[CANCER RESEARCH 30, 498–503, February 1970]

A Study of Surface Ionogenic Groups of Chick Embryo Cells Transformed by Rous Sarcoma Virus

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SUMMARY

The electrokinetic surface properties of chick embryo cell cultures transformed by Rous sarcoma virus have been studied for any alterations associated with the malignant transformation. In this system a rapid and efficient transformation *in vitro* takes place which enables a comparison to be made between normal and malignant cells derived from the same embryo and cultured under the same conditions. No difference could be detected between the normal and malignant cells for either the surface charge or pH-mobility relationship. The expected decrease in calcium-binding power of the malignant cells was not evidenced in this system, as both types of cells also had the same affinity to calcium. These data as well as other recent studies suggest that the relationship between calcium affinity and malignancy is in need of further clarification.

Neuraminidase treatment produced a mobility decrease in malignant cells that never surpassed that noted in normal cells but was always either equivalent to or less than that of their normal counterparts. It was concluded that no generalization could be made as to the correlation between malignancy, increased surface charge density, and increased surface sialic acid.

INTRODUCTION

In previous studies attempts have been made to correlate changes in surface properties of the malignant cells with their unique biological behavior. It has been suggested that the lack of "contact inhibition" of the tumor cells was related to their poorer mutual adhesiveness (1) and was also evidenced in a decreased calcium affinity of their surfaces (7). In some cases (1, 14, 25) the malignant properties were believed to be associated with a higher net surface negative charge due to an increased surface sialic acid (3, 14, 26). Conflicting results from the literature as to the relationship between increased surface charge density and increased surface sialic acid of neoplastic cells (9, 28, 30) have emphasized the need for further investigation of these suppositions.

In the present study an attempt has been made to examine the above problems in a well-controlled system of neoplastic transformation *in vitro*. The Rous sarcoma virus induces a rapid and almost complete transformation in chick embryo cell cultures in the course of a few days (29). In contrast other oncogenic viruses transform only a small fraction of the cells in culture, thus necessitating long-term serial passaging. In this investigation an electrokinetic characterization of normal and RSV[2]-transformed chick embryo cells has been undertaken to test the validity of the above assumptions. Special attention has been given to the effects of neuraminidase treatment and changing pH and calcium concentration on the electrophoretic mobilities of these cells.

MATERIALS AND METHODS

Cells. Primary cultures were prepared from 9- to 10-day-old chick embryos. Eggs were obtained from brown Leghorn chickens of a leukosis-free flock (obtained through the courtesy of Dr. A. Kohn of the Israel Institute for Biological Research).

Virus. The Bryan "high-titer" strain RSV (RAV-2) used here was obtained from Dr. A. Kohn. The viral stock was prepared from a 9-day-old tumor produced by wing-web inoculation.

Media. The standard culture media contained M-199-5% calf serum-10% Tryptose phosphate with the usual concentration of penicillin, streptomycin, and mycostatin.

Solutions. The standard solution used for electrophoretic measurement was 0.0145 M NaCl, made isotonic by the addition of 4.8% glucose and buffered to neutrality with 0.3 mM $NaHCO_3$. All other measuring solutions, also at an ionic strength of 0.0145, were prepared as previously described (24). The neuraminidase (EC 3 . 2 . 1 . 18 Behringwerke AG, Marburg-Lahn, Germany, Lot 11651) was diluted in calcium-NaCl solution to an amount giving the maximal decrease in mobility for human erythrocytes after a 30-min incubation at 37°.

Procedure. Chick embryo cell cultures were grown

[1]This investigation was supported by USPHS Research Grant CA-06582 from the National Cancer Institute.
Received March 21, 1969; accepted June 26, 1969.

[2]The abbreviation used is: RSV, Rous sarcoma virus.

according to the procedure described by Temin and Rubin (29). The virus was introduced either directly with the cell suspension or 24 hr after seeding. In the former case the virus was first incubated 1 hr with the cells in a small amount of medium at 37° and then plated in the standard amount (5 ml) of medium. When the virus was introduced after seeding, the cell cultures were incubated without medium for 45 min with 0.2 ml viral suspension. The usual amount of medium was then added. Cells were harvested from cultures within 1 or 2 weeks after viral inoculation, when microscopic examination indicated that most cells had undergone transformation. Corresponding normal chick fibroblast cultures derived from the same embryo were harvested at the same time as the malignant cells. Single-cell suspensions were prepared by exposure of the cultures to freshly made 0.05% trypsin (Nutritional Biochemicals Corporation, Cleveland, Ohio) at about 30° for 10 min. In some experiments cells were dissociated by incubation of the cells 0.1% EDTA in calcium- and magnesium-free phosphate buffer for 90 min at 37°. Detachment of the cells from the monolayer after both EDTA and trypsin treatment was accomplished by scraping with a rubber policeman and gentle pipetization of the cell sheets to free the cells. Enzyme action was inhibited by cooling the cell suspensions in ice. Cells were centrifuged for 3 min at 350 X *g* and prepared for electrophoresis by the methods described in the previous article (24).

Comparative experiments performed to test the effect of EDTA and trypsin on the mobility of cells showed that cells dissociated by enzymic treatment had mobilities about 10% lower than those of cells dissociated by the chelator. Trypsin was still used in preference to EDTA in most experiments because of the better viability (dead cells <5%) of the cells after this treatment.

Electrophoretic Measurements. All measurements were made with a rectangular microelectrophoretic chamber modified (16) for use in a lateral position. In this method, Ag-AgCl electrodes are immersed in a saturated KCl solution which is separated from the electrophoretic cell by means of sintered glass discs.

RESULTS

No difference was found in the electrophoretic mobilities of normal and RSV-transformed chick embryo cells. The mean mobility for normal cells was 1.80 ± 0.31 and that for transformed cells was 1.86 ± 0.32 ($p > 0.05$). A typical experiment depicting the similarity between these cells for distribution in mobility (expressed in μ/sec/V/cm is shown in Chart 1. Cells suspended and measured in the original medium for growth of the cultures exhibited the same equivalence of values as those measured at ionic strength 0.0145. The mean mobilities (not corrected for viscosity) were 1.18 ± 0.27 and 1.14 ± 0.30 for normal and malignant cells, respectively. A tabulation of mean mobility values for the majority of individual experiments performed on both normal and malignant cells has been made in Table 1. An examination of these data indicates that there is a considerable variation in mobility between the different experiments,

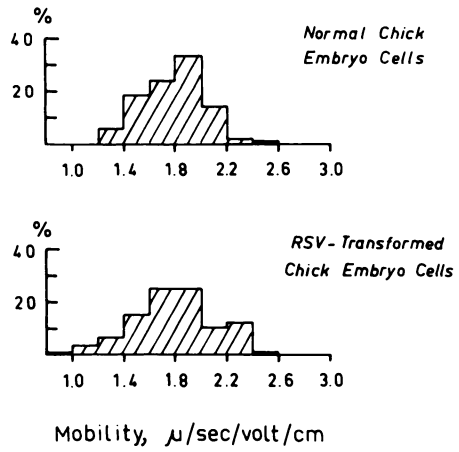

Chart 1. Histogram of number of cells (% of total) as a function of the electrophoretic mobility of normal and RSV-transformed chick embryo cells. All measurements were made in 0.0145 M NaCl solution, made isotonic with 4.8% glucose and buffered to neutrality with 0.3 mM $NaHco^3$

Table 1

The mean electrophoretic mobilities of normal and RSV-transformed chick embryo cells

Each pair of data represents one experiment on normal and transformed cells derived from the same egg, cultured in the same way, and measured on the same day. All measurements were carried out in 0.0145 M NaCl solution made isotonic with 4.8% glucose and buffered to neutrality with 0.3 mM $NaHCO_3$.

Mobility of normal cells (μ/sec/V/cm ± S.D.)	No. of cells	Mobility of transformed cells (μ/sec/V/cm ± S.D.)	No. of cells
1.93 ± 0.25	25	2.03 ± 0.38	26
1.96 ± 0.21	27	2.14 ± 0.46	28
1.61 ± 0.14	15	1.89 ± 0.17	15
1.82 ± 0.17	30	1.79 ± 0.11	25
2.12 ± 0.39	15	2.17 ± 0.24	15
1.76 ± 0.15	15	1.86 ± 0.19	15
1.64 ± 0.24	15	1.70 ± 0.25	15
1.83 ± 0.18	15	1.79 ± 0.22	15
1.73 ± 0.25	25	1.93 ± 0.24	26
1.67 ± 0.24	15	1.78 ± 0.32	15
1.76 ± 0.28	15	1.99 ± 0.24	35
2.30 ± 0.22	15	2.09 ± 0.36	10
1.71 ± 0.28	99	1.66 ± 0.34	42
1.57 ± 0.11	15	1.86 ± 0.27	15
1.67 ± 0.24	15	1.78 ± 0.32	15
1.80 ± 0.22	15	1.74 ± 0.37	15
1.78 ± 0.28	15	1.80 ± 0.27	15
1.94 ± 0.20	15	1.82 ± 0.19	15
1.86 ± 0.25	15	2.06 ± 0.41	15
1.84 ± 0.15	15	1.83 ± 0.14	8
2.09 ± 0.14	10	1.83 ± 0.26	20

in spite of the fact that the cells were grown and harvested under similar conditions. Most of the results above were obtained by mild treatment with trypsin which yielded suspensions of rounded and predominantly viable cells. In experiments where the monolayers were dissociated by EDTA (Table 2), the normal and transformed cells again showed no difference in their mobilities. The mean mobility

Table 2

The electrophoretic mobilities of normal and RSV-transformed chick embryo cells

The number of cells measured are in parentheses. A: Cell suspensions were prepared by treatment of the cultures with 0.05% trypsin for 10 min at 30°. B: Cell suspensions were prepared by treatment of the cultures with 0.1% EDTA for 90 min at 37°. All measurements were carried out in 0.0145 M NaCl solution made isotonic with 4.8% glucose and buffered to neutrality with 0.3 mM $NaHCO_3$.

	Mobility (μ/sec/V/cm ± S.D.)	
	Normal cells	Transformed cells
A	1.80 ± 0.31 (385)	1.86 ± 0.32 (321)
B	2.03 ± 0.23 (85)	2.16 ± 0.34 (85)

Neuraminidase-treated cells

	Control	Enzyme-treated	Mobility reduction (%)	Control	Enzyme-treated	Mobility reduction (%)
A	1.86 ± 0.22 (141)	1.49 ± 0.26 (115)	20	2.00 ± 0.28 (192)	1.68 ± 0.29 (105)	16
B	2.08 ± 0.24 (55)	1.42 ± 0.22 (48)	32	2.10 ± 0.32 (55)	1.64 ± 0.35 (51)	22

for the normal cells was 2.03 ± 0.23 and that for the transformed cells was 2.16 ± 0.34 ($p > 0.05$).

pH-Mobility Relationship. The pH-mobility relationship of both the normal and transformed cells was virtually identical (Chart 2). Both types of cells exhibited positive mobility between pH 2 and 3.6 and negative mobilities between pH 3.9 and 8. The isoelectric point of the normal and malignant cells was estimated at pH 3.8. The reversal of charge observed at low pH's is considered to be significant since cells resumed their original mobilities in the standard medium after immersion in pH 2.

Calcium Binding. Chart 3 shows the effect of increasing calcium concentrations on the mobility of the normal and transformed cells. A slight reduction in mobility is already evident at 5×10^{-6} M calcium as compared to the control without this cation. From this point on there is a gradual decline in mobility values up to 4.8×10^{-3} M, the maximum concentration permissible at the ionic strength of 0.0145. It can be seen from the chart that the calcium affinity of normal chick embryo cells is almost identical with that of the same cells transformed by Rous sarcoma virus.

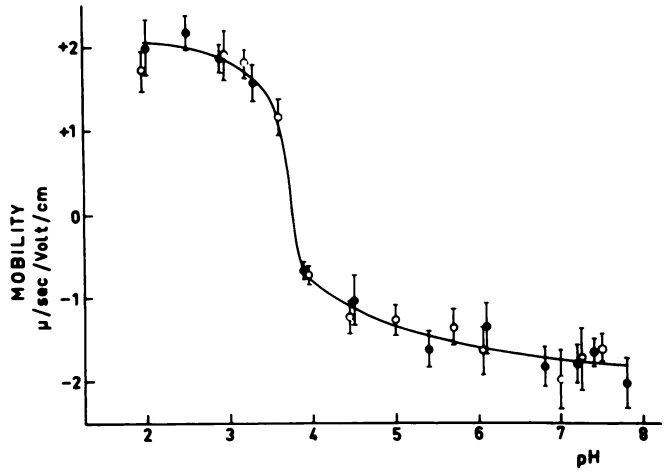

Chart 2. pH-mobility relationships of normal and RSV-transformed chick embryo cells. All measurements were carried out at an ionic strength of 0.0145. Isotonicity was maintained with 4.8% glucose. Each point on the curve represents the mean mobility of at least 50 cells ± S.D. ○, normal cells; ●, transformed cells.

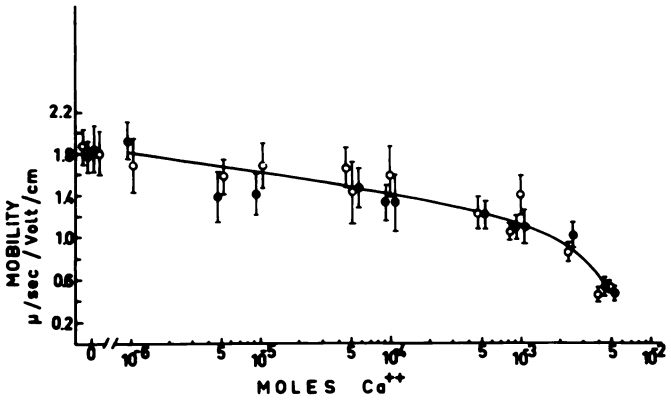

Chart 3. The effect of calcium on the electrophoretic mobility of normal and RSV-transformed chick embryo cells. All measurements were carried out at an ionic strength of 0.0145. Isotonicity was maintained with 4.8% glucose. Each point on the curve represents the mean mobility of at least 50 cells ± S.D. ○, normal cells; ●, transformed cells.

Neuraminidase Treatment. Exposure of the normal cells to neuraminidase reduced their mobility by about 10 to 20% and produced an equivalent or smaller reduction in the malignant cells. The enzyme caused about a 30% decrease in mobility in normal cells dissociated by EDTA and again a similar or lesser reduction in correspondingly treated

malignant cells. In no case was the degree of charge reduction of the transformed cells greater than that of the normal controls for any given experiment. These results are summarized in Table 2.

DISCUSSION

Basically, the unique biological behavior of malignant cells, as evidenced in their altered contact relationships, invasiveness, and metastatic ability, can be viewed as stemming from their inability to form normal cell associations. One approach to the study of these properties has been to determine the structural components of the cell surface in both normal and malignant cells (1). The method of microelectrophoresis has afforded a means of analyzing the most external chemical moieties of the cell periphery with the minimum amount of manipulation and damage to the cells. Implicit to this approach has been the supposition that the ionogenic groups on the surface are those important for cellular interactions and that certain changes in one or more of these groups might result in the "disturbed" state of malignancy. To this end the early studies in this field concentrated on comparisons of surface charge density (2), as derived from the electrophoretic mobility, and enzymic removal of sialic acid or other charged components of the membrane. In a number of cases (14, 26, 27) the higher net surface charge was found to be due to an increased amount of sialic acid in certain types of tumor cells as opposed to corresponding normal cells. The hypothesis that there was a direct correlation between these factors and the state of malignancy was strengthened by the finding of Defendi and Gasic (11) that embryonic hamster cells transformed *in vitro* by polyoma virus had a thicker coat of sialomucin than their normal counterparts when stained by the Hale reaction. Forrester, Ambrose, and Macpherson (14) corroborated the above results for the same system by demonstrating the appearance of a cell population with a higher charge density than that of the normal cells. This additional charge was found to be eliminated upon treatment with neuraminidase. In a later study, however, Forrester, Ambrose, and Stoker (15) showed the above charge enhancement to be true for only some clones out of a group of transformed hamster kidney fibroblast clones. Subsequent investigators were unable to support these findings for various types of human carcinomas (30) nor for tissue cells of normal and malignant origin grown as monolayer cultures (28). In recent studies on murine leukemias Cook and Jacobson (9) and Patinkin *et al.* (24) also found no increased surface charge in the leukemic cells when compared to their normal counterparts.

A stumbling block that has consistently hampered the study of the properties of neoplastic cells has been the difficulty in obtaining true normal counterparts for such cells. This problem has been overcome in large part by the use of chick embryo cell cultures infected with Rous sarcoma virus where there is a rapid and nearly complete transformation of normal populations into malignant ones. In this system the normal cells have been seen to undergo striking changes in patterns of growth and cell surface morphology with transformation (31). It might have been expected that if changes in surface ionogenic groups were at all involved in cellular associations, they would be most evident in a system such as this. Surprisingly enough no difference could be detected between the normal and malignant cells for either the surface charge, pH-mobility relationship, or calcium affinity. Furthermore in no case was a greater reduction in electrophoretic mobility observed for the malignant cells than for the normal ones after both had been subjected to neuraminidase. On the contrary, their mobility decrease was always either equivalent to or less than that of their normal counterparts. These observations are in line with the findings of Vassar (30), Simon-Reuss *et al.* (28), Cook and Jacobson (9), and Patinkin *et al.* (24) for a variety of normal and malignant tissues, both embryonic and adult. They are also in agreement with the results of Ohta *et al.* (23) who examined the sialic acid content of a number of normal and virus-transformed cell lines differing in their degree of contact inhibition. All the transformed lines had slightly lower amounts of sialic acid than did their normal counterparts. Quantitative determinations of the sialic acid released upon neuraminidase action from the plasma membranes of normal rat liver and hepatoma also revealed no difference in this regard between the normal and the neoplastic cells (5). All these results do not confirm the postulated correlation between sialic acid and cellular neoplastic properties as a general phenomenon but on the other hand do not exclude the possibility that other glycoproteins might be involved. In addition to the above groups it may well be that the uncharged components, which are not detected by the electrophoretic method, are also of importance. The studies by Cox and Gesner (10) on the role played by surface sugars as sites for cell interaction and recognition may be relevant here.

Previous work equated the lower total calcium content of malignant tissues with a decreased calcium-binding capacity of the surface of the tumor cell which was in turn responsible for a diminished adhesiveness (7). As has been shown in the present work, the capacity of the cell surface to bind calcium was the same for both normal and transformed cells. This is in agreement with our previous observations (24) on normal lymphocytes and leukemic cells. The finding is of considerably more significance for the chick embryo cell system where the normal fibroblasts form stable cell adhesions, believed to involve calcium bonds. The degree of calcium binding for both the normal and Rous-transformed fibroblasts is approximately 2 orders of magnitude higher than that found for either the lymphocytes, macrophages, or erythrocytes. This is suggestive of the presence of additional anionic groups at the surface of cells of this system which endow them with increased calcium affinity. The sulfate groups of the acid mucopolysaccharides are likely candidates, since they are known to exhibit a calcium-binding power higher than those of the carboxyl groups (6). Another group that may be considered is the RNA phosphate, shown by Weiss and Mayhew (32) also to possess a higher affinity to calcium than does the sialic acid carboxyl.

In a critical work on the calcium content of neoplastic tissues, Hickie and Kalant (18) found an increase in calcium and magnesium content of Morris hepatoma cells as opposed

to normal rat liver cells. In a recent study (13) on the effect of changing the serum calcium level on the calcium content of tumor tissues, neither of these 2 factors was found to influence the incidence of metastases in the host. In none of these investigations was any appraisal made of the amount of calcium bound to the surface, which would be the relevant site involved in the adhesiveness of cells to one another. The method used here permits such an analysis of sites to be made by providing a measure of the number of ionogenic groups at the surface which combine with calcium. The fact that no difference between normal and malignant cells was demonstrated in our systems does not necessarily invalidate the postulates of Coman (7). It is conceivable that the calcium needed for cell association is localized only at certain specific areas of the cell surface and therefore cannot be detected by the measurement of net surface charge. That this possibility is plausible is brought out by Armstrong's (4) observation that the amount of calcium sufficient for reaggregation of embryonic tissues is far less than that producing an observable decrease in charge density. The importance of the localization of this cation at only certain parts of the membrane in the maintenance of communication between various associated cells has been stressed by Loewenstein (21). It is apparent that the entire problem of the relationship between adhesiveness and calcium affinity in both normal and malignant systems is in need of further clarification.

The general pattern emerging from these studies is that cells of diverse species, tissues, states of differentiation, and function have surprisingly similar patterns of electrokinetic behavior. This resemblance may be a reflection of those cell surface components that are common to all cells, whatever their origin or function. It would seem that the biological distinctivenesss of the various cell types is not expressed in those charged groups that are measured at the electrophoretic plane of shear. The sialoglycoproteins, known to contribute significantly to the peripheral charge, have been found on the surface of all cells examined so far (8). Nevertheless attempts to correlate the presence of these substances with various specialized cell functions such as patterns of growth or malignancy have generally not been successful (19). The trend of recent investigations has been to implicate this group in the basic physiological processes of the cell such as active transport rather than in specific cellular functions (5, 12, 20, 22).

Clearly, much remains to be done towards the elucidation of those properties of the cell surface responsible for cell adhesion and its variation in malignancy. Further advances in this field may be dependent on the development of methods for studying the charged and uncharged components at specific areas of the cell surface.

ACKNOWLEDGMENTS

We extend our gratitude to Mr. Z. Ben-Sasson for his helpful suggestions and cooperation in various phases of the experiments and to Mrs. Y. Ingster for her excellent technical assistance. We also thank Dr. A. M. Kohn and Dr. M. Ionconesco for their interest and advice.

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