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# Rates of aggregation, loss of anchorage dependence, and tumorigenicity of cultured cells

**(concanavalin A/methyl cellulose)**

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ABSTRACT **The net rate of spontaneous aggregation of cells suspended with EDTA was measured for various cell types including spontaneous transformants and cells transformed with DNA and RNA viruses. The anchorage dependence as determined by growth in methyl cellulose and the tumorigenicity *in vivo* were also determined. All cells that had lost their anchorage dependency and were tumorigenic showed a high net rate of spontaneous adhesion. A31 was the only nontransformed cell line to have a high net rate of adhesion. The net rate of spontaneous aggregation of cells is a quick and reliable index of tumorigenicity and offers a new approach to understanding the mechanisms of cell surface changes associated with transformation.**

One important approach to understanding the altered biology of malignant cells is comparison of the properties of cells grown in tissue culture with the characteristics of the same cells injected into experimental animals. Studies of this type have shown that malignant transformation is often associated with changes in the surface properties of cells (1, 2). Loss of postconfluence inhibition of cell division (3, 4), loss of contact inhibition of cell movement (5), and changes in surface antigenicity (6, 7) are thought to be related to such *in vivo* properties of tumors as loss of growth control, invasiveness, metastasis, and escape from immunological surveillance. In this regard, *in vitro* studies of cell adhesion have been of interest. It has been shown that transformation is associated with a decrease in cell adhesion to surfaces such as glass or plastic (8, 9). The effects of transformation on cell-to-cell adhesion are less clear, and different studies have produced conflicting results (10–13).

Another surface property of cells that is affected by transformation is the ability to be agglutinated by plant lectins such as concanavalin A (Con A). Transformed cells brought into suspension with EDTA show an increased sensitivity to agglutination by plant lectins (14). Several aspects of the lectin agglutination phenomenon led us to propose recently that lectin agglutination might be superimposed on a spontaneous cell-to-cell adhesive process that is more active in transformed than in normal cells (15). Indeed, Cassiman and Bernfield have recently shown that two simian virus 40 (SV-40) tranformed cell lines suspended with EDTA showed higher net rates of spontaneous aggregation than did the corresponding normal cell lines (16).

We measured the initial net rate of spontaneous aggregation of cells suspended with EDTA for various cell types including spontaneous transformants and cells transformed by DNA and RNA viruses. In order to correlate this membrane property with other aspects of the transformed state, we tested the same cells for loss of anchorage dependence (growth in methyl cellulose), which has been shown to be closely linked to tumorigenicity (17, 18). We also tested the tumorigenicity of the cells directly in the strain of animal from which they were originally derived.

The results of these experiments show that the initial net rate of spontaneous aggregation of our cells was consistently higher in cells that showed loss of anchorage dependence and produced tumors in animals. The initial net rate of spontaneous aggregation of cells is a quick and reliable index of tumorigenicity and offers a new approach to understanding the mechanisms of cell surface changes associated with transformation.

Abbreviations: Con A, concanavalin A; SV-40, simian virus 40; PBS-CMF, Ca- and Mg-free phosphate-buffered saline

## EXPERIMENTAL PROCEDURES

**Cells.** Primary cultures of Swiss mouse and Syrian hamster cells were obtained by trypsinizing decapitated 16- to 18-day-old mouse and hamster embryos. Cultures of these cells were grown to confluence, trypsinized, transferred to medium containing 10% dimethyl sulfoxide, and stored frozen at −70° until used. After thawing, the cells were passed twice in culture prior to use in experiments.

3T3 clone 1 (3T3-MIT) (19) and 3T3 clone 2 (3T3d) (19) are subclones of the original Swiss mouse 3T3 line. A31 is a clone of Balb/c 3T3 cells obtained from D. Livingston. NIH cells are a clone of 3T3 cells derived from NIH mice provided by C. Scher.

KK cells are a line of SV-40 transformed hamster fibroblasts isolated by G. Diamandopoulos (20). SV-40 transformed mouse embryo cells (VLMT3) (21) were the gift of S. Tavethia. SV-40 transformed Balb-c A31 cells (SVT2)(19) were obtained from T. Benjamin. Swiss 3T3 cells doubly transformed by SV-40 and polyoma (SV-Py 3T3) were obtained from H. Green.

Nil-1C1 (22) cells, obtained from H. Amos, are derived from a spontaneous transformant arising in cultured hamster cells. Polyoma transformed Norwegian brown rat fibroblasts (Py-B4) were isolated by T. Benjamin. NIH 3T3 cells transformed by the Kirsten leukemia virus (KNIH) were the gift of C. Scher. Cultures of the B16 mouse melanoma (23) were obtained from J. Folkman.

Stock cultures of cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% serum (fetal calf serum for primary cultures, A31, NIH, KNIH, and KK cells; donor calf serum for the remaining cell lines) and antibiotics.

Cultures used in these experiments were free of contamination by mycoplasma as judged by the uridine phosphorylase assay (24) and direct observation of cells by scanning electron microscopy.

**Aggregation Assay.** The rate of aggregation of suspended cells was measured by the method described by Skehan and Friedman (25). Cells from stock cultures were plated at $10^5$ cells/100-mm plastic petri dish (Corning) and allowed to grow for 48 hr prior to use in experiments. Cells were brought into suspension by washing the monolayers twice with Ca- and

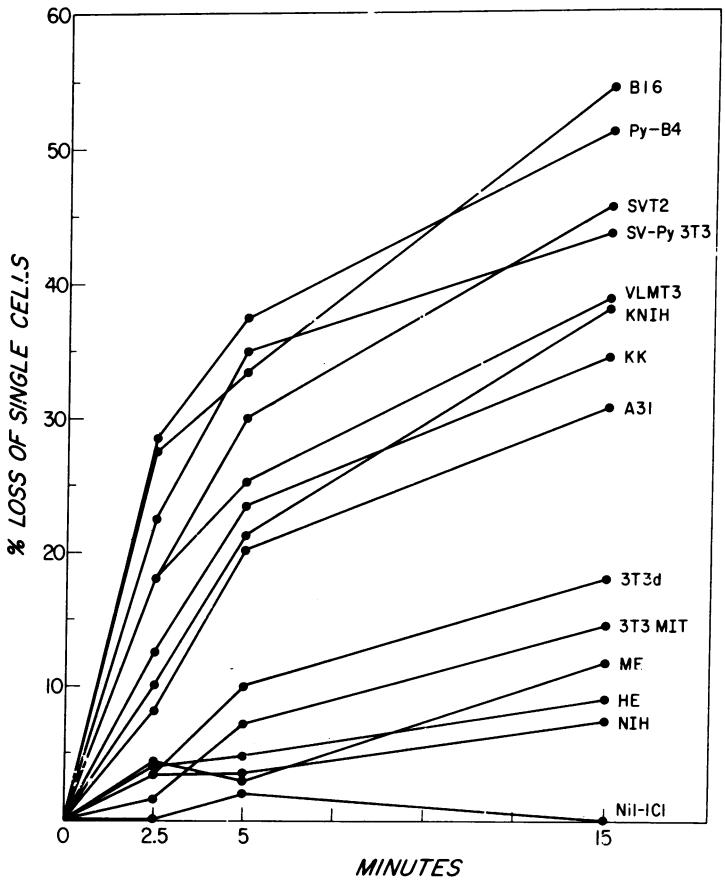


FIG. 1. Net rate of adhesion of the 14 cell lines tested, expressed as the percentage loss of single cells with time. The percentage loss of single cells is that percentage of the total number of cells counted which have gone into aggregates of two or more cells at each time period. All data points are the mean values for at least three experiments at various times over a 3-month period. ME, mouse embryo; HE, hamster embryo.

Mg-free phosphate-buffered saline (PBS-CMF) and incubated for 10 min at 37° in 5 ml of 0.02% (wt/vol) solution of EDTA in PBS-CMF. The suspended cells were then transferred to siliconized glass centrifuge tubes (Siliclad, Dow-Corning), pelleted by gentle centrifugation, resuspended in PBS-CMF, and diluted to a final concentration of $10^5$ cells per ml. The viability of the suspended cells as judged by trypan blue exclusion was always greater than 85%. After gentle pipetting to obtain a suspension consisting primarily of single cells, 1.5 ml of the cell suspension was immediately transferred to siliconized 35 × 15 mm nontissue-culture petri dishes (Falcon no. 1008) that were placed on a reciprocating shaker (60 oscillations/min) in a 37° warm room. At designated times, petri dishes were removed from the shaker and kept stationary for 2–3 min to allow the cells to settle to the bottom of the dish. The extent of aggregation was then determined by counting 300 successive cells with an inverted phase microscope using a 40× water-immersion objective and scoring cells as singles, pairs, groups (three to five cells), or aggregates (more than five cells).

Counts were made at predetermined areas along a diameter of the dish to control for nonrandom distributions of cells. Repeat counts, made at points along another diameter of the dish, agreed to within 5% of the original counts. In some experiments, DNase (Sigma) was included in the incubation medium at a concentration of 50 μg/ml. The results shown here represent the mean of at least three different experiments done on different days. The net rate of adhesion is obtained from plots of the percent loss of single cells against time. The percent loss of single cells is that percentage of the total number of cells counted that have gone into pairs, groups, or aggregates at each time period. The effect of Con A (Pharmacia) on the rate of aggregation of cells was determined by mixing 50 μl of concentrated stock solution of Con A with the cell suspension just prior to placing the dishes on the reciprocating shaker.

**Growth of Cells in Semisolid Media.** The ability of cells to grow in medium containing methyl cellulose was tested according to the procedure of Risser and Pollack (17). Cells were suspended in a mixture of 10% serum, 90% Dulbecco's modified Eagle medium, and 1.2% methyl cellulose (Mallinckrodt) at densities of $10^5$, $10^4$, $10^3$, and $10^2$ cells per 4 ml of medium. This mixture was layered onto a plate containing 6.0 ml of 0.9% agar (Gibco), 90% Dulbecco's modified Eagle medium, and 10% donor calf serum or 10% fetal calf serum. Cultures were weekly fed with 4.0 ml of methyl cellulose medium and were scored for growth after 4 weeks by counting the fraction of originally plated cells that formed colonies. Only colonies visible without magnification (>0.3 mm) were counted.

**Tumorigenicity of Cells.** Tumorigenicity of cells was assessed by subcutaneous injection into the animals from which the cells were originally derived. (NIH and KNIH cells were not tested for tumorigenicity because we were unable to obtain the correct strain of mice.) The presence of palpable tumor nodules 1 month after injection of $10^5$ and $10^6$ cells under the skin of the back was considered positive tumorigenicity. Mouse cells that did not form tumors in the original experiment were retested by injecting them into animals that received intraperitoneal injections of rabbit anti-mouse-thymocyte serum (Microbiological Assoc.) according to the schedule described

Table 1. Percentage loss of single cells into aggregates

| Cell line | Mean percentage of loss of single cells | |
|---|---|---|
| | 5 min | 15 min |
| Mouse embryo | 2.8 ± 2.4 | 13.0 ± 3.5 |
| Hamster embryo | 4.8 ± 0.9 | 9.0 ± 2.4 |
| 3T3d | 10.0 ± 3.2 | 18.0 ± 2.7 |
| 3T3-MIT | 6.9 ± 2.0 | 14.6 ± 1.6 |
| NIH | 3.6 ± 1.9 | 7.3 ± 3.4 |
| A31 | 20.3 ± 5.5 | 30.8 ± 5.0 |
| SVT2 | 32.5 ± 3.0 | 49.7 ± 2.1 |
| SV-Py 3T3-MIT | 34.8 ± 2.2 | 43.5 ± 4.9 |
| Py-B4 | 37.7 ± 5.6 | 51.3 ± 3.2 |
| B16 | 33.6 ± 0.5 | 54.5 ± 15.5 |
| KNIH | 21.2 ± 3.3 | 38.2 ± 3.3 |
| KK | 23.5 ± 2.7 | 34.4 ± 3.4 |
| VLMT3 | 25.2 ± 2.9 | 38.8 ± 2.9 |
| Nil-1C1 | 2.0 ± 2.0 | 0.0 ± 0.0 |

Percentage loss of single cells is that percentage of the total number of cells that have gone into aggregates of two or more cells at each time period, determined as described in *Experimental Procedures*. Data are expressed as mean ± SEM for at least three experiments.

by Stanbridge *et al.* (26). Animals were reinjected with antithymocyte serum twice weekly and were scored for tumors at 1 month after injection of the cells.

## RESULTS

### Aggregation of suspended cells

Fig. 1 and Table 1 show the net rates of aggregation for cells suspended with EDTA. The aggregation assay used in these experiments is reproducible among multiple samples and over periods of several months' time. On the basis of net rates of adhesion, the cells we studied fall into two groups, one showing relatively high net rates of aggregation and one showing relatively low net rates. The rapidly aggregating cells include all of the virus-transformed cells, the B-16 melanoma, and one clone of 3T3 cells (A31). The poorly aggregating cells include all of the embryonic cells, three of the four clones of 3T3 cells, and Nil-1C1 cells.

The difference in net rates of aggregation between the two groups of cells was apparent after 2.5 min of shaking and increased as the incubation continued to 15 min. The size distribution of the aggregates revealed another difference between the two groups. As shown in Fig. 2, cells with low net rates of aggregation (e.g., hamster embryo) were rarely found in aggregates of more than five cells whereas up to 50% of the cells from a rapidly aggregating cell line (e.g., Py-B4) were found in aggregates of greater than five cells after a 15-min incubation.

The net rate of cell aggregation in our assay varied with cell concentration, duration of shaking, and cell line tested. A low net rate of aggregation of a cell line could therefore be due to a decrease in the number of cells in suspension, as a result of either cell lysis or adherence of single cells to the petri dish. The following experiments demonstrate that these variables are not responsible for the observed difference in net rate of adhesion. Hemocytometer counts of the number of cells in suspension before and after shaking for 15 min were identical. Viability of cells as measured by trypan blue exclusion decreased by no more than 1–2% during 15 min of shaking. When adherence of single cells to the petri dish was assessed by shaking cell suspensions for 15 min and then gently washing the nonadherent cells from the dish with buffer and counting the number of adherent cells by phase contrast microscopy, no difference in the number of adherent cells was found between the two groups. Finally, inclusion of DNase at a concentration of 50 μg/ml did not change the difference in net rate of aggregation between the two groups.

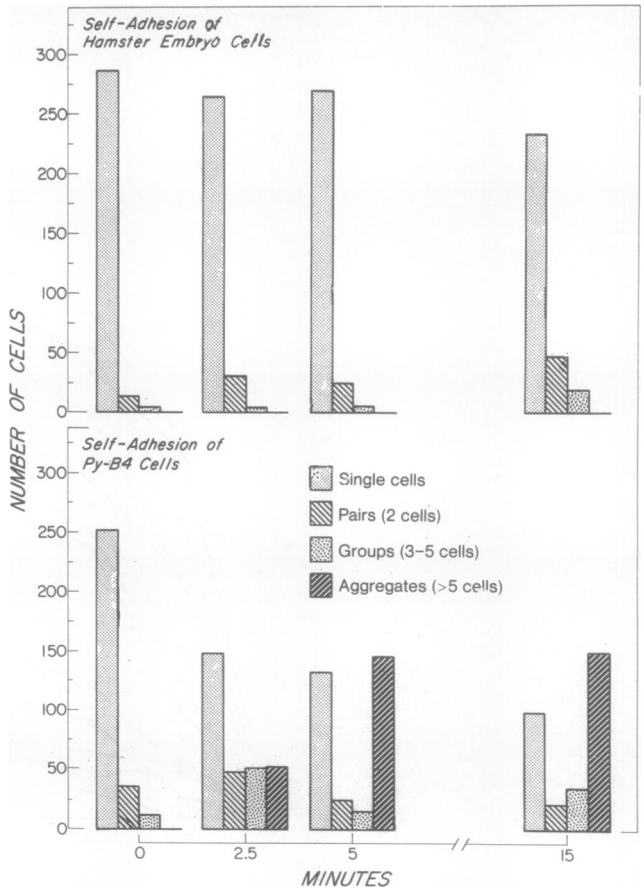


FIG. 2. Distribution of aggregate sizes with hamster embryo and Py-B4 cells. At each time period, duplicate counts of 300 cells were made on a sample as described in *Experimental Procedures*. Each bar represents the mean value of the total number of cells in a given aggregate size at a given time period.

### Effect of Con A on aggregation

Because agglutinability of cells by the plant lectin, Con A, has been correlated with transformation, we studied the effect of Con A on aggregation in our system. Preliminary experiments showed that the effects of Con A on aggregation only became significant when the aggregation assay was modified by shortening the incubation time and decreasing the concentration of cells in the dishes by 50%. These modifications were necessary to reduce the level of spontaneous agglutination to a point such that relatively small levels of induced agglutination induced by Con A could be observed. Addition of Con A to the aggregation mixture under these conditions did cause increases in aggregation of some cell types, but this effect was not consistently correlated with rates of spontaneous aggregation. Nil-1C1 cells, which showed minimal spontaneous aggregation, were strongly agglutinated by Con A whereas VLMT3 cells, which always showed rapid rates of spontaneous aggregation, were not affected by Con A over a dose range of 20–100 μg/ml. These experiments indicate that, at least in some cells, Con

Table 2. Correlation between high net rate of adhesion and tumorigenicity

| Cell lines | High net rate of adhesion | Methocel growth (%) | Formation of tumors *in vivo* |
|---|---|---|---|
| SV-Py 3T3 MIT | + | 100 | 5/5 |
| B16 | + | 40 | 5/6 |
| Py-B4 | + | 100 | 5/5 |
| VLMT3 | + | 100 | 5/5 |
| KNIH | + | 40 | (Not tested)* |
| KK | + | 80 | 4/5 |
| SVT2 | + | 100 | 4/4 |
| Nil-1C1 | − | 0 | 0/5 |
| Hamster embryo | − | 0 | 0/5 |
| Mouse embryo | − | 0 | 0/5 |
| 3T3-MIT | − | 0 | 0/5 |
| 3T3d | − | 0 | 0/5 |
| NIH | − | 0 | (Not tested)* |
| A31 | + | 0 | 0/5 |

A high net rate of adhesion is defined as >25% loss of single cells at 15 min. Net rate of adhesion, growth in methyl cellulose, and tumorigenicity were determined as described in *Experimental Procedures*.

* NIH and KNIH cells were not tested directly for tumor formation because the strain of mice from which the cells were derived was not available. However, the KNIH cell line used in these studies has been shown to form invasive tumors when cells are inoculated into the chick chorioallantoic membrane (32). The NIH cell line did not form tumors under these conditions.

A-induced and spontaneous aggregation proceed by separate mechanisms.

### Loss of anchorage dependence

Several studies have shown that there is an excellent correlation between tumorigenicity of cells in nude mice and their ability to form colonies when grown in medium containing methyl cellulose (17, 18). Seven of our cell lines (KK, SVT2, VLMT3, SV-Py 3T3, Py-B4, KNIH, and B16) formed colonies in methyl cellulose (Table 2). This group includes all but one (A31) of the cells in the high adhesion group. None of the cells showing low rates of adhesion grew in methyl cellulose. Of the seven lines that formed colonies, all but two had plating efficiencies of nearly 100%; 40% of both the B16 melanoma cells and the KNIH cells formed colonies. From these experiments we conclude that only cells that show a high initial net rate of adhesion formed colonies in methyl cellulose.

### Tumorigenicity

In order to assess the tumorigenicity of our cells directly, we injected them subcutaneously into the same species of animal from which the cells were originally derived. The test was considered positive if nodules greater than 0.5 cm in diameter were found 1 month after injection of the cells. A nonspecific inflammatory response was ruled out by dissecting out the tumors and reculturing cells derived from trypsinized tumor tissue.

Of the seven cell lines that formed colonies in methyl cellulose (see above), six were tested for tumorigenicity and all six formed tumors in animals (Table 2). As before, A31 was the only cell type showing a high initial rate of adhesion but no tumorigenicity. None of the cells in the poorly adhesive group formed tumors. In order to control for suppression of tumorigenicity by the host's immune response, we injected the various mouse cells used in our experiments subcutaneously into mice of the appropriate species that had been immunosuppressed by injection of rabbit anti-mouse-thymocyte serum (26). This treatment accelerated the growth of some of the tumorigenic cells but did not alter the *in vivo* behavior of any of the originally nontumorigenic cells. These experiments extend the correlation between aggregation rates and loss of anchorage dependence to include tumorigenicity.

## DISCUSSION

Our experiments indicate that the net rate of spontaneous aggregation is increased in cells that have lost anchorage dependence and become tumorigenic. Of a total of 16 cell types examined, only 1 nontumorigenic cell line had a net rate of aggregation falling within the range of the tumorigenic group; none of the tumorigenic cells showed a low net rate of aggregation. Furthermore, it is important to point out that the one nontumorigenic cell that showed a high rate of aggregation (the A31 clone of Balb 3T3 cells) appears in several respects to be partially transformed. A31 cells attached to glass beads are tumorigenic in animals (27), and the fibrinolytic activity of this line is in the range normally found in tumorigenic cells (D. Rifkin, personal communication). It has also recently been shown that A31 produces tumor angiogenesis factor (28). Thus, it seems possible that the high net rate of adhesion found in this cell line is another demonstration of its premalignant state. The fact that A31 does not grow in methyl cellulose indicates that the change that causes increased rate of aggregation is not in itself sufficient to cause tumorigenicity or loss of anchorage dependence.

In contrast to our results showing an increase in net rate of aggregation associated with tumorigenicity, other reports have shown a decrease in cell-to-cell adhesion after transformation (12, 13). It is important to note, however, that those studies used trypsinized cells. Cassiman and Bernfield (16) have shown that trypsinization decreases the cell-to-cell adhesion of normal or transformed cells and also renders both cell types equally adhesive. Thus, the real significance of experiments using trypsinized cells is difficult to assess (29).

The system used to measure aggregation in our experiments determines the net initial rate of intercellular aggregation. The net rate is a function of both the rate of formation of intercellular adhesions and the rate of dissolution of adhesion. It is not necessarily related to the strengths of intercellular adhesion in solid tumors. Any relationship between the increased rate of aggregation of tumorigenic cells and the *in vivo* properties of tumors is complicated by the fact that the aggregation assay is performed under highly artificial conditions with cells that have been suspended by EDTA treatment and incubated in PBS-CMF without added divalent cations. In fact, some studies have indicated that cells in solid tumors are less tightly bound together than are cells in normal tissues (10) and have decreased numbers of junctional complexes between cells (30). These alterations in intercellular adhesion have been thought to correspond to the tendency of the cells to metastasize (31). Further experiments are necessary to determine the relationship between the initial adhesive interactions measured in our system and the intercellular interactions occurring in solid tumors.

The results of our experiments with Con A are interpreted to imply that Con A-induced agglutination and spontaneous aggregation, at least in some cases, do not proceed by the same mechanism because not all cells that show high rates of spontaneous aggregation are agglutinated by Con A. Spontaneous aggregation is not usually detected with the hanging drop technique commonly used to measure Con A agglutination. This is probably due to the fact that the hanging drop procedure

uses mild agitation of the cell suspension, minimizing the number of cell-to-cell collisions that could lead to spontaneous aggregation. The most important conclusion to be drawn from our experiments with Con A is that the net rate of spontaneous aggregation is apparently more closely correlated with tumorigenicity than is the rate of Con A-induced agglutination.

The results reported here indicate that the spontaneous aggregation assay detects a difference in the initial net rate of aggregation between normal and transformed cells that is closely linked with tumorigenicity and loss of anchorage dependence. Control experiments show that cell loss or damage cannot account for this difference.

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1. Robbins, J. G. & Nicolson, G. L. (1975) in *Biology of Tumors*, ed. Becker, F. F. (Plenum Press, New York), Vol. IV, pp. 3–40.
2. Nicolson, G. L. (1976) *Biochim. Biophys. Acta* **458**, 1–72.
3. Holley, R. W. & Kiernan, J. A. (1968) *Proc. Natl. Acad. Sci. USA* **60**, 300–304.
4. Martz, E. & Steinburg, M. S. (1973) *J. Cell. Physiol.* **81**, 25–38.
5. Abercrombie, M. & Heaysman, J. E. M. (1954) *Exp. Cell Res.* **6**, 293–306.
6. Coggin, J. H. & Anderson, N. G. (1974) *Adv. Cancer Res.* **19**, 105–165.
7. Old, L. J., Boyse, E. A. & Stockert, E. (1965) *Cancer Res.* **25**, 813–819.
8. Sanford, K., Barker, B. E., Woods, M. W., Parsbod, R. & Law, L. W. (1967) *J. Natl. Cancer Inst.* **39**, 705–720.
9. Shields, R. & Pollard, K. (1974) *Cell* **3**, 31–38.
10. Coman, D. R. (1961) *Cancer Res.* **21**, 1436–1438.
11. Halpern, B., Pejsachowicz, B., Febvre, H. L. & Barski, G. (1966) *Nature* **209**, 157–159.
12. Dorsey, J. K. & Roth, S. (1973) *Dev. Biol.* **33**, 249–256.
13. Edwards, J. G., Campbell, J. A. & Williams, J. F. (1971) *Nature New Biol.* **231**, 147–148.
14. Lis, H. & Sharon, N. (1973) *Annu. Rev. Biochem.* **43**, 541–574.
15. Ukena, T. E. & Karnovsky, M. J. (1976) *J. Supramol. Struct.*, in press.
16. Cassiman, J. J. & Bernfield, M. R. (1975) *Exp. Cell Res.* **91**, 31–35.
17. Shin, S., Freedman, V. H., Risser, R. & Pollack, R. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4435–4439.
18. Freedman, V. H. & Shin, S. (1974) *Cell* **3**, 355–359.
19. Todaro, G. J. & Green, H. (1963) *J. Cell Biol.* **17**, 299–313.
20. Diamandopoulos, G . & Enders, J. F. (1966) *Am. J. Pathol.* **49**, 397–417.
21. Tevethia, S. S. & McMillan, V. L. (1974) *Intervirology* **3**, 269–276.
22. Sakiyama, H., Gross, S. K. & Robbins, P. W. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 872–876.
23. Green, E. L., ed. (1968) in *Handbook on Genetically Standardized Jax Mice* (Bar Harbor Times Publ. Co., Bar Harbor, Maine), 2nd ed., pp. 57–58.
24. Levine, E. M. (1974) *Exp. Cell Res.* **74**, 99–109.
25. Skehan, P. & Friedman, S. J. (1975) *Exp. Cell Res.* **92**, 350–360.
26. Stanbridge, E. J., Boulger, L. R., Franks, C. R., Garrett, J. A., Reeson, D. E., Bishop, D. & Perkins, F. T. (1975) *Cancer Res.* **35**, 2207–2212.
27. Boone, C. W. (1975) *Science* **188**, 68–70.
28. Klagsbrun, M., Knighton, D. & Folkman, J. (1976) *Cancer Res.* **36**, 110–114.
29. Weiss, L. (1958) *Exp. Cell Res.* **14**, 80–83.
30. McNutt, N. S. & Weinstein, R. S. (1969) *Science* **165**, 597–598.
31. Coman, D. R. (1953) *Cancer Res.* **13**, 397–404.
32. Scher, C. D., Haudenschild, C. & Klagsbrun, M. (1976) *Cell* **8**, 373–382.