines and tissue samples using the technique of Blin and Stafford (1976). The DNA content of the purified samples was measured spectrophotometrically. For analysis, equal amounts of DNA (15 µg) from each sample were digested to completion with EcoR1 restriction endonuclease (which cuts the neomycin resistance gene only once). The samples were loaded on an agarose (0.8%) gel and electrophoresed to separate the digested DNA fragments. Equal loading of the tracks was checked by staining with ethidium bromide and u-v transillumination of the gel. Denaturation and transfer of DNA in the gel, to Hybond N^+ membranes (Amersham International, Little Chalfont, UK), was performed by alkaline blotting. Filter membranes were then probed, according to the following protocol, with a ^{32}P-labeled 2.2kb Bam H1/Hind III DNA fragment containing the whole neomycin resistance gene excised from plasmid pSV_2 neo. Prehybridization was performed for 3 h at 65°C in 5 x Denhardt's; 5 x SSPE; 0.5% SDS solution containing 20 µg/ml denatured salmon sperm DNA. Hybridization was performed overnight in fresh prehybridization solution with the addition of denatured labeled probe (1-2ng/ml). Filters were then washed 6 times (20 min each) at increasing stringency to a final stringency of 0.2xSSC; 0.1% SDS at 65°C. Autoradiography was performed overnight at -70°C using Kodak x-o-mat 's' film with intensifying screens.

Growth rates in vitro

In vitro growth rates of K5 and T3 cell lines were determined by plating ($1x10^4$) cells per well in 24-well plates, 4 wells per clone. On 5 consecutive days 20ml of a ^{125}I-UdR solution in DMEM were added to each well of one plate to give a final concentration of 0.5 µCi/mL. After 2 h incubation the cells were washed 3 times with PBS and lysed with 0.1N NaOH (120 µl/well). The cell lysate was absorbed onto cotton swabs, and cpm incorporated were determined with a gamma counter.

Growth rates in vivo

In vivo growth rates of K5 and T3 cell lines were determined by injecting 10^6 cells of each clone subcutaneously into the right flank of nude mice and calculating the means of 2 orthogonal diameters of the tumors measured twice weekly up to 9 weeks after injection.

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