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Independence of Normal Phenotypic Properties and Cell Surface Receptor Mobility in Variant Cell Lines

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We report the use of three classes of variants from the long-established, malignantly transformed LM cell line to demonstrate that the apparent mobility of cell surface receptors need not be dependent on the expression of the transformed phenotype in vitro.

Key words: variant cell lines, receptors, cell surface properties, concanavalin A, colchicine, tumorigenicity, growth

A principal interest of this laboratory over the past several years has been in the effects of membrane lipid fatty acid composition on various cell surface properties [1–3]. Of particular interest to us have been the effects of membrane lipid physical state on those in vitro cellular attributes which have been correlated with malignant potential in vivo, including decreased growth control, decreased anchorage dependence of growth, and cell surface alterations [4, 5].

The LM cell line which we selected for use has evolved over a 35-year period from the methylcholanthrene-treated culture established by Earle (L cells) into a line adapted for growth in serum-free medium; it was thus ideally suited for our initial lipid modification studies [6]. However, this long-established, malignantly transformed cell line has no normal, nonmalignant counterpart which would enable us to make the comparative studies necessary for a meaningful evaluation of lipid effects on the transformed phenotype. We have therefore taken an alternative approach to establish a "normal" control and have isolated and characterized several "variant" cell lines [7, 8] which have lost one or more of the phenotypic characteristics of the transformed parental cell line.

While characterizing these variants, we became aware that there is considerable confusion in the literature as to whether an increased apparent mobility of cell surface Con A

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binding sites is [9, 10] or is not [11, 12] a phenotypic property characteristic of the transformed cell. In an attempt to resolve this discrepancy, we have examined our variant cell lines to determine if the loss of phenotypic characteristics of the transformed cell can be correlated with a decrease in the apparent mobility of the Con A binding sites.

In this communication, then, we report the isolation and characterization of several variants, isolated by independent procedures, from the transformed LM cell line. We use these variants to demonstrate that the apparent mobility of Con A binding sites is not a reliable attribute of the transformed cell, since it is easily uncoupled from other, more established indicators of transformation.

MATERIALS AND METHODS

Cell Lines

LM cells derived from NCTC clone 929 were obtained from the American Type Culture Collection. Each variant was isolated from a separate, individual clone of this strain, as described below.

General Protocol for Variant Isolation

Variants were selected by a modification of the methods of Ozanne [13] and Vogel and Pollack [8]. LM cells were grown to 80–100% confluency and then challenged with a selecting agent. Most cells assumed a rounded shape and detached from the plate. The remaining cells were throughly washed with minimum essential medium (Eagle), with Hank's salts (MEM, Gibco) and grown to 80–100% confluency in MEM + 10% calf serum and then challenged again. Resistant cells were regrown to confluency and were then cloned by serial dilution into microtest wells (Linbro) to achieve an average of one cell per well. While the frequencies of reversion were not rigorously determined, we estimate that no variant arose at a frequency of greater than 10^{-3} or 10^{-4}. Only clones exhibiting a flat morphology were selected for study.

Selection Procedures

We have isolated three classes of variant cells from LM, in each case using selective agents which tend to eliminate from a population those cells displaying a phenotypic trait commonly associated with malignant transformation. Because of the observed preferential toxicity to transformed cells of concanavalin A (Con A) [13], a variant was selected for resistance to this lectin. Microtubules have been implicated in a variety of cell surface phenomena associated with the transformed state [4, 14] and are known to be disrupted by colchicine [15]. We therefore isolated a colchicine-resistant variant, Co.12, by a procedure similar to that of Vogel and Pollack [8]. Trypsin was used to obtain a third class of variants by selection for more adhesive cells, since normal cells appear more adhesive to a substratum than their transformed counterparts [4].

Specifically, each variant class was isolated as follows:

a. Con A variants. LM Cells were challenged for two cycles of treatment with 600 μg/ml of Con A (Miles) in MEM + 10% calf serum for 24 h at 37°C. The clone used in these studies has been designated R1.

b. Colchicine variants. Cells were challenged for two cycles of treatment with 10^{-2} M colchicine (Sigma) in MEM for 6 h. A colchicine-resistant clone, designated Co.12, has been used for this study.

c. Trypsin variants. LM cells were challenged for two cycles of treatment with 0.05% trypsin for 10 min at 37°C. A clone of trypsin-resistant cells, T4, was selected for use in this study.

Culture Procedures

Stock cultures of LM cells were grown in MEM supplemented with 0.5% bacto-peptone (MEM + P). Stock cultures of variants were maintained on MEM + 10% calf serum. Monolayer cultures were established in 75-cm^2 Corning tissue cultures flasks containing 12 ml of media. At confluency, cells were passed as previously described [16]. Comparisons between lines were always made using cultures grown under identical conditions; except where noted the media used for all experiments was MEM + 1% calf serum. Variants were passed at subconfluency to avoid reversion. Frozen stocks of all lines were made at an early passage, and all lines were studied within the 5th and 15th passages of the initial clone or the frozen stock. The cell lines were maintained at 37°C in an atmosphere of humidified air and 6% CO_2.

Analysis of Cellular Growth

Cell growth was followed by counting in a hemocytometer, as previously described. The saturation densities presented in Table I are the maximal cell densities achieved by the respective cell line when plated and grown in the appropriate medium without changes. Under these culture conditions, saturation density is roughly independent of the initial innoculum. The plating efficiencies of all variants were roughly equivalent to that of the parental line.

Measurement of Cell Adhesion to Substratum

The adhesion of cells to a substratum was assayed as a resistance to treatment with trypsin. LM cells and variants were grown to 90–95% confluency in MEM + 1% calf serum. Great care was employed in using LM cultures which were not overconfluent. Cultures were treated with various concentrations of trypsin in Hanks's salts for 10 min at 37°C. Detached cells were collected and counted in a hemocytometer. The cells remaining attached to the dish were removed by incubation with 0.25% trypsin in Hanks's salts for 10 min at 37 °C and they also were counted. The ratio of cells removed after the initial trypsin treatment to the total number of cells was taken as a measure of the adhesiveness of cells to their substratum.

Con A Mediated Agglutinability

Agglutinability was measured by a hemadsorption assay which determines the Con A-induced association of erythrocytes to a confluent fibroblast monolayer [1].

Tumorigenicity

Cells were grown in Dulbecco-Vogt modified MEM (Gibco) + 10% fetal calf serum prior to injection. After dispersion with 0.5% trypsin in Hanks's balanced salt solution, cells were washed with MEM plus fetal calf serum, and viability was determined by the trypan blue exclusion technique. Finally, 10^4, 10^5, and 10^7 viable cells were suspended and injected subcutaneously into male C3H/NIH mice, 6–10 weeks old. Animals were palpated weekly for evidence of tumors, and the tumor weights were determined at 28 days after injection.

Electron Microscopy

The cell surface labeling procedure used was essentially that of Smith and Revel [12]. Cells were grown to near confluency in MEM + 1% calf serum on glass coverslips or plastic petri dishes. Monolayers were incubated with Con A (100 μg/ml) for 10 min and then, after thorough washing with phosphate-buffered saline (PBS), labeled with 1 mg/ml of

TABLE I. Phenotypic Properties of Variants Selected From a Transformed Mouse Cell Line

Phenotypic properties	Parental line (LM)	Variants R1	Co.12	T4
Growth control				
A. Saturation density[a] (cells/cm^2)				
Supplement: 0.5% peptone	3.0×10^5	$2,3 \times 10^3$	4.2×10^3	1.0×10^4
1% calf serum	3.0×10^5	2.0×10^4	1.0×10^4	1.2×10^4
5% calf serum	3.9×10^5	1.0×10^5	6.4×10^4	1.0×10^5
10% calf serum	3.7×10^5	1.5×10^5	6.4×10^4	1.9×10^5
B. Generation time[b] (h)				
Supplement: 0.5% peptone	24	0	96	77
1% calf serum	29	36	36	36
5% calf serum	24	28	30	30
10% calf serum	24	24	27	24
Anchorage dependence				
A. Adhesion[c] (% cells detached with 0.05% trypsin)	94	29	37	32
B. Growth in suspension[d]	+	–	–	–
Tumorgenicity				
Incidence of tumors[e]	High	Medium	Medium	Low
Cell surface alterations				
A. Agglutinability by Con A (μg/ml of Con A for max. Con A-mediated hemadsorption)	150	300	300	300
B. Con A-induced redistribution of cell surface receptors[f]	+	+	+	+

[a] Measured as described in Materials and Methods.
[b] Measured as initial rate of cell growth and expressed as the time needed for a doubling of cell number. In this experiment, when variants were maintained in 0.5% peptone, R1 did not grow at all; Co.12 doubled only 0.5 times; and T4 doubled only 1.5 times. As mentioned in the Results section, variant lines could not be maintained continuously in the absence of serum.
[c] Measured as resistance to trypsin treatment as described in Materials and Methods.
[d] Spinner flasks were innoculated at 8×10^5 cells/ml in MEM and 1% calf serum, and growth was followed until stationary phase was reached.
[e] See Figure 1 and Table II.
[f] See Figures 2–4.

hemocyanin for an additional 10 min. Hemocyanin was purified from the hemolymph of Busycon canaliculata (personal communication, Susan Brown, University of California, San Francisco). All washings and incubations were done at 22°C. The samples were fixed with with 2.5% glutaraldehyde (TAAB Lab) in PBS, postfixed in 1% OsO_4 (Polysciences) in PBS, and dehydrated with ethanol. Cells grown on glass were either dried at the critical point of Freon 13 or were dried from amyl acetate [17]. Cells grown on plastic were removed from the plate with amyl acetate, placed on glass coverslips, and dried from the same solvent. Shadow-cast replicas were made of the dried cells, as described by Smith and Revel [12].

To detect the native distribution of Con A-binding sites on the cell surface, cells were fixed with 2.5% glutaraldehyde before labeling. Prefixed cells were washed with PBS and incubated over night with 0.15 M NH_4Cl at 4°C in order to block unreacted aldehyde

groups. Cells were then labeled as above and, following another fixation with 2.5% glutaraldehyde, were processed exactly as described above for postfixed cells.

Replicas were examined in either a Hitachi HU11A or a Philips EM200 electron microscope. The photographs presented in this paper are printed as negative images.

RESULTS

We have compared three classes of variant cell lines to the transformed parental line with respect to a number of characteristics commonly associated with malignant transformation. The results are summarized in Table I.

Growth Control

In contrast to LM, all variant lines show an obligate serum requirement for growth. While the saturation density and growth rate of LM are generally independent of serum concentration, all variants have saturation densities and growth rates which vary with serum concentration.

Anchorage Dependence

The variant lines generally displayed phenotypic properties consistent with an increased anchorage dependence for growth. All variants were considerably more adhesive to their substratum than the parental line, as measured by a trypsinization assay. A more thoroughly documented study of this property for lines R1 and LM has been published [18]. No variant line grew in suspension culture, while LM grew vigorously. In addition, when compared with LM, all variants showed a reduced ability to grow in semisolid medium (agar, data not shown).

Tumorigenicity

All variants were at least slightly tumorigenic, although, as Figure 1 demonstrates, the tumors induced by the higher dilutions of variant cells tended to develop more slowly

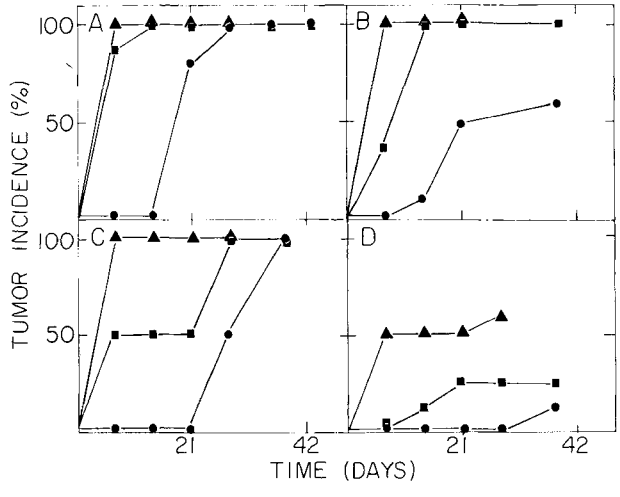

Fig. 1. Tumor incidence. Six to eight mice were injected with 10^7 (▲), 10^5 (■), and 10^4 (●) cells subcutaneously as described in Materials and Methods. A) LM, B) R1, C) Co.12, D) T4.

TABLE II. Tumor Weight at 28 Days

Cell strain	Mean tumor weight ± SE
LM	2.61 ± 0.71 g
R1	0.99 ± 0.15 g
Co.12	0.86 ± 0.26 g
T4	0.42 ± 0.28 g

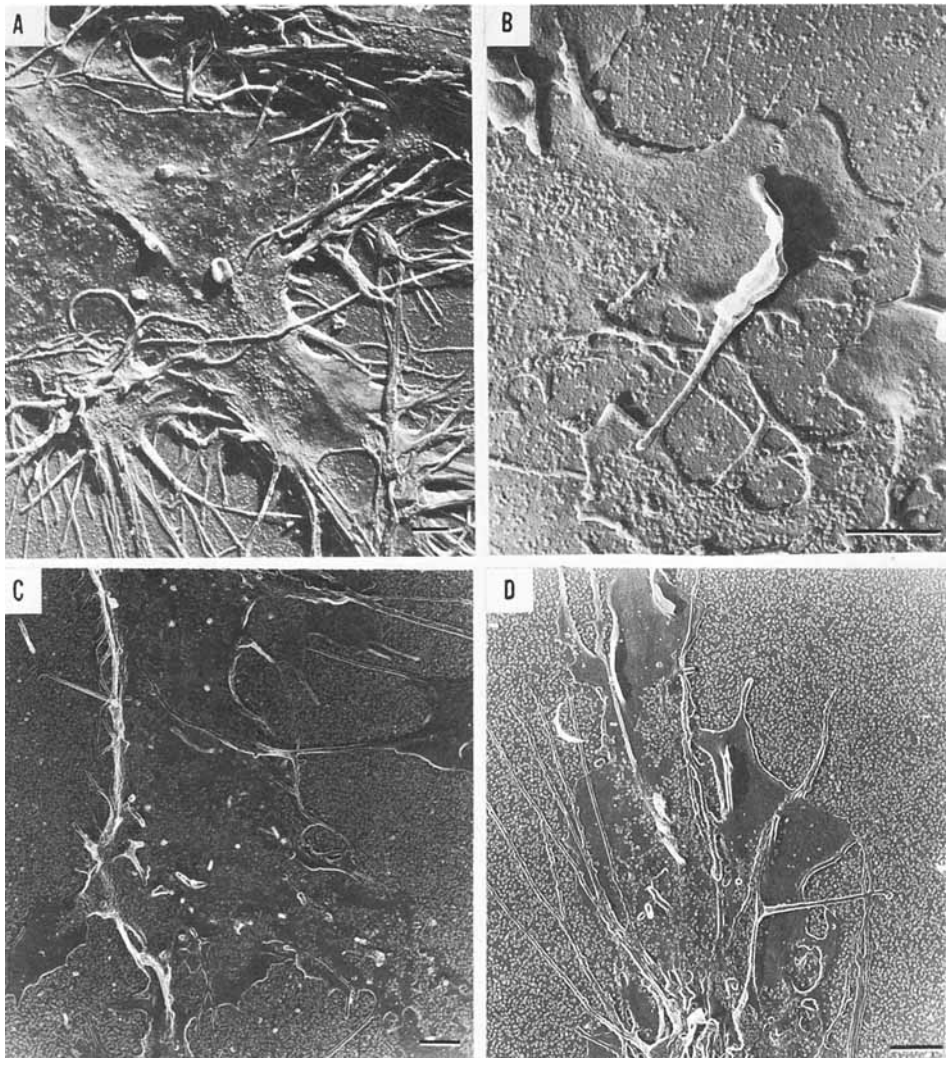

Fig. 2. Lectin-induced redistribution of cell surface receptors on parental and variant cell lines. Cells were fixed following labeling with Con A/hemocyanin as described in Materials and Methods. Bar equals 1 μ. A,B) The parent cell line, LM; C,D) R1, Con A-selected variant.

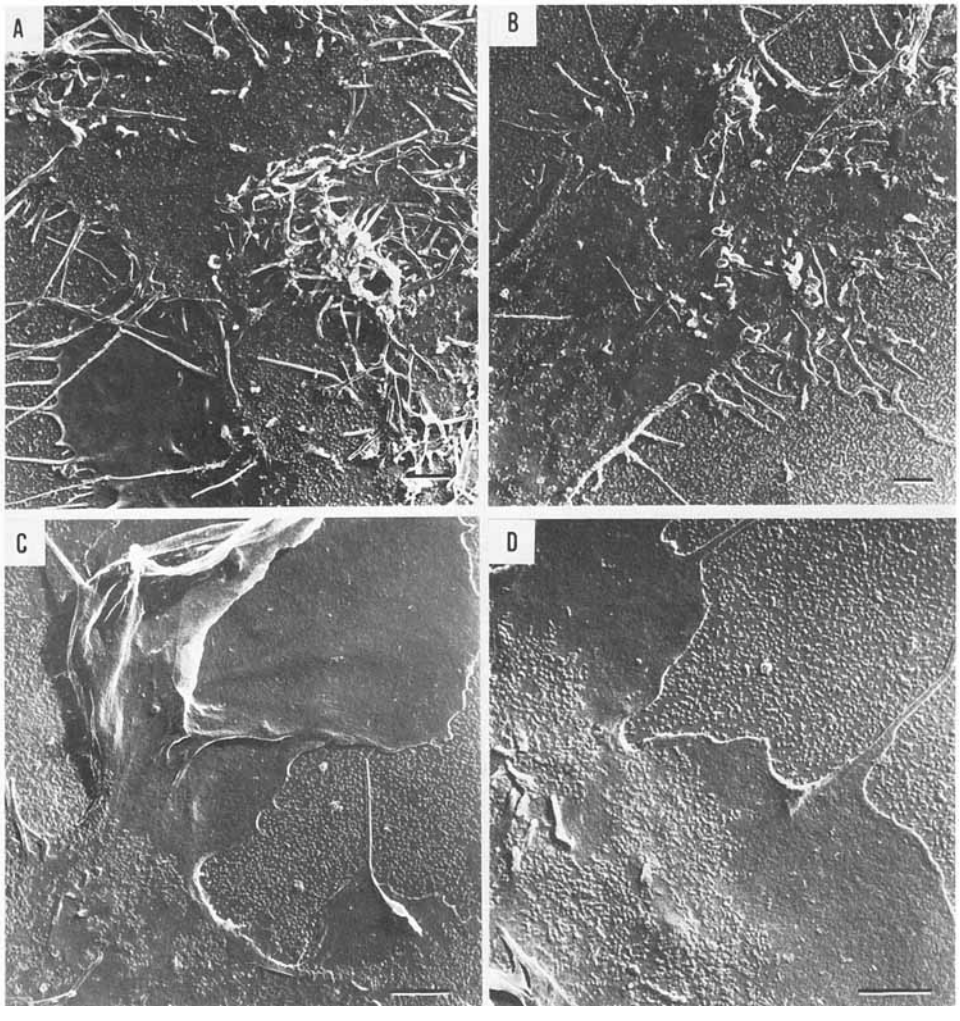

Fig. 3. Lectin-induced redistribution of cell surface receptros on variant cell lines. Cells were fixed following labeling with Con A/hemocyanin as described in Materials and Methods. Bar equals 1 μ. A,B) Co.12, Colchicine-selected variant; C,D) T4, trypsin-selected variant.

than those induced by LM. The trypsin variant was clearly less tumorigenic than the parental line at all dilutions tested. In addition, the tumors induced by all variant lines were much smaller than those induced by the parental line, as shown in Table II.

Cell Surface Properties

The variants and the parental line were examined for cell surface properties generally correlated with transformation. All variants were less agglutinable by Con A than was LM. Unexpectedly, however, the Con A-binding sites of all the variants and the parental line had an identical tendency to cluster in response to lectin treatment, as shown in Figures 2 and 3. That this phenomenon was lectin-induced was confirmed by the random distribution of Con A bound to the prefixed cells, as depicted in Figure 4.

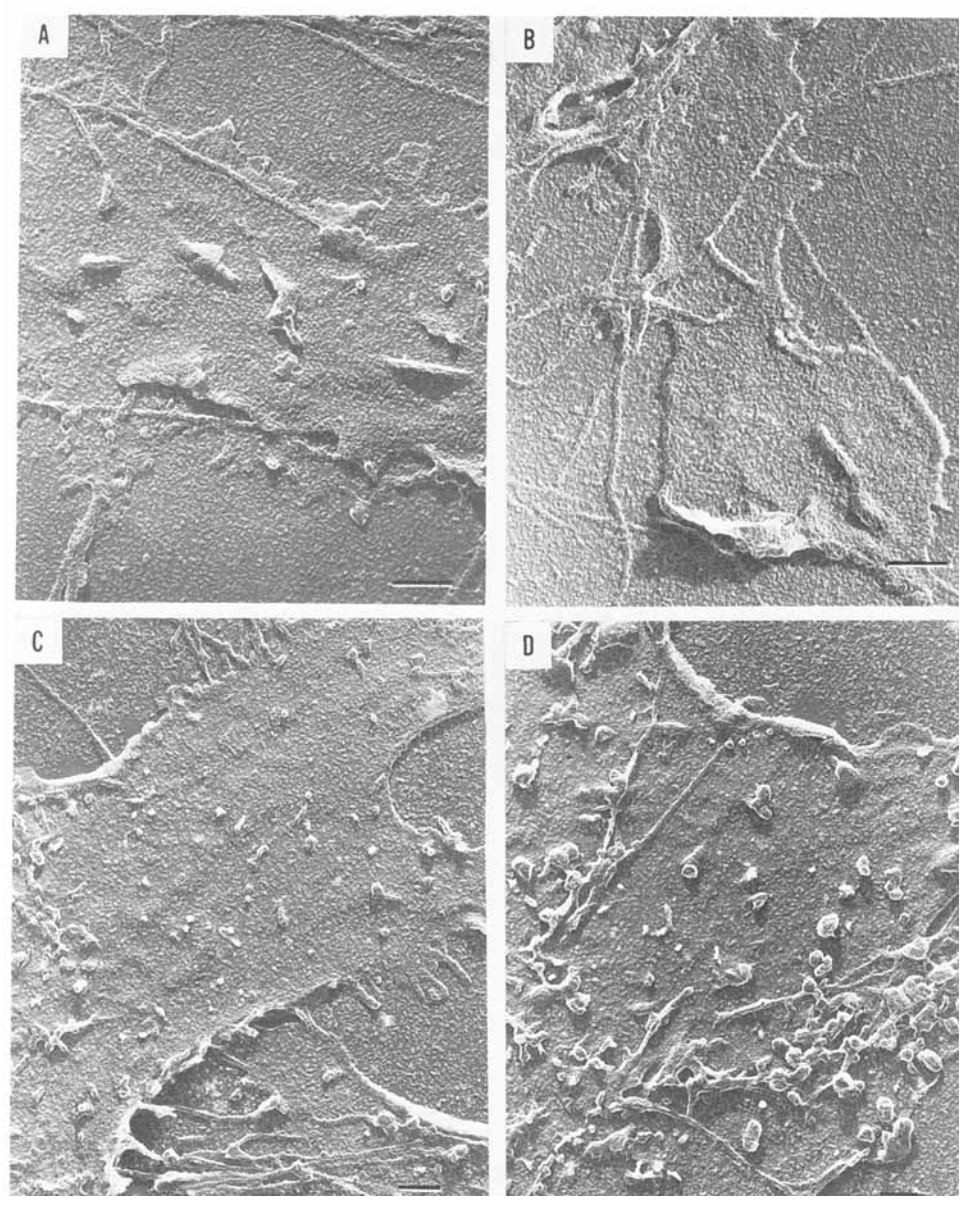

Fig. 4. Native cell surface receptor distribution on parental and cell surface variants. Cells were prefixed with glutaraldehyde prior to Con A/hemocyanin labeling. Bar equals 1 μ. A,B) LM, parent cell line; C,D) Co.12, colchicine-selected variant.

DISCUSSION

In our attempt to generate a normal counterpart to the long-established, malignantly transformed LM line, we have isolated three classes of variants which regained many of the phenotypic properties of "normal" cells in culture. When compared with LM, three variant lines appeared to have two well-documented characteristics of normal cells in vitro: growth control [4] and anchorage dependence for growth [19] (Fig 1). In addition, all variant lines were much less agglutinable by Con A than was the parental line, and agglutinability is a property commonly associated with transformed cells. Paradoxically, with the exception of the trypsin variant, the variant lines do not display a clear-cut reduction in oncogenicity which can be correlated with their reversion to a normal phenotype. Since it seems that tumorigenicity is not strictly synonymous with the in vitro transformed phenotype, great caution should be taken not to infer a malignant potential in a cell from its in vitro behavior. We emphasize that the only ultimately reliable test for malignancy is a direct test for oncogenicity.

There are many reports in the literature of an apparently greater mobility (or ability to form clusters) of lectin-binding sites on the transformed cell surface [9, 10]. We thus expected that the Con A-binding sites on the variant membranes might be displayed in a more diffuse or randomized fashion than those of the LM cell line after Con A treatment, but in no case was this observed. In addition, a sizable proportion of "normal" Balb/C 3T3 (NCTC clone A31) cells displayed patterns of Con A binding interpretable in terms of lectin-induced topographic redistribution (data not shown). Thus, there does not seem to be a strict correlation between the transformed phenotype in vitro and the apparent mobility of surface lectin-binding sites. While our findings are in agreement, in this respect, with previous reports [11, 12] and the more recent data of Ukena and Karnovsky [20], we believe our study is unique in the use of flat variants to demonstrate the dissociability of this trait from other phenotypic characteristics of the transformed state.

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REFERENCES

1. Rittenhouse HG, Williams RE, Wisnieski B, Fox CF: Biochem Biophys Res Commun 58:222, 1974.
2. Li JK-K, Williams RE, Fox CF: Biochem Biophys Res Commun 62:470, 1975.
3. Wisnieski BJ, Parkes JG, Huang YO, Fox CF: Proc Nat Acad Sci USA 71:4381, 1974.
4. Robbins JC, Nicolson GL: In Becker EF (ed): "Cancer: A Comprehensive Treatise." New York: Plenum, 1975, vol 3.
5. Shields R, Pollack K: Cell 3:31, 1974.
6. Williams RE, Wisnieski BJ, Rittenhouse HG, Fox CF: Biochemistry 13:1969, 1974.
7. Pollack R, Green H, Todaro G: Proc Nat Acad Sci USA 60:126, 1968.
8. Vogel A, Pollack R: In Prescott DM (ed): "Methods in Cell Biology." New York: Academic, 1974, vol 8.

9. Inbar M, Sachs L: FEBS Lett 32:124, 1973.
10. Rosenblith J, Ukena TE, Yin HH, Berlin RO, Karnovsky MJ: Proc Nat Acad Sci USA 70:1625, 1973.
11. Raff MC, dePetris S, Mallucci L: In Clarkson B, Baserga R (eds): "Control of Proliferation in Animal Cells." Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, 1974.
12. Smith S, Revel JP: Devel Biol 27:434, 1972.
13. Ozanne B: J Virol 12:79, 1973.
14. Rittenhouse HG, Williams RE, Fox CF: J Supramol Struct 2: 629, 1975.
15. Vogel A, Rissen R, Pollack R: J Cell Physiol 82:181, 1973.
16. Williams RE, Li JK-K, Fox CF: Biochem Biophys Res Commun 62:462, 1975.
17. Revel JP, Wolken K: Exp Cell Res 78:1, 1973.
18. Rittenhouse HG, Williams RE, Fox CF: Biochem Biophys Res Commun 71:975, 1976.
19. Shin S-I, Freedman UH, Risser R, Pollack R: Proc Nat Acad Sci 72:4435, 1975.
20. Ukena TE, Karnovsky M: J Supramol Struct Suppl 1, 1976, pp 261–273.