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Cell Adhesion and Experimental Metastasis: a Study Using the B16 Malignant Melanoma Model System*

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Abstract—*The adhesive properties of B16F1 and B16F10 cells have been studied following their rates of attachment to various substrates and by analysis of their rates of aggregation within the defined environment provided by a cone and plate viscometer. Contrary to previous reports, we found that the B16F10 cells (with significant lung-colonizing potential following tail vein injection) were less homotypically adhesive than B16F1 cells (which colonize lungs poorly). The adhesiveness of B16F10 cells approached that of B16F1 cells only under conditions (low shear, low cell number) where cell collisions were thought to be so few that quantitative differences in aggregation rate could not be determined. B16F1 cells also adhered more to lung cells than did B16F10 cells when assessed by aggregation rate. However, analysis of aggregate composition in which one cell type had been fluorescently labelled showed that B16F10 cells actually formed more mixed aggregates with lung cells than did B16F1 cells. There was no significant difference in the adhesiveness of B16F1 or B16F10 cells to liver cells as assessed by aggregation rate. Analysis of aggregate composition under these circumstances, however, showed that B16F10 cells formed fewer mixed aggregates with liver cells than did B16F1 cells. These results are consistent with the possibility that metastatic cells need to display poor homotypic adhesiveness in order to detach from the primary but enhanced heterotypic adhesiveness in order to colonize specific organs.*

INTRODUCTION

THE ADHESIVE interactions of tumour cells either with themselves (homotypic interaction) or with host cells (heterotypic interaction) are thought to be important in determining the eventual outcome of the metastatic process. It is possible to identify three phases during the pathogenesis of metastasis where adhesive interactions might influence the eventual outcome of the process: detachment of the metastasizing cell from the primary, interaction with host cells during transport to a remote site, and interaction with host cells to localize the tumour cell prior to secondary growth. Consider a tumour cell spreading by a haematogenous route. Intuitively, such a cell should display poor homotypic adhesiveness in order to detach from the primary, poor heterotypic adhesiveness to leukocytes in order to avoid cytotoxic cells, and significant heterotypic adhesiveness at some remote site in order to localize. Unfortunately, available data suggest that such an interpretation may represent a gross oversimplification of the metastatic process. Thus B16F10 malignant melanoma cells, which display significant lung colonizing ability following tail vein injection (an experimental system employed to model in part the metastatic process), are thought to adhere more homotypically than B16F1 cells, which colonize lungs poorly [1–4]. Furthermore, B16F10 cells adhere more to splenic lymphocytes than do B16F1 cells [1], and even though the B16F10 cells are more sensitive to immune lymphocyte cytotoxicity [5], they still form significantly more lung colonies than do B16F1 cells. Although it seems obvious that more than cell adhesiveness *per se* is involved in metastasis, it remains entirely conceivable that changes in adhesiveness could contribute to the

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overall phenomenon. Indeed, the non-random patterns of tumour cell arrest seen in certain experimental systems [6] and the organ-specific growth of particular metastatic tumours in both human [7] and animal systems [8, 9] may involve selective and possibly specific heterotypic cell–cell adhesive interactions.

We have re-examined the adhesive properties of B16F1 and B16F10 cells using two different techniques. The first is based on the rotary aggregation method but employs the principles of viscometry to provide more standard conditions [10]. An indication of the physical conditions of the aggregation environment is an absolute requirement for the comparison of the adhesiveness of different cell types. The second technique is based on the collecting lawn method, in which the adhesiveness of suspended cells is measured by following their attachment rate to a lawn or monolayer of adherent cells. Our results provide evidence in support of the role of cell adhesiveness in the detachment of a metastasizing tumour cell from the primary and in its localization at particular secondary sites.

MATERIALS AND METHODS

Maintenance of cell lines

B16 mouse melanoma cells lines, F1 (low lung colonization) and F10 (high lung colonization), were kindly supplied by Dr. I. J. Fidler (Frederick Cancer Research Center, Frederick, MD, U.S.A.). Melanoma cells were grown in CMEM, which consisted of Eagles minimum essential medium (Flow Labs) supplemented with 10% foetal calf serum (Gibco Ltd), 2mM sodium pyruvate, non-essential amino acids, vitamins, 50IU/ml benzyl penicillin and 50 μg/ml streptomycin sulphate (Flow Labs). Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Subconfluent monolayers were subcultured every third day by the transfer of 0.2×10^6 cells per 9-cm diameter Petri dish (Nuclon, Gibco Ltd). Monolayers were detached following a 1-min incubation at room temperature in Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (CMF PBS) containing 2mM EDTA (ethylenediamine tetra-acetic acid, disodium salt). Removal of cells was facilitated by vibrating the Petri dish on a rotamixer for approx. 15 sec. The cells were removed by pipetting with 4 vol. CMEM and were sedimented in the cold for 3 min at 150 ***g***. The cells were then resuspended and counted using a Coulter counter (Model ZB) in conjunction with a Channelyzer C1000.

Melanoma cell lines were maintained as described for up to 20 consecutive transfers before returning to cryogenic stocks to initiate fresh cultures. Cell cultures were regularly monitored and found to be free of mycoplasma contamination as assessed by the fluorescent stain Hoechst 33258 [11].

Preparation of tissue cells

Single-cell suspensions of lung and liver were obtained from syngeneic C57B16 mice (Olac 1980 Ltd). Whole organs were removed under aseptic conditions and were washed with phosphate-buffered saline containing 50IU/ml benzyl penicillin and 50 μg/ml streptomycin sulphate. Tissues were cut into a fine mince and incubated with 10 ml of an enzyme solution containing 0.025% trypsin (Difco 1:250), 0.025% collagenase and 0.01% deoxyribonuclease 1 (Sigma) in CMF PBS at 37°C for 45 min on a rotary mixer at 1 cps. Single cells were then obtained by filtration through nylon bolting cloth (pore size, 20 μm), enzyme activity was halted by the addition of 2 vol. CMEM to the filtrate, and the cells obtained by sedimentation in the cold for 3 min at 150 ***g***. The resulting cell pellet was washed twice with CMEM, finally resuspended in a known volume of CMEM and counted using the Coulter counter. The single cells were then seeded into 9-cm diameter Petri dishes at approx. 10^5 cells per dish. Tissue culture cells were maintained in CMEM under identical conditions to those used for the melanoma cell lines except that they were maintained as primary cultures only and were refed every fourth or fifth day until confluent monolayers were produced. At confluence the monolayers were washed with 5 ml CMF PBS and then incubated at 37°C for 5 min with 2 ml CMF PBS containing 0.1% trypsin plus 0.5 mM EDTA. Cell lines were removed by gentle pipetting with 2 vol. CMEM and were sedimented in the cold for 3 min at 150 ***g***. The tissue cells were then resuspended in CMEM, counted using the Coulter counter and adjusted to the desired concentration for use.

Substrate adhesion

Substrate adhesion was measured by following the rate of attachment of single-cell suspensions to plastic and cellular substrates. B16 melanoma variants were recovered as described and re-suspended in CMEM containing 20 mM HEPES at 4×10^5 cells/ml. A 0.2-ml sample of cell suspension (8×10^4 cells) was added to each well of a Falcon (3047) 24-well tissue culture plate (Becton-Dickinson Ltd). At timed intervals the cell suspension was aspirated from duplicate wells with a Pasteur pipette, the wells washed gently with 1 ml Isoton (Coulter Electronics Ltd) and the aspirated suspensions and washings made to 10 ml with Isoton. The number of cells recovered from each well was then determined

using the Coulter counter and the proportion of adherent cells obtained by difference.

For use in homotypic and heterotypic monolayer attachment assays, B16 variants or C57B16 lung cells respectively were grown to confluence in Falcon 24-well plates. Growth medium was aspirated from the wells and replaced with 0.2 ml of the appropriate cell suspension. Rates of attachment of B16 variants were then determined as decribed above.

Cell aggregation

The aggregation of the B16 variant cell lines was carried out on low- and high-density cultures. Low-density cultures were seeded at 10^5 cells per 9-cm diameter dish, which yielded 1.5×10^6 (B16F1) or 1.6×10^6 (B16F10) cells after 3 days. High-density cultures were seeded at 5×10^5 cells per dish, which yielded confluent monolayers by 3 days containing 6×10^6 (B16F1) or 8×10^6 (B16F10) cells. The aggregation of a single-cell suspension was carried out within the confines of a Wells-Brookfield cone and plate viscometer (model LVT-C/P, cone angle 0.8°) at shear rates between 4.5 and 450/sec essentially as described before [10]. For homotypic aggregation assays, cells were suspended in CMEM (supplemented with 20 mM HEPES buffer) to a constant volume fraction of 0.37% based on Coulter Channelyzer nodal volumes. A 1-ml sample was added to the viscometer cup and cell aggregation was measured by the fall in total particle number with time. The results are expressed as an aggregation index N_t/N_0, where N_0 is the number of particles (single cells plus aggregates) at time 0 and N_t is the number of particles at time t min.

Heterotypic aggregation assays involving C57B16 lung or liver cells and melanoma variant cell suspensions were carried out under similar conditions except that a 0.5-ml aliquot of each cell type (10^6 cells/ml) was added to the sample cup and mixed at time 0. Cell aggregation was then measured as described above.

Cell labelling

For heterotypic aggregation assays C57B16 tissue cells were labelled as follows: cells were harvested as described and suspended at 10^6 cells/ml in CMEM at 4°C. The fluorescent lipid probe AFC_{16} (5-(*N*-hexadecanoyl) aminofluorescein; Molecular Probes Inc.) at 1 mg/ml in dimethylsulphoxide was added to the cell suspension (10 μl/ml), mixed immediately and allowed to react with the cells for 1 min at room temperature. The cells were then sedimented in the cold for 3 min at 150 *g* and washed twice with CMEM. Cells were then resuspended in CMEM containing 20 mM HEPES at 10^6 cells/ml and kept at 4°C on ice until required. The extent of labelling as assessed before use was always 100%. Aggregate size and composition were determined microscopically using a Zeiss fluorescence microscope.

RESULTS

Substrate adhesion

The substrate adhesiveness of B16F1 and B16F10 cells was studied by following their attachment to homotypic monolayers, heterotypic monolayers or to the plastic substrate. In all three systems the adhesiveness was initially rapid over the first 5 min, after which the adhesive rate generally tended to decline (Fig. 1). In the homotypic monolayer and plastic substrate attachment assays B16F1 cells were significantly more adherent ($2P < 0.05$) than B16F10 cells. Both cell lines, however, were more adherent towards a homotypic monolayer than to the plastic tissue culture substrate (Fig. 1). In the heterotypic monolayer attachment system to syngeneic lung cells both B16 variants showed a significant increase in the early rate of attachment relative to that for the plastic substrate or for homotypic monolayers. In contrast to homotypic monolayer and plastic substrate attachment, there were no significant differences in the rate or overall attachment of B16F1 cells and B16F10 cells to lung monolayers (Fig. 1). When considered relative to plastic substrate or to homotypic monolayer attachment, however, it was apparent that B16F10 cells displayed a more pronounced adhesive change than B16F1 cells did towards lung cells.

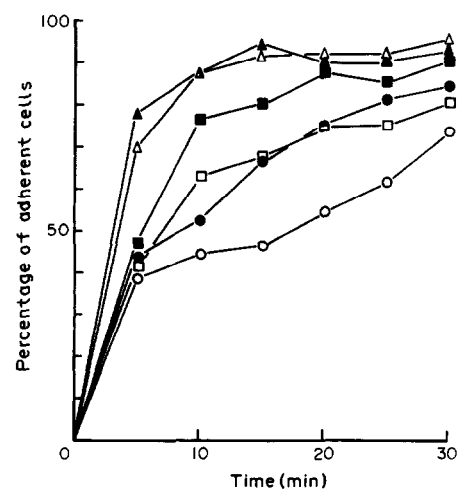

Fig. 1. Adhesion of B16F1 (solid symbols) and B16F10 cells (open symbols) to plastic (○,●), homotypic (□,■) and lung (△,▲) substrates. Each point is the mean of 6 determinations.

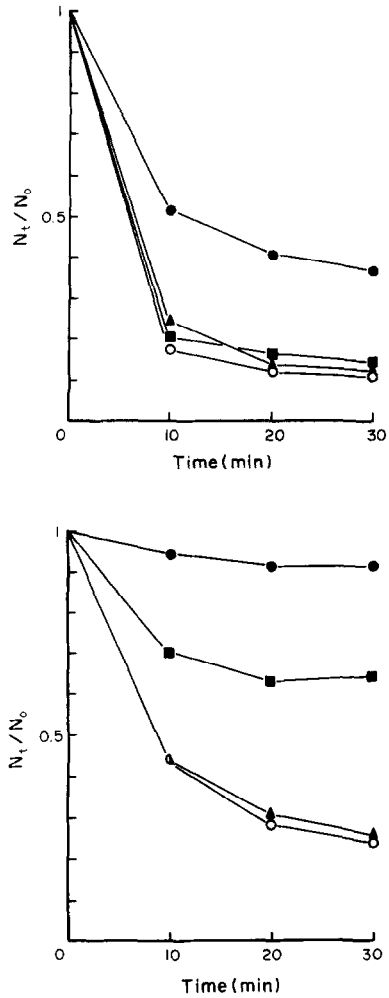

Fig. 2. Aggregation kinetics of (a) B16F1 and (b) B16F10 cells at different shear rates. ● 450/sec, ■ 225/sec, ○ 90/sec, ▲ 45/sec. *Each point is the mean of* 12 *determinations.*

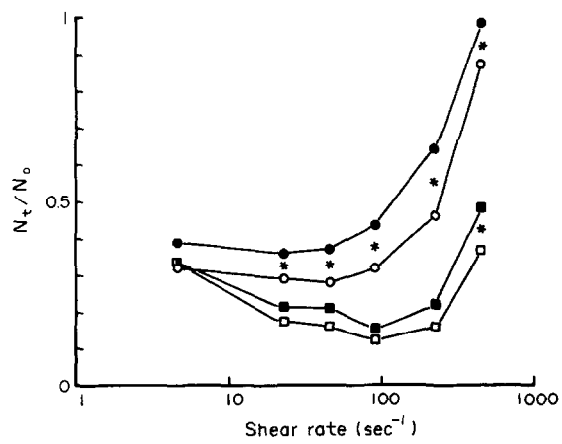

Fig. 3. Effect of shear rate on the aggregation of B16F1 (□,■) *and B16F10 cells* (○,●) *grown to low (open symbols) or high culture densities (solid symbols). Each point represents the mean of at least* 4 *determinations.* *$2P < 0.05$ (*Student's t test*).

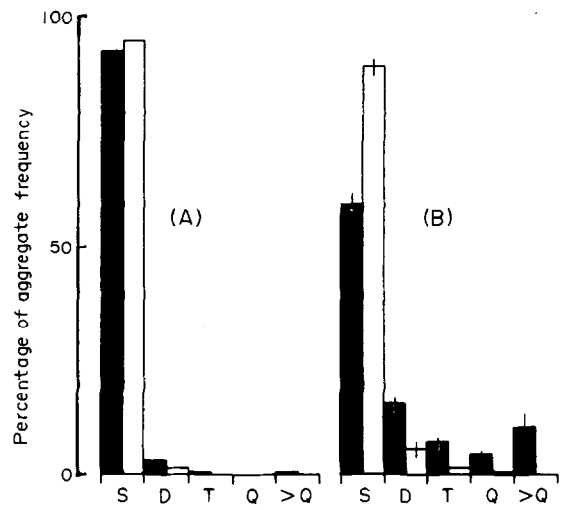

Fig. 4. Aggregate distribution profile of B16F1 (solid columns) and B16F10 cells (open columns) before (a) and after (b) 10 min *of aggregation at a shear rate of* 450/sec. *S = single cells, D = doublets, T = triplets, Q = quadruplets, >Q = aggregates of 5 or more cells.*

Aggregation

The homotypic aggregation kinetics of the B16 melanoma variants at shear rates of 45–450/sec are shown in Figs 2 (a) and (b) and are summarized in Fig. 3. Aggregation was initially rapid and a plateau phase was established after about 10 min. The extent of aggregation was shear-dependent, a decline in early aggregation rate being displayed with increasing shear rates. Cell culture density also affected the extent of aggregation, as shown in Fig. 3. B16F10 cells from low-density cultures were significantly more adherent ($2P < 0.05$) than B16F10 cells from high-density cultures at all shear rates tested. B16F1 cells, however, displayed similar culture-density-related effects only at a shear rate of 450/sec. At a low shear rate (4.5/sec) there was no significant difference in the adhesiveness of B16F1 and B16F10 cells. Although both melanoma variant cell types displayed shear rate and culture-density-dependent effects in their rate and extent of aggregation, B16F1 cells were more adherent than B16F10 cells under all conditions except aggregation at 4.5/sec. The greater homotypic adhesiveness of B16F1 cells relative to B16F10 cells was confirmed by aggregate distribution analysis after 10 min of aggregation at 450/sec (Fig. 4). At this shear rate, B16F1 cells were still capable of forming a significant number of multicellular aggregates, whereas B16F10 cells remained largely as single cells.

The extent of homotypic aggregation of both B16F1 and B16F10 cells was also dependent on the dilution of the sample under test (Fig. 5). At a shear rate of 90/sec and over a concentration range of 3×10^5–5×10^6 cells/ml (equivalent to volume

fractions of 0.12–1.89%) maximum aggregation for both B16 variant cell lines was observed at a concentration of 1.5×10^6 cells/ml (equivalent to a volume fraction of 0.37%). Both cell types were less adhesive at cell concentrations above or below this value. At the shear rate employed in this particular study (90/sec) B16F1 cells were significantly more adhesive ($2P < 0.001$) than B16F10 cells except at the lowest cell concentration studied (3×10^5 cells/ml), where no significant difference was observed ($2P > 0.05$).

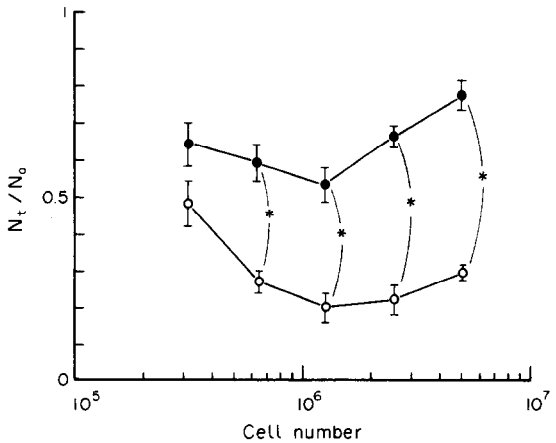

*Fig. 5. Effect of cell number on the extent of aggregation of B16F1 cells (○) and B16F10 cells (●) after 10 min at a shear rate of 90/sec. Each point is the mean of 6 determinations (±S.E.M.). *2P < 0.001 (Student's t test).*

Table 1. Homotypic and heterotypic aggregation of Bl6 variants and C57Bl6 tissue cells

System	N_{10}/N_0	
Homotypic		
B16F1	0.21 ± 0.01	(26)
B16F10	0.60 ± 0.03	(25)
C57B16 lung	0.87 ± 0.04	(6)
AFC_{16}-labelled lung	0.81 ± 0.05	(6)*
C57B16 liver	0.84 ± 0.08	(3)
AFC_{16}-labelled liver	0.84 ± 0.08	(3)*
Heterotypic		
AFC_{16} lung + B16F1	0.24 ± 0.01	(8)†
AFC_{16} lung + B16F10	0.28 ± 0.01	(8)‡
AFC_{16} liver + B16F1	0.41 ± 0.03	(5)§‖
AFC_{16} liver + B16F10	0.44 ± 0.02	(5)§¶

All experiments performed at a shear rate of 225/sec. Figures in parentheses refer to the number of repeat experiments.

*No significant difference compared to unlabelled controls. †No significant difference compared to homotypic B16F1. ‡$2P < 0.001$ compared to homotypic B16F10. §No significant difference. ‖$2P < 0.001$ compared to homotypic B16F1. ¶ $2P < 0.05$ compared to homotypic B16F10. Significance was determined from Student's *t* test (two-tailed).

In order to facilitate comparison, the cell types used in heterotypic aggregation were first tested in a homotypic system to establish conditions where lung and liver cell aggregation were not significantly different. At a shear rate of 225/sec lung and liver cells were both poorly adhesive (Table 1), although under similar conditions B16F1 cells were still significantly more adherent than B16F10 cells ($2P < 0.001$).

In heterotypic assays towards sygeneic lung cells, B16F1 cells were found to be marginally more adhesive than B16F10 cells (Table 1). However, when compared to their rate of homotypic aggregation, B16F1 cells did not display any significant increase in adhesiveness towards lung cells. This feature contrasted with B16F10 cells, which displayed a significant increase ($2P < 0.001$) in adhesiveness towards lung cells when compared to their homotypic adhesiveness (Table 1).

In heterotypic assays towards syngeneic liver cells there was no significant difference in the extent of aggregation exhibited by B16F1 and B16F10 cells. When compared to the extent of homotypic aggregation, however, B16F1 cells were significantly less adhesive and B16F10 cells were significantly more adhesive to liver cells (Table 1).

Since the kinetic analyses described above provide no information on the specificity of interaction in heterotypic aggregate systems, we carried out a visual comparison using fluorescently labelled lung or liver cells in order to

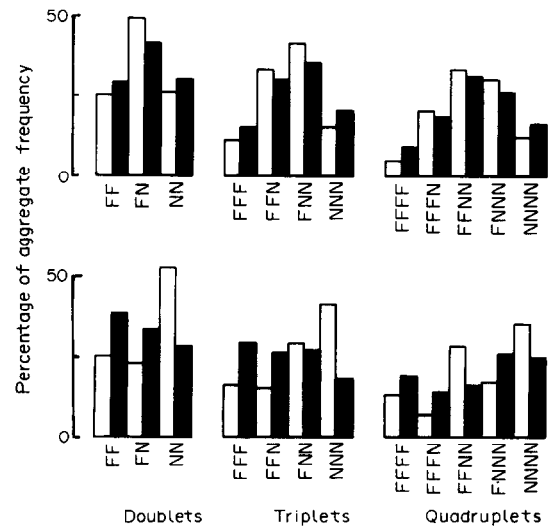

Fig. 6. Composition of aggregates formed during the heterotypic aggregation of B16F1 and B16F10 cells with syngeneic lung and liver cells. Top: tumour and lung; bottom: tumour cell + liver. B16F1 cells (shaded columns); B16F10 cells (open columns); F = fluorescent cell (lung or liver labelled with AFC_{16}); N = non-fluorescent B16F1 or B16F10 cell. Cells were aggregated for 10 min at a shear rate of 225/sec.

identify the cellular composition of aggregates. Control experiments showed that the fluorescent probe AFC_{16} used to identify lung or liver cells did not affect the adhesive properties of these cells or of both tumour cell variants. B16F10 cells formed more mixed aggregates with lung cells than did B16F1 cells (Fig. 6). A similar analysis following heterotypic aggregation with liver cells showed that B16F10 cells formed fewer mixed aggregates in this case than did B16F1 cells (Fig. 6).

DISCUSSION

The initial event in the metastatic process usually involves local invasiveness of surrounding tissues by the neoplastic cells. Significant spread to remote sites, however, is associated with the detachment of the metastasizing cells from the primary tumour and their relocation following transport predominantly by the blood and/or lymphatic routes [12]. Coman and his colleagues [13, 14] were amongst the first to provide experimental evidence in support of the hypothesis that the decreased homotypic adhesiveness of malignant cells relative to that of normal cells might contribute to their invasive properties. We have questioned the general applicability of this hypothesis since the quantitative analysis of cell adhesion is critically dependent upon the techniques employed, and under some circumstances tumour cells might appear to be more adherent than normal cells [10]. The significance of this feature of adhesion assays is illustrated by the comparison of our results on the homotypic adhesiveness of B16 variants with the results of other groups using the same cells but different experimental techniques. Rotary aggregation of B16F1 and B16F10 cells in multiwell trays [1–3] or in rolling tubes [4] has shown that B16F1 cells are less adherent than B16F10 cells. Adhesiveness in all of these studies was essentially assessed by scoring the percentage of single cells remaining following aggregation. It may be argued, however, that the number of single cells remaining after aggregation does not reliably reflect the extent of aggregation. Consider an aggregation system involving, for example, 10 cells. If all of the cells aggregate, a spectrum of aggregate sizes may result from a 1×10 cell aggregate to 5×2 cell aggregates. Assessing single-cell number alone does not provide any information on what is an obvious difference in the way the cells have aggregated, and in fact this method equates the two results. Which, if any, method of assessment provides the most accurate information on adhesiveness has not been resolved, although most studies employ aggregation kinetics [15] or aggregate distribution profiles [16] as criteria. We have used both of these methods of assessment in the present study and conclude, contrary to previous reports, that B16F10 cells are generally less adhesive than B16F1 cells. Indeed, when assessed by their exceedingly rapid rate of initial aggregation, we find that B16F1 cells are amongst the most adhesive cells we have studied. Surprisingly, other workers have claimed virtually no homotypic aggregation of B16F1 cells over a 30-min period [1]. Our results are not peculiar to cells grown in our laboratory since B16 variants obtained from another laboratory displayed the same aggregation behaviour (results not shown). Furthermore, in all other respects the B16 variants behaved as expected, with the B16F10 line colonizing the lungs significantly more than B16F1 cells following tail vein injection (not shown). The adhesiveness of B16F10 cells approached that of B16F1 cells only under two conditions: low shear rate (4.5/sec) and low cell concentration (3×10^5 cells/ml). The most likely interpretation of these results is that under such conditions relatively few cell collisions result and thus quantitative differences in aggregation cannot be reliably assessed.

Our conclusion on the higher adhesiveness of B16F1 cells relative to B16F10 cells as assessed by aggregation rate was confirmed by analysis of aggregate distribution. Thus B16F1 cells formed more aggregates of greater size classes than did B16F10 cells. Furthermore, B16F1 cells adhered to homotypic monolayers and to the plastic substrate at a significantly greater rate than did B16F10 cells under comparable conditions. Similar results (not shown) were obtained from other B16F10-derived cell lines such as B16BL6, which was originally selected for invasive ability and is known to be more metastatic than the B16F1 cell line [17]. Our results thus argue in favour of a correlation between decreased homotypic adhesiveness and metastatic ability for the B16 melanoma cell system, although we caution on the generality of this phenomenon.

The other significant feature of our work relates to the relevance of heterotypic adhesiveness in the entrapment and subsequent localized growth of B16 cells in specific organs. Nicolson and Winklehake [1] provided the original evidence suggesting that B16F10 cells adhere more to lung cells than do B16F1 cells and that this parallels their lung-colonizing ability following tail vein injection. Since these variants adhere somewhat differently in our experimental system, we have re-investigated this phenomenon utilizing the defined physical conditions provided by viscometry [10, 16, 18]. The most significant problem involved in studying heterotypic aggregation concerns relative rates of adhesiveness. If two

different cell types aggregate at different rates in homotypic suspensions, then some adjustment must be made when the two cell types are aggregated together to account for this difference in adhesiveness. Unfortunately the aggregation process is exceedingly complex and no adequate theoretical treatment is available. Despite this limitation, an indication of the events occurring during heterotypic aggregation can be obtained following the analysis of aggregate composition. Thus absolute specificity of adhesion, for example, would be shown by the lack of mixed aggregates and selectivity by the percentages of mixed aggregates of particular composition.

When we analysed heterotypic adhesiveness by aggregation rate we found that B16F1 cells were marginally more adhesive to lung cells than were B16F10 cells. The extent to which this result reflected simultaneous homotypic adhesiveness during heterotypic aggregation could only be determined by analysis of aggregate composition. We indentified the cell types in a heterotypic aggregation system by labelling one with the fluorescent lipid probe AFC_{16}, which by itself had no effect on adhesiveness. On subsequent analysis by fluorescence microscopy we found that B16F10 cells formed more mixed aggregates with lung cells but fewer with liver cells than did B16F1 cells. These results were essentially confirmed using the less sensitive substrate attachment assay, where the adhesiveness of B16F10 cells to a heterotypic lung monolayer was seen to be promoted more significantly relative to homotypic attachment than was that for B16F1 cells. Since primary cultures of lung cells as used in this study probably contain a variety of cell types, including fibroblasts, epithelial and endothelial cells, it is conceivable that the promotion of B16F10 cell adhesiveness is a general property of lung tissue rather than of one particular cell type, such as the lung capillary endothelial cell. Such a possibility, however, awaits clarification using the methods employed in this study. Nevertheless, while highlighting the need for caution in comparative studies, our results indicate that the potential of adhesive selectivity as a mechanism contributing to organ-specific metastasis warrants further consideration.

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