Eur J Cancer Clin Oncol, Vol. 18, No. 7, pp. 669–675, 1982.
Printed in Great Britain.

0277-5379/82/070669-07$03.00/0

The Adhesiveness of Normal and SV40-transformed BALB/c 3T3 Cells: Effects of Culture Density and Shear Rate*

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Abstract—*The adhesiveness of BALB/c 3T3 cells and their SV40 virally transformed counterparts as assessed by aggregation kinetics was found to vary as a function of cell culture density and aggregation conditions. In culture, SV3T3 cells were found to have a faster growth rate and a smaller cell volume than 3T3 cells. Although both cell types displayed increasing adhesiveness with increasing culture density, the adhesiveness of SV3T3 cells was consistently lower than that of 3T3 cells of comparable culture density when the cells were aggregated under shear rate conditions* ≤ 45/sec. *When the shear rate was increased from 90 to 450/sec, however, the aggregation profile inverted, with the 3T3 cells becoming less adherent than the SV3T3 cells. The ability of the transformed SV3T3 to remain adherent under conditions of relatively high shear may facilitate extravasation during the process of tumour spread.*

INTRODUCTION

THERE has been a pervading view in tumour cell biology that decreased cellular adhesiveness is correlated in some way with tumorigenic or metastatic potential. This view has its foundations in the earlier work of Ludford[1] and Cowdry[2], but it was not until the studies of Coman[3–5], who measured the force required for cell detachment from the substrate, that the proposed correlation gained tacit acceptance. It has become apparent over the last few years, however, that the adhesive behaviour of both normal and transformed cells is more complex than was originally thought. It now seems likely that the generalization that decreased adhesiveness is a property of tumorigenic cells is a misconception which has arisen from too few studies on too few cell types. Wright *et al.*[6], for example, have found that a number of transformed tumorigenic cell lines are in fact more adhesive than their non-transformed, non-tumorigenic counterparts as assessed by the rate of aggregation of reciprocally shaken EDTA-dissociated cells. A similar correlation between lung-colonizing potential of tumour cells and their adhesiveness has been demonstrated by Winkelhake and Nicolson[7] and Raz *et al.*[8]. These authors found, using the collecting lawn method[9] or a modification of the aggregation technique to induce cell collisions[10], that B16F10 malignant melanoma cells (with a relatively high lung-colonizing potential) are more adhesive than B16F1 cells (with a relatively low lung-colonizing potential). This correlation between colonizing potential and cell adhesiveness is in contrast to the results reported by Bubenik *et al.*[11], which imply an inverse correlation between cell surface adhesiveness (measured by latex particle adhesion) and malignancy in certain murine fibroblastoid cell lines. In contrast to all of these reports, Dorsey and Roth[12] found no correlation in their study of adhesiveness and malignancy using the collecting aggregate technique[13] to examine the adhesiveness of 3T3, SV-3T3 and 3T12 murine cell lines.

The few examples described above illustrate the variations which underlie current research techniques employed in measuring cellular adhesiveness. Although adhesiveness might be expected to vary with method of assessment[14], it also varies with cell type[12], dissociation procedure[15–17], culture density[18], serum concentration[19], cell products [20], temperature [21], time [14, 22],

Accepted 16 February 1982.
*This work was supported by the Medical Research Council (U.K.).
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junctional development[20] and various ions, drugs and routine media additives such as glucose and glutamine [16]. The effects of many of these variables are of obvious importance when attempts are made to generalize between the results of different laboratories. In the present report we have employed standardized procedures to study the effects of culture density and shear rate on the adhesiveness of BALB/c 3T3 cells (clone A31) and their SV40 virally transformed counterparts. Adhesiveness was measured kinetically by following changes in total particle number as normal or transformed cells were aggregated within the confines of a Wells–Brookfield cone-plate viscometer.

MATERIALS AND METHODS

Maintenance of cell lines

BALB/c 3T3 cells (clone A31) and their 3T3B/SV40-transformed counterparts were obtained from Flow Laboratories (Scotland) and were maintained as monolayer cultures in plastic 75 cm^2 tissue culture flasks (Sterilin Ltd) in a culture medium consisting of Dulbecco's minimum essential medium supplemented with 10% foetal calf serum, 2mM L-glutamine, 50 IU/ml benzyl penicillin and 50 μg/ml streptomycin sulphate (Flow Labs). Cultures were kept 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 75 cm^2 flask. To remove cells from culture flasks, monolayers were washed with 5 ml Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (CMF-PBS) and subsequently treated for 5 min at 37°C with 2 ml of CMF-PBS containing 0.1% trypsin (Difco 1:250) and 0.5 mM EDTA (ethylenediamine tetra-acetic acid, disodium salt). The resulting cell suspension was treated with 4 vols of culture medium and centrifuged in the cold (4°C) for 3 min at 150 *g*. The cells were then resuspended as required and a 40-μl sample was added to 20 ml Isoton (Coulter Electronics Ltd) before counting on a Coulter counter model ZB (aperture size 200 μm, attenuation = 8, aperture current $\approx$ 0.25 mA, lower threshold = 10, upper threshold = 100) coupled to a Coulter Channelyzer model C1000 with XY recorder. Cell suspensions prepared in this manner were consistently > 85% single cells and > 95% viable as assessed by trypan blue dye exclusion. Nodal cell volumes were determined directly from the Coulter Channelyzer volume distribution curves, whereas mean cell volumes were determined from the distribution curves by the application of Simpson's rule [23].

Samples of cell suspensions in culture medium at various passage numbers were supplemented with 10% dimethylsulphoxide, adjusted to 2×10^6 cells/ml and stored in liquid N_2 vapour until required.

For experimental purposes both 3T3 and SV3T3 cells were seeded at either 0.2×10^6 (low density) or 0.9×10^6 (high density) cells per 9 cm diameter petri dish (Nuclon, growth area 60.8 cm^2) in 10 ml culture medium. For cell adhesion studies, single cell suspensions were prepared as described above except that the final resuspending medium was supplemented with 20 mM Hepes buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid).

Assessment of adhesiveness

Cell adhesiveness was measured by following the aggregation kinetics of single cell suspensions subjected to known laminar shear conditions with the confines of a Wells–Brookfield cone and plate viscometer (model LVT-C/P; cone angle 0.8°). The shear rate was varied between 4.5 and 450/sec in 7 steps and the sample chamber of the viscometer was water jacketed and maintained at 37°C by a Colora closed system temperature circulator (model K4). All working surfaces of the viscometer were siliconized prior to use with 5% DC1107 silicone oil (Asschem Ltd) in ethyl acetate.

Single cell suspensions were adjusted to a constant volume fraction of 37.92% based on nodal cell volumes. The volume fraction was equivalent to about $1–4 \times 10^6$ cells/ml and takes into account the fact that cells change in volume during culture *in vitro*. A 1-ml sample of cell suspension was placed in the viscometer cup and aggregation was assessed by the drop in total particle number (cells plus aggregates) with time as the cells adhered to each other [24]. Results are expressed as the aggregation index N_t/N_0, where N_0 = number of particles at time $t = 0$ and N_t = number of particles at t = 10, 20 or 30 mins.

RESULTS

Growth characteristics

The growth characteristics of 3T3 and SV3T3 cells seeded at low (0.2×10^6) or high (0.9×10^6) densities are shown in Fig. 1. The SV3T3 cells have faster growth rates at both low and high densities when compared to 3T3 cells. Although both cell types show a decrease in nodal cell volume with increasing culture density, SV3T3 cells are considerably smaller than their non-transformed counterparts at comparable densities (Fig. 2).

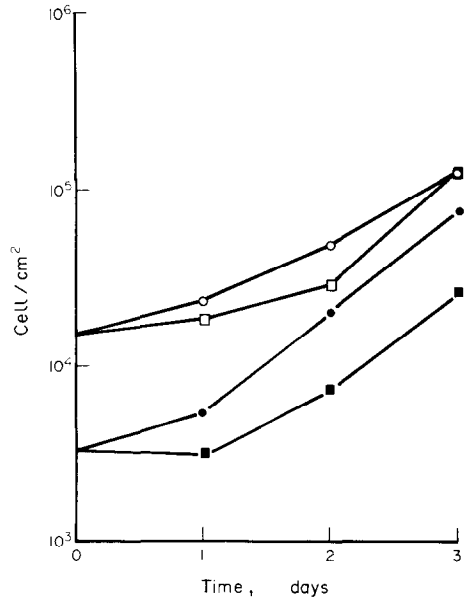

Fig. 1. Growth characteristics of 3T3 (squares) and SV3T3 cells (circles) seeded at low (solid symbols) and high cell densities (open symbols). SV3T3 cells have faster growth rates at both low and high cell densities. Results shown in Figs 1 and 3–5 are from representative experiments carried out in duplicate. Qualitatively similar results were obtained in 2–4 repeat experiments. Data are not pooled because of quantitative variation resulting from different final cell densities and different foetal calf serum batches (37 and our unpublished observations).

Adhesion as a function of cell culture density

SV3T3 cells are less adhesive than 3T3 cells of comparable culture density (Figs 3a, b). Both cell lines, however, display increasing adhesiveness with increasing culture density.

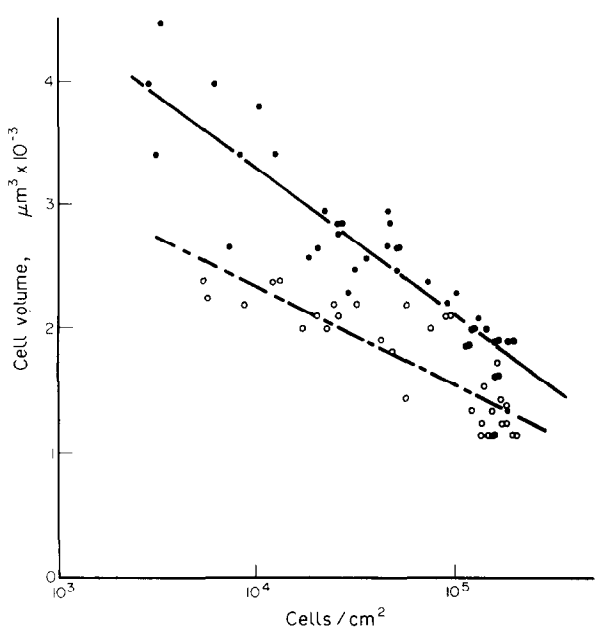

Fig. 2. Changes in nodal cell volume of 3T3 (●) and SV3T3 cells (○) as culture density increases. 3T3 cells are larger than their transformed counterparts at comparable densities. Lines of the form $y = -1209.5 \log_{10}x + 8153.9$ *and* $y = -791.5 \log_{10}x + 5504.6$ *were fitted to the 3T3 and SV3T3 data, respectively, by regression analysis.*

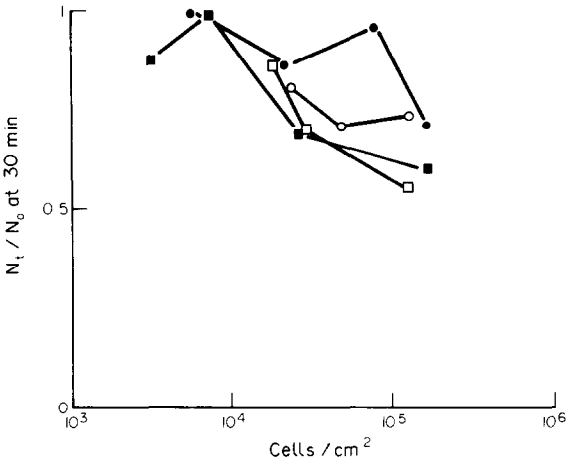

Fig. 3. Adhesiveness of 3T3 (squares) and SV3T3 cells (circles) as a function of culture density. 3T3 cells are generally more adhesive than SV3T3 cells at comparable culture densities whether original seeding was from low (solid symbols) or high innocula (open symbols).

These studies were carried out at a fixed shear rate of 45/sec.

Adhesion as a function of shear rate

When cells were subjected to shear rates varying between 4.5 and 450/sec, the aggregation profile was found to alter dramatically (Figs 4a, b). Adhesiveness decreased as the shear rate increased, but SV3T3 cells were less sensitive to the effects of increasing shear. These differences in shear-related adhesive behaviour between 3T3 and SV3T3 are illustrated in Fig. 5, where the aggregation profiles for both cell types are compared at $N_t = 20$ min. SV3T3 cells are generally less adhesive than 3T3 cells at low shear rates ($\lesssim 45$/sec) but become relatively more adhesive at high shear rates ($\lesssim 90$/sec). In these experiments, cultures of 3T3 and SV3T3 cells were set up under identical conditions and final cell densities were within the range of $1.2–2.3 \times 10^5$ cells/cm^2.

DISCUSSION

In our analysis of the adhesive behaviour of 3T3 cells and SV3T3 cells we have adopted a set of standard procedures to prepare >85% single cell suspensions. We subsequently aggregated these cells in a serum-containing medium within the defined conditions generated by a cone and plate viscometer. We chose a convenient and well-studied cell line for our experiments and employed a 0.1% trypsin/0.5 mM EDTA harvesting procedure routinely. According to Cassiman and Bernfield [15], however, 0.05% trypsin/0.02% EDTA treatment masks any differences between the adhesiveness of 3T3 and SV3T3 cells

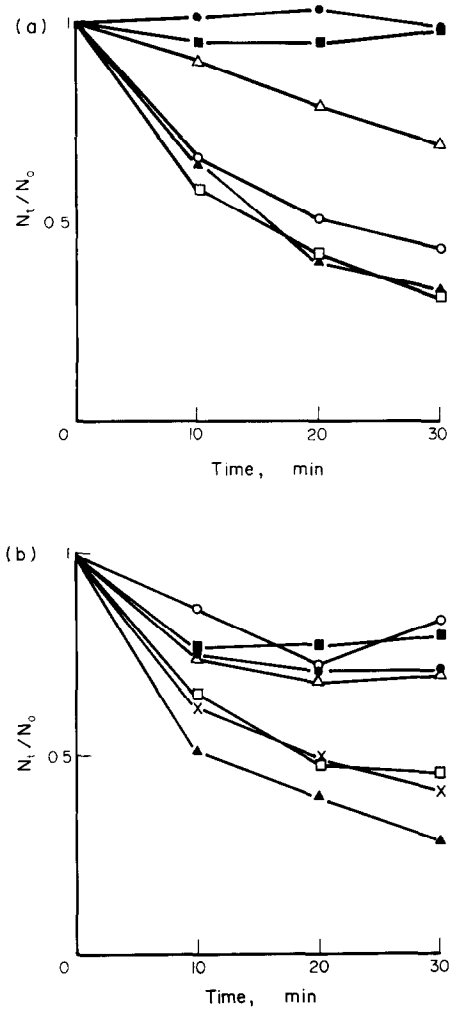

Fig. 4. Adhesiveness of (a) 3T3 and (b) SV3T3 cells as a function of aggregation shear rate. 3T3 cells are poorly adherent at shear rates > 225/sec, *whereas SV3T3 cells remain adherent up to shear rates of* 450/sec. × = 4.5/sec; ▲ = 11.25/sec; □ = 22.5/sec; ○ = 45/sec; △ = 90/sec; ■ = 225/sec; ● = 450/sec. *In Fig. 4a the results from* 4.5/sec *overlie those from* 11.25/sec.

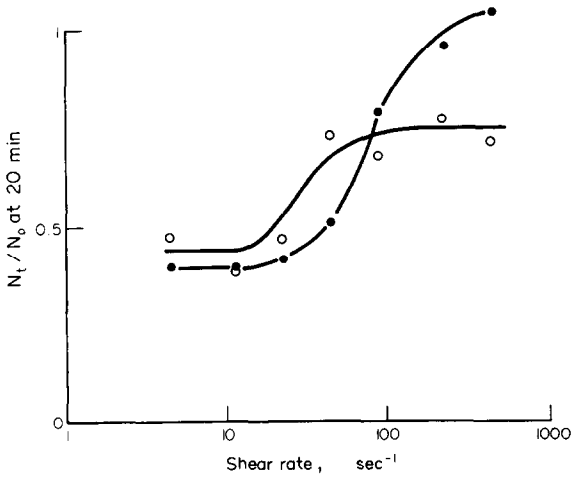

Fig. 5. Adhesiveness of 3T3 (●) and SV3T3 (○) cells as a function of aggregation shear rate. Adhesiveness of each cell type is compared after 20 min *of aggregation to illustrate the greater adhesiveness of SV3T3 cells at higher shear rates.*

(as assessed by the rate of rotary aggregation) since both cell types become of low adhesiveness. When harvested by 0.02% EDTA alone, however, the SV3T3 cells were significantly more adhesive than their non-transformed 3T3 cells. Unfortunately, EDTA treatment alone does not yield a single-cell suspension, and thus Cassiman and Bernfield [15] found it necessary to filter their preparation to remove aggregated cells. It could be argued that this process removes an adherent cell population, especially if the yield of single cells is low and that results obtained using this procedure do not reflect the adhesiveness of the cell population as a whole. Evans and Proctor [25] have provided evidence from a theoretical analysis of the collision processes occurring during aggregation that supposedly 'homogeneous' populations may in fact be composed of sub-populations of cells of varying adhesiveness. Furthermore, Cassiman and Bernfield [15] and numerous other authors have analysed aggregation under serum-free conditions which could be expected to affect both cell survival and the rate of aggregation. In some cell systems, for example, serum decreases cell adhesion [26], whereas in others, serum promotes it [27].

Using a culture medium containing 15% foetal calf serum, Gail and Boone [28] measured cell contact time for both 3T3 and SV3T3 cells, and found that 3T3 cells spent about 3 times as long in contact as SV3T3 cells. The authors argued that contact time reflects mutual adhesivity, although alternative behavioural processes such as contact paralysis independent of adhesiveness may be in operation. Nevertheless, these basic results reflect the observations of McNutt and Weinstein (quoted in [28]) that SV3T3 cells have fewer observable junctions than 3T3 cells, and thus may be expected to display lower adhesiveness.

It is clear that our understanding of the adhesive behaviour of normal and virally transformed cells remains controversial and that the extent to which this may be attributable to variations in technique is uncertain. Furthermore, our results suggest that even when comparable systems are employed (e.g. rotary aggregation), disparate results may be obtained if there is any deviation in shear conditions. We have found that the relative adhesive values of 3T3 and SV3T3 cells may invert at higher shear rates (> 90/sec), with 3T3 cells becoming relatively less adhesive. The ability of SV3T3 but not 3T3 cells to adhere under high shear conditions may reflect fundamental differences in membrane rigidity. SV3T3 cells have 50% more cholesterol content than 3T3 cells

and thus are more rigid than their non-transformed counterparts [29]. This increased rigidity of transformed cells may allow them to adhere under conditions of high shear which would otherwise inhibit adhesiveness. Several studies [30–34] have provided evidence that increases in membrane rigidity are associated with increases in cell adhesiveness. However, this relationship may not be straightforward since, despite significant variation in rigidity between 3T3 and SV3T3 cells, differences in adhesiveness only become apparent at shear rates above about 90/sec. The biological significances of our observation that SV3T3 cells remain adhesive under relatively high shear conditions can only be alluded to, but the possibility exists that during tumour spread this adhesive behaviour of transformed cells might facilitate extravasation from blood vessels where high shear rates are to be expected.

We have also found that cell adhesiveness increases along with an increase in culture density up to about 10^5 cells/cm^2. Edwards and Campbell [21] and O'Neill [18] found that confluent BHK21 Clone 13 cells harvested with trypsin/EDTA aggregated more than cells from less dense cultures. In a later paper, however, O'Neill and Burnett [19] found that EDTA-harvested 3T3 cells aggregated more in sparse than in dense cultures whereas the aggregation of SV3T3 cells was relatively unaffected by culture density. All of these studies were carried out in serum-free medium using different assay methods, so that direct comparison is not possible. Nevertheless, it is of interest that Inbar *et al.* [29] found that membrane rigidity increases with culture density. Our results showing an increase in cell adhesiveness with culture density are thus not unexpected in the light of the proposal discussed earlier that increased rigidity may be correlated with an increase in adhesion. Furthermore, Bosmann and Lione [35] found that the ability of B16 malignant melanoma cells to form lung cononies increased with culture density. This increased frequency of lung colony formation may reflect an underlying increase in cell adhesiveness as the cells become more densely distributed.

Our observation that a decrease in cell volume occurs as the cells become more densely distributed may be pertinent to the mechanisms underlying the accompanying increase in cell adhesiveness. For example, a decrease in cell volume may lead to a concentration of the postulated cell surface moieties responsible for cell adhesiveness, in much the same way as has been argued for concanavalin A binding sites [36], thus resulting in an increased probability of adhesions developing.

It is apparent from the foregoing that cell–cell adhesiveness is an exceedingly complex phenomenon and that the underlying cellular and molecular events are poorly understood. Using the standardized procedures outlined above, we have presented evidence which correlates adhesiveness with both the conditions of culture and of aggregation. Because these variables are often not taken completely into account, it is not surprising that confusion exists as to whether virally transformed cells are more or less adhesive than their non-transformed counterparts. Our results suggest that in unsynchronized cultures of similar culture density, SV3T3 cells are less adhesive than 3T3 cells at low shear rates ($\leqslant 45$/sec) but become more adhesive at higher shear rates ($\leqslant 90$/sec). Apart from suggesting a possible explanation for disparate results in different studies, high adhesiveness of some transformed cells at shear rates near those expected in blood vessels might facilitate extravasation during tumour spread.

REFERENCES

1. LUDFORD RJ. Differences in the growth of transplantable tumours in plasma and serum culture media. *Proc R Soc Lond* 1932, **112**, 250–263.
2. COWDRY EV. Properties of cancer cells. *Arch Pathol* 1940, **301**, 1245–1274.
3. COMAN DR. Decreased mutual adhesiveness, a property of cells from squamous cell carcinomas. *Cancer Res* 1944, **4**, 625–629.
4. COMAN DR. Mechanisms responsible for the origin and distribution of blood borne tumour metastases: a review. *Cancer Res* 1953, **13**, 397–404.
5. COMAN DR. Adhesiveness and stickiness: two independent properties of the cell surface. *Cancer Res* 1961, **21**, 1436–1438.
6. WRIGHT TC, UKENA TE, CAMPBELL R, KARNOVSKY MJ. Rates of aggregation, loss of anchorage dependence and tumourigenicity of cultured cells. *Proc Natl Acad Sci USA* 1977, **74**, 258–262.
7. WINKELHAKE JL, NICOLSON GL. Determination of adhesive properties of variant metastatic melanoma cells to Balb/3T3 cells and their virus transformed derivatives by a monolayer attachment assay. *J Natl Cancer Inst* 1976, **56**, 285–291.

8. RAZ A, MCLELLAN WL, HART IR *et al.* Cell Surface properties of B16 melanoma variants with differing metastatic potential. *Cancer Res* 1980, **40**, 1645–1651.
9. WALTHER BT, OHMAN R, ROSEMAN S. A quantitative assay for intercellular adhesion. *Proc Natl Acad Sci USA* 1973, **70**, 1569–1573.
10. GERISCH G. Zellfunktionen und Zellfunktionswechsel in der Entwicklung von *Dictyostelium discoideum* I. Zellagglutination und induktion der fruchtkorperpolaritat. *Arch Endwickl Org* 1960, **152**, 632–654.
11. BUBENIK J, PERLMAN P, FENYO EM, JANDLOVA T, SUHAJOVA E, MALKOWSKY M. Inverse correlation between cell surface adhesiveness and malignancy in mouse fibroblastoid cell lines. *Int J Cancer* 1979, **23**, 392–396.
12. DORSEY JK, ROTH S. Adhesive specificity in normal and transformed mouse fibroblasts. *Dev Biol* 1973, **33**, 249–256.
13. ROTH SA, WESTON JA. The measurement of intercellular adhesion. *Proc Natl Acad Sci USA* 1967, **58**, 974–980.
14. EDWARDS JG, DYSART J MCK, EDGAR DH, ROBSON RT. On the reduced intercellular adhesiveness of virally transformed BHK21 cells. *J Cell Sci* 1979, **35**, 307–320.
15. CASSIMAN JJ, BERNFIELD MR. Transformation induced alteration in fibroblast adhesion: masking by trypsin treatment. *Exp Cell Res* 1975, **91**, 31–35.
16. EDWARDS JG, CAMPBELL JA, ROBSON RT, VICKER MG. Trypsinized BHK21 cells aggregate in the presence of metabolic inhibitors and in the absence of divalent cations. *J Cell Sci* 1975, **19**, 653–667.
17. BRUGMANS M, CASSIMAN JJ, VAN DEN BERGHE H. Selective adhesion and impaired adhesive properties of transformed cells. *J Cell Sci* 1978, **33**, 121–132.
18. O'NEILL CH. Growth induction by serum or polyoma virus inhibits the aggregation of trypsinized suspensions of BHK21 tissue culture fibroblasts. *Exp Cell Res* 1973, **81**, 31–39.
19. O'NEILL CH, BURNETT ME. Inhibition of spontaneous aggregation by wheat germ agglutinin in suspensions of Balb/c-3T3 and BHK21 tissue culture fibroblasts. *Exp Cell Res* 1974, **83**, 247–254.
20. HAYASHI H, ISHIMARU Y. Morphological and biochemical aspects of adhesiveness and dissociation of cancer cells. *Int Rev Cytol* 1981, **70**, 139–215.
21. EDWARDS JG, CAMPBELL JA. The aggregation of trypsinized BHK21 cells. *J Cell Sci* 1971, **8**, 53–71.
22. WHUR P, KOPPEL H, URQUHART CM, WILLIAMS DC. Quantitative electronic analysis of normal and transformed BHK21 fibroblast aggregation. *J Cell Sci* 1977, **23**, 193–209.
23. BOARDMAN L, HUETT M, LAMB JF, NEWTON JP, POLSON JM. Evidence for the genetic control of the sodium pump density in HeLa cells. *J Physiol* 1974, **241**, ting protein. *J Cell Sci* 1975, **18**, 347–356.
24. EDWARDS JG, CAMPBELL JA, WILLIAMS JF. Transformation by polyoma virus affects adhesion of fibroblasts. *Nature (New Biol)* 1971, **231**, 147–148.
25. EVANS CW, PROCTOR J. A collision analysis of lymphoid cell aggregation. *J Cell Sci* 1978, **33**, 17–36.
26. KELLIE S, EVANS CW, KEMP GD. The effects of serum proteins on the adhesion of lymphocytes. *Cell Biol Int Rep* 1980, **4**, 777.
27. KLEBE RJ. Isolation of a collagen dependent cell attachment factor. *Nature (Lond)* 1974, **250**, 248–251.
28. GAIL MH, BOONE CHW. Density inhibition of motility in 3T3 fibroblasts and their SV40 transformants. *Exp Cell Res* 1971, **64**, 156–162.
29. INBAR M, YULI I, RAZ A. Contact mediated changes in the fluidity of membrane lipids of normal and malignant transformed mammalian fibroblasts. *Exp Cell Res* 1977, **105**, 325–335.
30. CURTIS ASG, CAMPBELL J, SHAW FM. Cell surface lipids and adhesion I. The effects of lysophosphatidyl compounds, phospholipase A_2 and aggregation inhibiting protein. *J Cell Sci* 1975, **18**, 347–356.
31. CURTIS ASG, SHAW FM, SPIRES VMC. Cell surface lipids and adhesion II. The turnover of lipid components of the plasmalemma in relation to cell adhesion. *J Cell Sci* 1975, **18**, 357–373.
32. CURTIS ASG, CHANDLER C, PICTON N. Cell surface lipids and adhesion III. The effects on cell adhesion of changes in plasmalemmal lipids. *J Cell Sci* 1975, **18**, 375–384.

33. HOOVER RL, LYNCH R, KARNOVSKY MJ. Decrease in adhesion of cells cultured in polyunsaturated fatty acids. *Cell* 1977, **12**, 295–300.
34. SHAEFFER BE, CURTIS ASG. Effects on cell adhesion and membrane fluidity of changes in plasmalemmal lipids in mouse L929 cells. *J Cell Sci* 1977, **26**, 47–55.
35. BOSMANN HB, LIONE A. Capacity for tumour cell implantation as a function of *in vitro* cell density. *Biochem Biophys Res Commun* 1974, **61**, 564–567.
36. INBAR M, BEN-BASSAT H, SACHS L. Membrane changes associated with malignancy. *Nature (New Biol)* 1972, **236**, 13–16.
37. BEN-BASSAT H, INBAR M, SACHS L. Changes in the structural organization of the surface membrane in malignant cell transformation. *J Membrane Biol* 1971, **6**, 183–194.