Eur J Cancer Clin Oncol, Vol. 18, No. 10, pp. 1017–1026, 1982.
Printed in Great Britain.

0227-5379/82/101017-09$03.00/0

Properties of a Herpes Virus-transformed Hamster Cell Line—I. Growth and Culture Characteristics of Sublines of High and Low Metastatic Potential*

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Abstract—*A Herpes virus hominis type 2-induced tumour line of the Syrian hamster, designated HSV-2-333-2-26, exhibited a low level of spontaneous metastasis from the primary subcutaneous tumour mass. Two lung foci, met A and met B, were resected and transplanted s.c.; both lines showed an elevated metastatic potential compared with the parent cell line such that all animals inoculated with these cells developed metastatic disease within 40 days after resection of the primary tumour. Neither the rapidity of cell growth in vivo or in vitro, nor anchorage-independent growth or degree of attachment to a solid substrate in vitro are implicated in the altered behaviour of the metastatic cell lines.*

INTRODUCTION

THE METASTATIC spread of cells from the primary tumour to distant locations has become recognised as the central problem in malignant disease. The traditional view of a progression from a relatively homogeneous tumour mass followed by spread to the regional lymph nodes and ultimately by widespread dissemination has been questioned in recent years, as metastatic cells are frequently shed from small primary tumours and may have properties distinct from those of non-metastatic cells. Thus, recent studies have shown that metastatic and primary tumour cell populations may differ in drug susceptibility [1], organ affinity [2], karyotype [3], biochemistry [4], surface antigens [5], immunogenicity [6] and lectin sensitivity [7]. These differences suggest the action of selective pressures in metastasis; cells which possess certain properties may be more likely to form metastases than other cells in the primary tumour mass. It is therefore important to determine the properties of tumour cells which allow the formation of metastatic deposits.

In the present communication the selection and characterisation of two highly metastatic variant cell lines of a Herpes virus hominis type 2 (HSV-2)-transformed hamster cell line is described. Some biologic properties of the variant lines are compared with those of the parent line; these include *in vivo* growth rate and incidence of metastases, *in vitro* growth rate, cell morphology and anchorage-independent growth. The results for the three cell lines are compared in an attempt to determine the biological features which characterise metastatic as compared to non-metastatic cells.

MATERIALS AND METHODS

Hamsters

Male Syrian golden hamsters, 4–6 weeks of age and 60–90 g in weight, were obtained from a closed, randomly bred colony at the University of Sheffield and were used for all experiments. Whilst these animals are not an isogeneic, inbred strain, their immunological homology is very substantial since a 100% success rate has been achieved in exchange skin grafts between animals (Potter and Jennings, unpublished observation).

Accepted 2 June 1982.
*This work was supported by grants from Weston Park Hospital Cancer Research Fund and the Yorkshire Cancer Research Campaign.

In vivo *tumour lines*

The parental cell line, designated HSV-2-333-2-26, was originally derived *in vitro* by the selection of a single focus of transformation after infection of hamster embryo fibroblasts with ultra-violet light-irradiated Herpes simplex virus type 2; these cells were kindly supplied by Dr. F. Rapp, Department of Microbiology, Pennsylvania State University, Hershey, PA, U.S.A. Details of the isolation procedures are described for a sister cell line (333-8-9) in Shin *et al.* [8].

Following inoculation of the cells into weanling hamsters, a transplantable tumour line was established and maintained by trocar implantation of tissue fragments at 2 to 3-week intervals.

Two metastatic sublines were isolated from tumour-bearing hamsters. Subcutaneous primary tumours were resected at 10–12 mm mean diameter; four weeks later the animals were killed and the lungs examined for metastatic foci. Two foci were excised from a single animal showing macroscopic metastasis, transplanted s.c. and designated met A and met B. Evidence that the met A and met B cell lines were isolated from parent cells and not the result of independent virus transformation events was obtained by karyotypic analysis; several abnormal chromosomes were identified by G-banding in all these cell lines (W. Middleton, personal communication).

The parent, met A and met B lines were maintained by s.c. implantation for a further six passages; tumour material was minced and frozen directly in vapour-phase liquid nitrogen for further studies.

In vitro *cell lines*

Tumour fragments were dissociated with trypsin (0.25% 1:250, Difco) and the resulting cell suspension seeded into 4-oz medical flats in Hams F10 medium supplemented with 5% v/v foetal bovine serum and 50 μg/ml gentamycin. Cell lines were subcultured when confluent and after 2 weeks were frozen in vapour-phase liquid nitrogen in growth medium containing 10% DMSO. Cells within 10 passages of this stock were used for all subsequent experiments.

Assessment of metastatic potential

Tumour cells (10^4 in 0.2 ml) from *in vitro* cultures of parent, met A and met B cell lines were injected s.c. into 4 to 6-week-old male hamsters. The developing tumours were resected approximately 3 weeks later, when the average diameter was 10–15 mm, and the animals were observed for a further 8 weeks. Any hamsters showing signs of illness or respiratory distress during this time were killed and examined for metastatic involvement of regional lymph nodes, lungs, kidneys and accompanying tumour growth in the pleural cavity. Experiments were terminated 8 weeks after tumour resection, all surviving hamsters killed and a post-mortem examination carried out. Analysis of the survival data was subjected to the non-parametric $2 \times k$ contingency test.

Growth rates of tumours in vivo

Cells (10^4) were inoculated subcutaneously into 4 to 6-week-old male hamsters. Tumours became palpable after a latent period of about 14 days and their mean diameter was measured at intervals thereafter. The growth rates of individual tumours were calculated by linear regression analysis and, where the difference between tumours was not statistically significant, the slopes were pooled, as described by Rees and Westwood [9]. Linear regression analysis was carried out to test the similarity in growth rates between the cell lines.

Growth rates of cell lines in vitro

Sixty-millimetre tissue culture-grade Petri dishes (Sterilin) were inoculated with 5×10^4 cells in 5 ml Hams F10 medium supplemented with 5% foetal bovine serum. At 24-hr intervals dishes were harvested in triplicate with trypsin and the cell numbers counted using a haemocytometer. Analysis of the data was carried out using linear regression analysis of $\log_2$ mean cell number per dish vs time in culture.

Anchorage-independent growth

The ability of the cells to grow in the absence of adhesion to a solid substrate was determined by a modification of the technique of MacPherson and Montagnier [10]. A 5-ml base layer of 0.4% tissue culture-grade agar in Hams F10 medium with 10% foetal bovine serum was poured in to 50-mm plastic Petri dishes and cooled to 4°C. Cells were removed from flasks by a brief treatment with trypsin, re-suspended at 1.5×10^5 cells/ml in growth medium and added to 0.4% agar in Hams F10 held at 45°C in the ratio of 1 vol. of cells to 2 vol. of medium. One millilitre was pipetted rapidly into each of 5 Petri dishes; the temperature of the pre-cooled dishes ensured that the cells spent a minimum time at an elevated temperature. The final cell suspension obtained was 500 cells/ml in 0.27% agar, Hams F10 medium and 10% foetal bovine serum; dishes were in-

cubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

After 24 hr incubation parallel lines 2 mm apart were scribed on the underside of the dishes and the number of single cells residing between these marks was counted under inverted phase contrast microscopy. The count was repeated 10 and 15 days later and the cells scored for non-growth (less than 4 cells) or growth (more than 4 cells); at least 100 cells were counted for each assay. Most cells which grew formed very loosely packed colonies by 10 days and no increase in anchorage-independent growth was observed by incubation for a further 5 days; cells which were unable to grow remained refractile and viable at 10 days, but many had degenerated by 15 days post-inoculation. This protocol eliminated inaccuracies inherent in cell counts and dilutions, as both growing and non-growing cells are scored.

Cell morphology in vitro

Cells from all three lines were suspended in growth medium and plated into plastic Petri dishes containing a sterile glass coverslip at a density of 10^5 cells per dish. After incubation for 48 hr the morphology of exponentially growing cells attached to a glass substrate was photographed with a Leitz inverted microscope.

RESULTS

Metastasis of parent, met A and met B tumours

The development of spontaneous metastasis from the three cell lines was assessed following surgical excision of the primary tumour transplant three weeks after s.c. inoculation of tumour cells. Hamsters showing signs of illness or respiratory distress were killed and examined for the presence of metastatic foci. A comparison of the survival times of hamsters inoculated with parent, met A or met B cells in two independent experiments is shown in Fig. 1(a, b). The results show clear differences between the parent line and the two metastatic sublines; thus in both cases all hamsters inoculated with met A or met B tumour cells had become morbid by day 47 following tumour resection, whilst only 1 of a total of 20 hamsters which received the parent line tumour cells had developed metastatic disease. In the first experiment met B cells appeared to be more metastatic than met A cells; this observation was reversed in the second experiment, and analysis shows that neither difference was statistically significant.

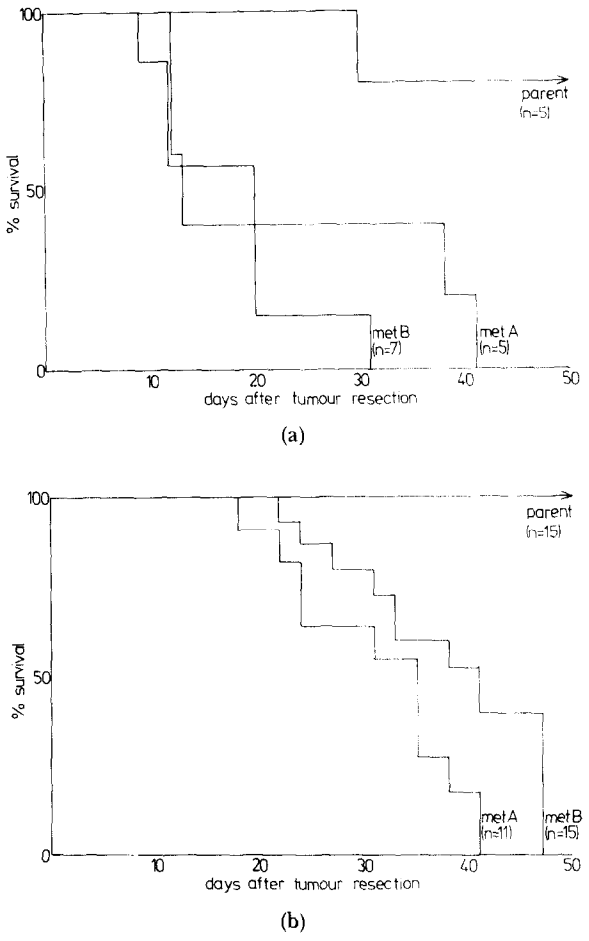

Fig. 1a, b. Survival of hamsters after subcutaneous inoculation of 10^4 cells of the parent, met A or met B cell lines, followed by resection of the primary tumours at 10–15 mm diameter. Differences between met A and met B are not statistically significant by 2 × k contingency test. (a) Experiment 1, $\chi_2^2 = 1.23$, P = > 50%; (b) experiment 2, $\chi_2^2 = 6.27$, P = > 10%.

The incidence and distribution of metastatic spread from the primary tumours induced by s.c. inoculation of the parent, met A and met B tumour cell lines is shown in Fig. 2(a, b). In the first experiment the single animal which died of metastatic disease following inoculation of the parent line showed involvement of all four sites examined; in addition, one further animal showed involvement of the regional lymph nodes when the experiment was terminated 8 weeks after excision. At the termination of the second experiment one animal inoculated with the parent cell line had macroscopic metastatic disease of the lungs, but all other hamsters were apparently disease-free (Fig. 2b). In contrast, generalised dissemination of the tumour cells was observed in hamsters inoculated with met A and met B tumour cells.

In one experiment metastatic deposits in the kidney were more often observed from the met A cell line, whilst involvement of the regional

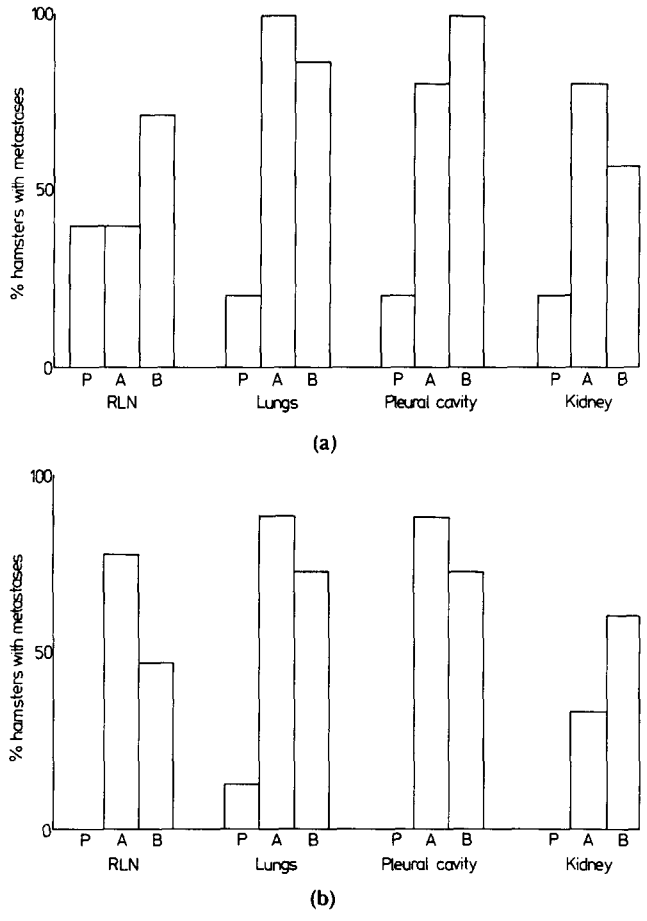

Fig. 2a, b. Incidence and organ distribution of metastatic deposits assessed up to 8 weeks *after resection of the primary tumour. Differences between met A and met B are not statistically significant by* $2 \times k$ *contingency test. (a) Experiment* 1, $\chi_2^2 = 0.90$, P = >80%; *(b) experiment* 2, $\chi_3^2 = 2.18$, P = >40%.

lymph nodes was more frequent in the met B line; however, this situation was reversed in the subsequent experiment, and in neither experiment was the difference statistically significant. Several animals which showed metastatic involvement of the lungs had no visible enlargement or involvement of the regional lymph nodes draining the primary tumour site; this observation infers that haematogenous as well as lymphatic spread of tumour cells occurred with these cell lines, although invasion via the body wall and colonisation of the lung tissue directly from the pleural cavity cannot be discounted.

The tumour-producing potential of the parent, met A and met B sublines following s.c. inoculation of viable tumour cells has also been examined. All three lines showed a tumour dose 50 (TD_{50}) of less than 10^2 cells, with no significant difference observed between them.

The rates of growth of tumours derived from the three cell lines is shown in Table 1. The growth kinetics most closely approximate logarithmic growth in common with those reported from similar studies [11]. Consequently, linear regression analysis was carried out on the $\log_{10}$ mean tumour diameter vs time, and the growth rate is shown as the slope of this line (i.e. the increase in $\log_{10}$ mean diameter per day). In addition, the mean diameter doubling time is tabulated. No significant difference was found in the growth rate between the individual tumours derived from either parent or met B lines. Consequently, pooled growth rates are presented for these two lines. The difference between parent and met B is not significant at a 5% level of confidence ($F_{1,134} = 3.010$, $P = <10\% > 5\%$). In all three experiments tumours derived from the met A cell line showed a large spread in growth rates; whilst the difference between individual tumours within each experiment is not significant, the range was greater than observed with met B or the parent cell lines. The variation was particularly evident in experiment 3, and the difference between tumour growth rates in all three experiments is significant at a level of confidence of 0.5%.

Table 1. *Rates of growth of tumours derived from the subcutaneous inoculation of the parent, met A and met B cell lines expressed as the linear regression slope of* $\log_{10}$ *mean tumour diameter vs time and the mean diameter doubling time in* days

Experiment No.	Parent		Met A		Met B	
	Slope	Growth rate	Slope	Growth rate	Slope	Growth rate
(1)	0.067	4.5	0.075	4.0	0.066	4.6
	0.062	4.9	0.069	4.4	0.079	3.8
	0.062	4.8	0.076	4.0	0.048	6.2
	0.069	4.4	0.092	3.3	0.046	6.6
			0.091	3.3	0.046	6.6
			0.082	3.7	0.066	4.5
	$F3,11 = 0.1497$		$F4,12 = 1.5551$		$F,5,18 = 2.1045$	
	$P = > 10\%$		$P = > 10\%$		$P = > 10\%$	
	PS = 0.064		PS = 0.081		PS = 0.066	
(2)	N.D.		0.087	3.5	0.046	6.5
			0.054	5.6	0.047	6.4
			0.056	5.4	0.041	7.4
			0.058	5.2	0.037	8.2
					0.047	6.4
					0.043	6.9
			$F3,6 = 1.9565$		$F5,10 = 0.1980$	
			$P = > 10\%$		$P = > 10\%$	
			PS = 0.061		PS = 0.043	
(3)	0.055	5.4	0.029	10.5	0.030	10.2
	0.054	5.5	0.063	4.8	0.050	6.1
	0.063	4.8	0.053	5.7	0.050	6.0
	0.052	5.8	0.043	7.1	0.052	5.8
			0.064	4.7	0.050	6.0
					0.045	6.7
					0.056	5.4
	$F3,14 = 0.7584$		$F4,10 = 2.4242$		$F8,27 = 0.8167$	
	$P = > 10\%$		$P = > 10\%$		$P = > 10\%$	
	PS = 0.057		PS = 0.050		PS = 0.048	
Cumulative	$F7,25 = 0.5591$		$F14,28 = 4.2049$		$F20,55 = 1.3685$	
statistics	$P = > 10\%$		$P = < 1\% > 0.5\%$		$P = > 10\%$	
	PS = 0.060				PS = 0.0506	

PS = pooled slope (where statistically tenable); ND = not done.

In vitro *growth rates*

The growth rates of the three cell lines when cultured *in vitro* in Hams F10 medium supplemented with 5% foetal bovine serum are shown in Fig. 3. Growth was exponential over the 3-day time course of the experiment, during which the population doubling time of the parent and met B cell lines was slightly in excess of 20 hr whilst met A doubled in 15.5 hr. Thus the growth rate of met A was significantly faster than the other two cell lines at a 1% level of confidence, whilst the parent and met B lines grew at indistinguishable rates.

Anchorage-independent growth

The plating efficiency of the three cell lines in semi-solid agar is shown in Table 2; a single determination at passage 3 was made for the parent line and two separate determinations at passages 3 and 8 were made for the metastatic sublines. The results show that the met A cells plated less efficiently than the parent line but that the met B cells were more able to grow in the absence of adhesion to a solid substrate.

Morphology

Photomicrographs of the three cell lines growing upon a glass substrate are shown in Fig. 4. The impression that the tumour cell lines were loosely adhesive, very easily trypsinised and readily strip from the substrate in alkaline or depleted medium is confirmed by micrography. All three lines showed a significant proportion of refractile, rounded cells barely attached to the substrate. The attached cells of the met B line had a fusiform appearance with numerous overlapping projections. In contrast, the adherent cells of the parent line exhibited flattened and more epithelial morphology; cells were arranged in a non-overlapping, regular array and formed a non-refractile cell sheet. The met A cells had a morphology intermediate between the other two lines, with a slightly greater similarity to met B. The hypothesis that a decreased level of substrate adherence *in vitro* may reflect a similar loss of adherence *in vivo*, which could predispose to the shedding of tumour cell emboli and the formation of micrometastases, is not supported by the three cell lines reported here as all three cell lines exhibit a similar and only loosely adherent morphology.

Table 2. Anchorage-independent growth of the parent, met A and met B cell lines in semi-solid agar culture

Cell line	Passage No.	Plating efficiency (%)
Parent	3	29
Met A	3	22
	8	19
Met B	3	35
	8	45

DISCUSSION

Several previous studies have reported the selection of tumour cell lines of increased metastatic potential from a parent line of lower potential. The two most intensively studied systems are the B16 melanoma and the RAW 117 murine lymphosarcoma; whilst these

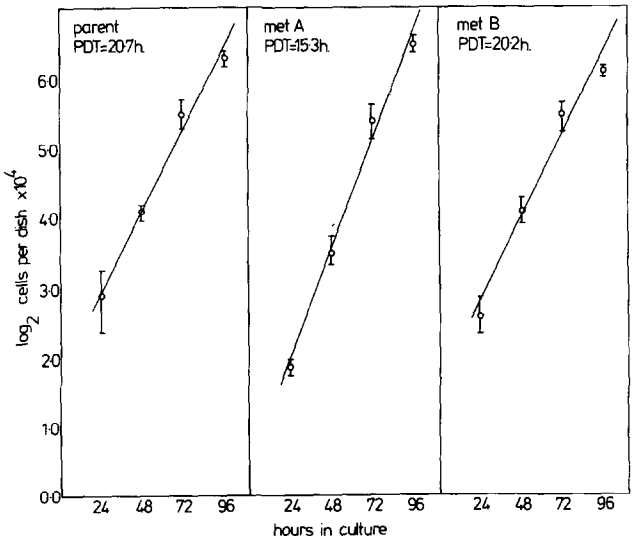

Fig. 3. Rate of growth of the parent, met A and met B cell lines in vitro. *Parent and met B lines grew at statistically indistinguishable rates (linear regression analysis,* $F_{1,19} = 0.130$, $P = 10\%$) *whilst met A grew more quickly (met A/parent,* $F_{1,19} = 10.513$, $P = < 0.5\%$; *met A/met B,* $F_{1,19} = 8.637$, $P = < 1\%$). *PDT = population doubling time.*

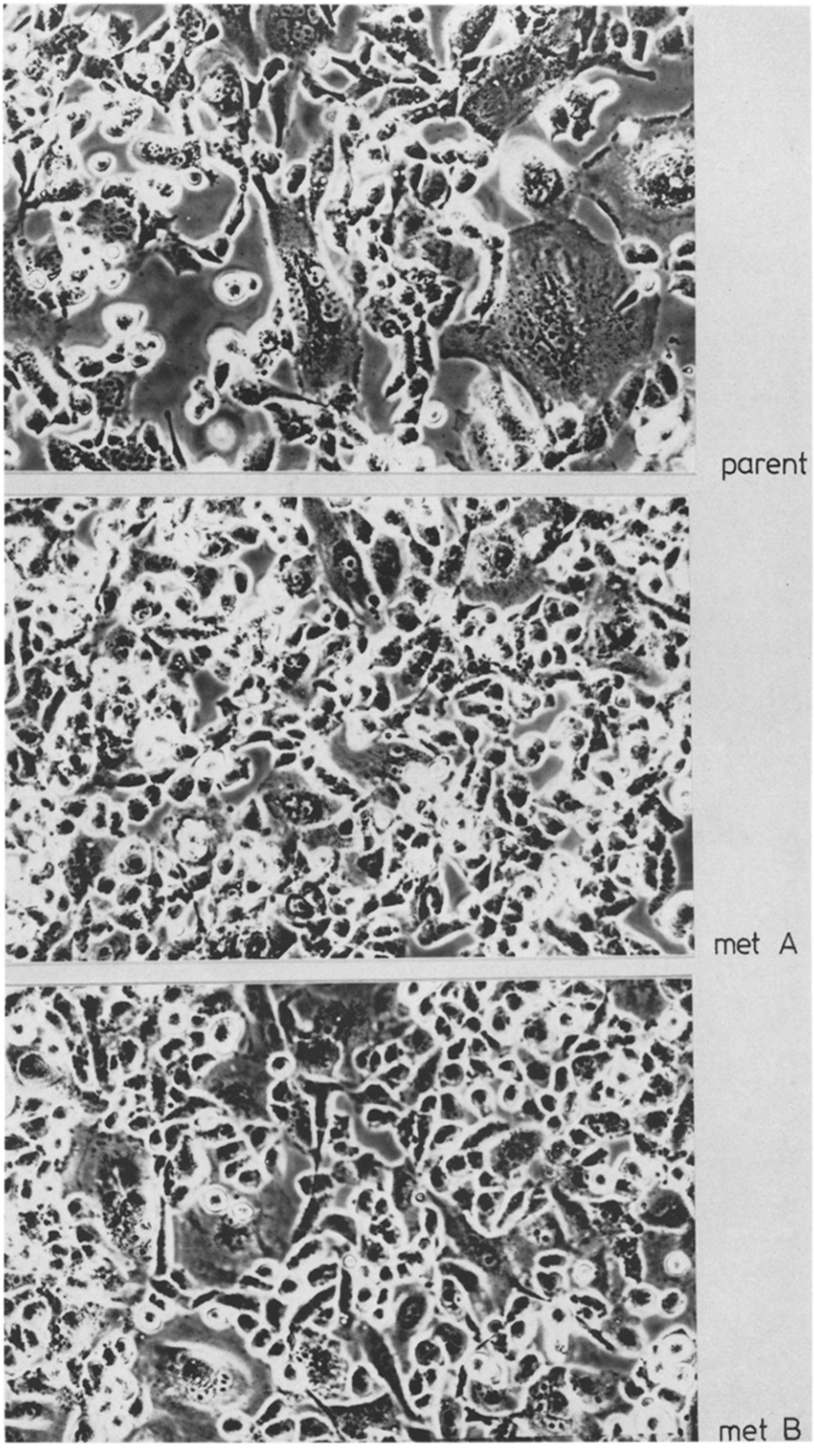

Fig. 4. Morphological growth characteristics of the parent, met A and met B cell lines attached to a glass substrate in vitro. *Whilst all cell lines exhibited a poorly adherent morphology, met B cells were more fusiform in nature than met A or parent.*

tumours are spontaneously metastatic, their selection was accomplished by 'artificial metastasis', the intravenous inoculation of a tumour cell suspension ([12, 13]). These sublines may not be ideal for the comparative study of metastatic cells; a system which was selected by spontaneous metastasis from a solid primary tumour is to be preferred. The 333-2-26 hamster tumour system described in this communication exhibits spontaneous metastasis at a low level. The technique of surgical removal of the primary tumour enabled the metastatic potential of tumour cells to be expressed in distant organs; recovery of tumour nodules from these secondary sites enabled isolation of two cell lines of high metastatic potential. This system probably represents a more realistic model of metastasis than the models described previously. After a single cycle of selection in the hamster 333-2-26 tumour cell system two sublines were isolated which showed a significant increase in metastatic potential. Further cycles of lung-to-subcutaneous transplant are being carried out at this time to determine whether a greater level of selection will result in a still higher metastatic potential of the met A and met B sublines.

One aspect of metastatic spread of human neoplastic disease is its organ-specific propensity. Selection of lung metastatic foci from the hamster 333-2-26 system resulted in an increase in overall metastatic potential but no predominant lung or renal localisation. In contrast, using i.v. inoculation Fidler and Nicolson have shown organ specific localisation of both B16 melanoma and RAW 117 lymphosarcoma. Further selection of both lung and kidney nodules is being carried out to determine the organ-specific metastatic potential of this system.

The growth rate of tumours derived from both the parent line and met B line were very similar. Firstly, linear regression analysis was carried out on the growth rates of tumours derived from inoculation of cells in each experiment; this analysis was repeated upon the combined data from all experiments for each cell line. In the case of the parent and met B cell lines no significant difference was observed between the growth rates of tumours within each experiment nor between experiments; consequently, pooled parameters were calculated which suggested that the parent line grew marginally faster than met B. However, this difference was not statistically significant. Tumours derived from the inoculation of met A cells were more heterogeneous in their growth rate; no significant difference was detectable between tumours within individual experiments, but regression analysis revealed highly significant differences between tumours when the data from all three experiments was combined. Despite this heterogeneity, the growth rate of met A was not grossly different from its parent or sister cell lines. Whilst it may be postulated that the successful growth of disseminated tumour cells into metastatic deposits may be influenced by their growth rate, there is no evidence of such an effect in this hamster tumour system.

The ability of transformed cells to grow and divide in the absence of anchorage to a solid substrate has been shown to correlate strongly with their tumorigenic potential *in vivo* [14]; it is of interest to determine whether the plating efficiency in soft agar is an indicator of the malignancy of tumorigenic cells. Whilst the parent line, an invasive but rarely metastatic tumour, plated at an efficiency of 29%, the two highly metastatic sublines showed a variant anchorage-independent growth, met A being lower at 21% and met B higher at 40%. Thus with a limited number of cell lines in this system, no apparent correlation between metastatic potential and growth in semi-solid agar is seen.

The data presented in this paper show that in the HSV-2-333-2-26 hamster tumour model it is possible to select very rapidly for increased metastatic potential and obtain cell lines which disseminate in all animals from a primary subcutaneous tumour site to form lethal secondary disease in distant organs, predominantly the lungs, kidneys and lymphatics. Several possible reasons for this new behaviour can be discounted from the results of the present study. Thus the rapidity of cellular growth either *in vivo* or *in vitro*, their independence of substrate attachment for division or their ease of detachment from the substrate do not appear to be increased in the metastatic cell lines. Other aspects of the behaviour and biochemistry of these cell lines are being studied to determine those characteristics implicated in their malignant potential; both the immunology of the cell lines (Teale *et al.*, in preparation) and the cell surface biochemistry (Walker *et al.*, in preparation) will be reported in subsequent communications.

Acknowledgements—We wish to thank the staff of the Department of Pathology, Weston Park Hospital, for their assistance during this investigation.

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