GANGLIOSIDE PATTERNS AND PHENOTYPIC CHARACTERISTICS IN A NORMAL VARIANT AND A TRANSFORMED BACK VARIANT OF A SIMIAN VIRUS 40-INDUCED HAMSTER TUMOR CELL LINE

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ABSTRACT

Ganglioside patterns of a cloned Simian virus 40- (SV40) induced hamster tumor cell (Cl_2TSV_5-S), its normal variant (Cl_2TSV_5-R) which are Cl_2TSV_5-S gradually adapted to grow in the presence of 2 μg/ml actinomycin D and exhibit certain normal phenotypic characteristics, and its back variant (Cl_2TSV_5-RR), which are Cl_2TSV_5-R cells grown in the absence of actinomycin D for more than 60 passages and which exhibit greater phenotypic similarity to Cl_2TSV_5-S cells, have been analyzed. All three cell lines contain *N*(acetylneuraminyl) galactosylglycosyl ceramide (hematoside, GM_3), *N*-acetylgalactosaminyl (*N*-acetylneuraminyl) galactosylglucosyl ceramide (GM_2), and a higher ganglioside tentatively identified as disialohematoside. However, Cl_2TSV_5-R have more GM_2 than Cl_2TSV_5-S whereas Cl_2TSV_5-RR contain an intermediate amount of GM_2. The amount of GM_2 is correlative with the activity of UDP-*N*-acetylgalactosamine: hematoside *N*-acetylgalactosaminyl transferase in the extract of the three cell lines and with their agglutination by wheat germ agglutinin.

Alterations in the pattern of gangliosides of cultured cells after transformation by oncogenic DNA viruses has been shown by a number of investigators (1–5). This is also true for chemically induced hepatomas which exhibit a simplified pattern of gangliosides compared to normal liver (6–8). In other studies, it has been reported that the ganglioside pattern changes when cultured normal cells become confluent (9, 10). These studies led Hakomori et al. (10) to propose that addition of glycosyl groups such as α-galactosyl, α-*N*-acetylgalactosaminyl, and neuraminyl to precursor glycolipids and gangliosides of growing cells renders the cells contact sensitive. Addition of these groups is considered to be due to an augmentation in the activities of nucleoside diphosphate sugars:glycolipid glycosyl transferases on cell-to-cell contact. Malignant cells are presumed to lack this response and hence exhibit loss of contact inhibition of growth and movement.

Using mouse cell lines, Cumar et al. (11) showed that simian virus 40 (SV40) and polyoma-transformed cells had a reduced content of higher ganglioside homologues and a lower activity of *N*-acetylgalactosaminyl transferase (UDP-*N*-acetylgalactosaminyl:hematoside *N*-acetylgalactos-

aminyl transferase) when compared to counterpart normal cells. More recently Mora et al. (12) have demonstrated that flat revertants of virally transformed cells that exhibit normal phenotypic growth properties in culture show a trend of reversion in ganglioside pattern and in the activity of the above enzyme. However, the ganglioside patterns of flat polyoma and SV40 revertants differ from one another and are not identical to that of normal mouse cells, thus leaving open the possibility of clonal selection during the isolation of flat revertants. Yogeeswaran et al. (13) have shown that different clones of virally transformed 3T3 cells exhibit different ganglioside patterns. In order to overcome this objection, it would be necessary to show that: (*a*) the variant cell contains the same gangliosides as the original transformed cells from which the variant was derived; (*b*) the alteration in the amount of a ganglioside in the variant agrees with the augmentation of the activity of the appropriate enzyme; and, most important of all, (*c*) demonstration of back reversion, so that the back variant cell shows the phenotypic behavior, ganglioside concentrations, and enzyme activity similar to that of the original transformed cells from which the variant was derived.

In this paper we wish to report results on the ganglioside patterns, *N*-acetylgalactosaminyl transferase activities, and phenotypic properties of three hamster cell lines which conform to the above requirements. Our data suggest that there is a relationship between alteration in the activity of an enzyme leading to an altered pattern of gangliosides and these modifications are expressed by the phenotypic properties of transformed cells.

MATERIALS AND METHODS

Cell Lines

CL$_2$TSV$_5$-S: This is a cell line which was established from a clone of a tumor obtained in a hamster after subcutaneous injection of SV40 virus. The isolation and properties of this cell line have been described by Tournier et al. (14).

CL$_2$TSV$_5$-R: This cell subline was derived from Cl$_2$TSV$_5$-S. It is resistant to growth inhibition in the presence of 2 μg/ml actinomycin D (15). Actinomycin D resistance was conferred on the cells by growing them in gradually increasing concentrations (0.01–2 μg/ml) through more than 80 passages (15).

CL$_2$TSV$_5$-RR: These are Cl$_2$TSV$_5$-R cells which have been grown through more than 60 passages in the absence of actinomycin D and are gradually recovering actinomycin D sensitivity.

BABY HAMSTER KIDNEY (BHK) CELLS: These were normal hamster cells which have been passaged several times and now grow to high cell densities. They were originally obtained from Dr. Tournier's laboratory, Villejuif, France.

Cells were grown in a constant temperature incubator at 37°C. Eagle's minimum essential medium (MEM) was supplemented with a fourfold concentration of amino acids and vitamins (Grand Island Biological Co., Grand Island, N. Y.) and 10% fetal calf serum (Flow Laboratories, Inc., Rockville, Md.). For biochemical studies, cells were grown in a number of plastic bottles. After the cells reached confluency, they were scraped with a rubber policeman and washed with phosphate buffered saline (PBS) in the cold (0°–5°C). Approximately 0.3–0.4 ml of packed cells was recovered from 20 bottles. The packed cells were frozen and kept at −20°C. In some experiments Cl$_2$TSV$_5$-R cells were recovered before they reached confluency in order to compare sialic acid content in growing and confluent cells.

Growth Characteristics of Cell Cultures

To study the growth and the saturation densities of the three cell lines used in these experiments, the cells were detached by trypsin and counted in a hemacytometer (Burker, Karl Hecht, Bayern, West Germany) 24 h after seeding and every 24 h thereafter until they reached confluency.

Viability of Cells

The viability of cells grown in the presence or absence of actinomycin D was established by two different methods. One of these was the dye exclusion test (16), and the other, the plating efficiency of cells (17). The plating efficiency of the cells was determined by plating 100, 200, and 500 cells per Petri dish and 2 wk later the cells were fixed in alcohol and stained with Unna blue. The number of colonies, their size, and the number of cells per colony were counted.

Growth in Agar

To study the colony-forming ability in agar of the three cell lines used in these experiments, 100, 200, 500, and 1,000 cells of each cell line were grown in MEM containing 0.3% of Bacto-agar (Difco Laboratories, Detroit, Mich.). The technique is that described by Montagnier and MacPherson (43).

Agglutination Assay

Cultured cells were removed from bottles after washing with a 0.2% solution of disodium EDTA in Ca^{++}-, Mg^{++}-free PBS. The suspended cells were

again washed three times with PBS and diluted with PBS to give 10^6 cells/ml. The agglutination tests were performed in small Petri dishes at 22°C. Cell suspensions (0.3 ml) were added to successive dilutions of 0.3 ml of wheat germ agglutinin (a gift from Dr. J. Weber, Department of Microbiology, University of Sherbrooke, Quebec). Agglutination was measured after 30 min incubation by directly observing the aggregates under a microscope.

Assay of Tumorigenicity

The cells were trypsinized and washed three times in PBS. They were resuspended in tissue culture medium containing no serum. Cell suspensions containing different numbers of cells were inoculated subcutaneously into suckling syrian hamsters. The development of the tumors was observed by the the appearance of palpable tumors during a 3 mo period. The tumor dose 50% (TD50) was calculated according to Reed and Muench (18).

Isolation of Gangliosides

Gangliosides were isolated according to Yogeeswaran et al. (19). The extraction procedure of Suzuki (20) was employed.

Separation, Identification, and Quantitative Estimation of Gangliosides

Aliquots of ganglioside fractions containing approximately 10–20 μg sialic acid (determined according to Miettinen and Takki-Luukkainen [21]) and obtained from an equal amount of tissue (on the basis of milligrams of protein) were spotted on thin-layer plates (silica gel G, Merck, Sharp & Dohme, Montreal) and the plates developed in chloroform-methanol-water (60:35:8, vol/vol/vol). The migration of various gangliosides was detected by resorcinol reagent (22). Human brain ganglioside mixture was used as a reference. The identification of the three major gangliosides in hamster cells was further confirmed by isolating a ganglioside mixture from Cl_2TSV_5-R cells (2 ml packed cells), separating them by thin-layer chromatography, cutting the ends of the plate with a diamond pencil, and spraying them with resorcinol reagent to locate the ganglioside bands. Each ganglioside band was removed from the unsprayed part of the plate by scraping the silica gel and it was eluted by treatment of the gel powder with $CHCl_3$-MeOH-H_2O (60:30:4.5). Sialic acid in each ganglioside was estimated according to Aminoff (23) after hydrolysis of the ganglioside in 0.1 N H_2SO_4 for 1 h at 80°C. After sialic acid determination in an aliquot, the concentration of H_2SO_4 in the hydrolysate was raised to 2 N, and the ganglioside was further hydrolyzed at 100°C for 2 h. Hexosamine was determined after neutralization with $NaHCO_3$, using the methods described by Strominger et al. (24) with galactosamine as standard. Neutral sugars were determined by anthrone reagent (25) after treatment of the hydrolysate with Amberlite MB_3.

Gangliosides were estimated after their separation on thin-layer plates as described by MacMillan and Wherret (26). *N*-acetylneuraminic acid was used as a standard (Sigma Chemical Co., St. Louis, Mo.).

Assay of UDP-N-Acetylgalactosamine: Hematoside N-Acetylgalactosaminyl-Transferase

For the assay of this enzyme, hematoside acceptor was isolated from dog erythrocytes as described by Yamakawa et al. (27) and Cumar et al. (11). Dog erythrocyte ganglioside has been shown to be *N*-acetylneuraminylgalactosylglucosyl ceramide. The same hematoside was also isolated as a major ganglioside from BHK cells. BHK cells contain only traces of higher ganglioside homologues.

UDP-*N*-acetylgalactosamine was used as a glycosyl donor. [^{14}C]UDP-*N*-acetylgalactosamine (sp act 40 mCi/mmol) was obtained from New England Nuclear Corp., Boston, Mass. Unlabeled UDP-*N*-acetylgalactosamine was a gift from Dr. H. Schachter, University of Toronto.

Packed frozen cells were defrosted, suspended in 5 vol of distilled water, and homogenized in the cold in a Potter-Elvehjem homogenizer. Homogenization was terminated when all cells were broken (30–60 s) as monitored by phase-contrast microscopy. Enough 1 M solution of NaCl was added so that the final concentration of NaCl in the homogenate was 0.15 M. The homogenate served as a source of the enzyme.

The procedure employed for the assay of the enzyme was essentially the same as described by Cumar et al. (11). The reaction mixture contained: dog erythrocyte hematoside, 0.1 μmol; Triton X-100 (12.5 mg/ml), 40 μl; Tris-HCl buffer (pH, 7.4), 50 μmol; $MnCl_2$, 2.5 μmol; [1-^{14}C]UDP-*N*-acetylgalactosamine (sp act 40 mCi/mmol), 2.4 nmol; and homogenate, 50 μl (approximately 100–300 μg protein). The control contained no hematoside. The total volume of the reaction mixture was 0.15 ml. The reaction mixture was incubated for 3 h at 37°C. It was inactivated by the addition of 2.4 ml of $CHCl_2$-MeOH (2:1) and the mixture left at 5°C overnight. The suspension was filtered through Whatman no. 50 filter paper and the retained protein washed twice with 2 ml of $CHCl_3$-MeOH-water (60:30:4.5). The filtrate was passed through a column (0.1 × 7 cm) packed with superfine Sephadex G-25 (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) and equilibrated with

$CHCl_3$-MeOH-H_2O (60:30:4.5). After all the filtrate had passed, the column was washed with 4 ml of the same solvent. The eluate was evaporated to dryness and the radioactivity was determined by liquid scintillation spectrometry.

To visualize the formation of *N*-acetylgalactosaminyl(*N*-acetylneuraminyl) galactosylglycosyl ceramide from *N*-acetylhematoside and UDP-*N*-acetylgalactosamine on thin-layer plate, the above reaction mixture was modified to contain 0.1 μmol hematoside from BHK cells (instead of dog erythocyte hematoside) and 0.1 μmol unlabeled UDP-*N*-acetylgalactosamine (instead of labeled UDP-*N*-acetylgalactosamine). The reaction mixture was incubated for 3 h at 37°C, inactivated by 2.4 ml of $CHCl_3$-MeOH (2:1), and filtered after overnight keeping in the cold. The precipitate was washed with 2 ml of $CHCl_3$-MeOH-H_2O (60:30:4.5). The filtrate was dried and chromatographed on a thin-layer plate. The gangliosides were detected by spraying resorcinol spray reagent.

RESULTS

Characteristics of the Cell Lines

Simard and Cassingena (15) have described growth characteristics of Cl_2TSV_5-S and Cl_2TSV_5-R cells. The actinomycin D-resistant cells grow much more slowly than actinomycin D-sensitive cells. We have confirmed this finding. Resistant cells grown in the absence of actinomycin D have a higher growth rate than the cells grown in the presence of the antibiotic (not shown). The saturation densities of Cl_2TSV_5-S, Cl_2TSV_5-R, and Cl_2TSV_5-RR are shown in Table I.

Both the actinomycin D-sensitive and -resistant cells produced tumors when injected into hamsters (Table I). However, the number of cells required for tumor production by actinomycin D-resistant cells is 25 times greater than is required for the production of tumors by actinomycin D-sensitive cells. It should be noted that actinomycin D is not administered to the animals after the injection of actinomycin D-resistant cells, thus leaving open the possibility for back reversion. The tumors produced could be sensitive to toxicity of actinomycin D, but this has not been tested.

The agglutination behavior of these cell lines with wheat germ agglutin shows that actinomycin D-sensitive cells are agglutinated by a much lower concentration of the agglutinin than is required for the agglutination of actinomycin D-resistant cells (Table I). Resistant cells which have been grown for 60 passages in the absence of actinomycin D require an intermediate concentration of agglutinin for their agglutination. Agglutination of these cells is reversed by *N*-acetylhexosamine. Further, it is to be noted that Cl_2TSV_5-R cells

TABLE I

Biological Characteristics of the Three Cell Lines

Cell line	Saturation density (cell/cm² × 10^{-5})*	Tumorigenicity‡	Concentration of wheat germ agglutinin for 100% agglutination (μg/ml)§	Plating efficiency‖		Colony efficiency in agar
				In the absence of actinomycin D	In the presence of actinomycin D (2 μg/ml)	
				%	%	%
Cl_2TSV_5-S	1.17	10^5	50	54	0	12.7
Cl_2TSV_5-R	0.47	2.5 × 10^6	370	30	31	0
Cl_2TSV_5-RR	1.18	ND	100	48	4	13.1

* Calculated at the end of the exponential growth phase after seeding 7 × 10^5 cells per Petri dish. The results are an average for 10 Petri dishes.

‡ TD50 calculated 3 mo after inoculation of cells into suckling hamsters (eight animals per dilution).

§ 100% agglutination means that all the cells are clumped after 30 min in contact with wheat germ agglutinin and clumps are formed by at least 25 cells.

‖ 100, 200, and 500 cells of each cell line were seeded per Petri dish. Colonies were counted 2 wk after seeding. The results are an average for ten Petri dishes.

¶ The technique employed was that described by Montagnier and MacPherson (43). 100 and 500 cells of each cell line were seeded. Colonies were counted 3 wk after seeding. The results are an average for ten Petri dishes.

ND, not determined.

grown in the absence of actinomycin D for 60 passages resulting in the development of Cl_2TSV_5-RR cells have gained partially their sensitivity to actinomycin D as well as their ability to grow in agar (Table I). The resistant cells (Cl_2TSV_5-R) neither grow in agar in the presence or absence of actinomycin D nor do they allow actinomycin D entry into the cell resulting in their death. In these respects the behavior of Cl_2TSV_5-RR is intermediate between the behavior of Cl_2TSV_5-R and Cl_2TSV_5-S cells.

The plating efficiency of Cl_2TSV_5-R is the same in the presence or absence of actinomycin D, whereas that of Cl_2TSV_5-S is 0 in the presence of actinomycin D and 54% in the absence of actinomycin D. The back variant (Cl_2TSV_5-RR) cells exhibit 48% plating efficiency in the absence and 4% in the presence of actinomycin D. The fact that plating efficiency of Cl_2TSV_5-R cells is the same in the presence or absence of actinomycin D is indicative that slow growth rate of these cells is not due to the derivation of actinomycin D-sensitive cells from actinomycin D-resistant cells. Furthermore, it is apparent that Cl_2TSV_5-RR has not completely reverted to the character of actinomycin D-sensitive cells, since it still has 4% of the cells which are viable in the presence of actinomycin D.

Morphologically, the three kinds of cells are similar to one another. However, when the cells occur in groups they are spindle shaped whereas single cells are round in shape. Since actinomycin D-resistant cells are sparsely populated on the culture dish, apparently there are more round-shaped cells and a few groups of spindle shaped in this cell line as compared to actinomycin D-sensitive cells and those grown in the absence of actinomycin D, which have mostly groups of large numbers of spindle-shaped cells and a few round-shaped single cells.

The data described in Table I are consistent with similar observations made in a paper by Wicker et al. (28), with the actinomycin D-sensitive and -resistant hamster tumor cells, which appeared after our work had been submitted for publication.

Sialic Acid and Galactosamine Contents of the Cell Lines

It has been observed by a number of investigators that the sialic acid content of cells decreases after transformation by oncogenic DNA viruses (29–31). In Table II are shown the amounts of sialic acid present in lipid-bound and protein-bound fractions in the four cell lines used in the present investigation. The amount of ganglioside-bound and protein-bound hexosamine is also given. Since the only ganglioside containing hexosamine present in these cell lines is GM_2, ganglioside-bound hexosamine can be estimated from the data presented for GM_2 content in Table III. It can be seen that the amounts of sialic acid present in resistant and sensitive cells is parallel to that reported for normal and DNA oncogenic virus-transformed cells (32). Resistant cells grown in the absence of actinomycin D give an intermediate value between the above two cell lines, indicating that they are reverting back to the transformed cell behavior. BHK cells, on the other hand, contain sialic acid and hexosamine whose content is similar to that of sensitive cells, and apparently their phenotypic behavior is similar to that of spontaneously transformed cells.

TABLE II

Total Sialic Acid and Hexosamine Content of the Cell Lines

Type of cells	nmoles sialic acid/mg protein		nmoles hexosamine*/mg protein	
	Lipid bound	Protein bound	Ganglioside bound	Protein bound
BHK	4.0	31.4	<0.5	19.0
Cl_2TSV_5-S	3.88‡	37.2	0.6	18.6
Cl_2TSV_5-R	5.82‡	60.0	2.1	29.0
Cl_2TSV_5-RR	4.5	45.0	0.8	20.5

The values are an average of two separate experiments except those marked with ‡ where an average of three experiments is given. Details of the determination procedure are given under Materials and Methods

* Calculated with galactosamine as standard.

TABLE III

Distribution of GM_3 and GM_2 in Hamster Cell Lines

Type of cells	GM_3	GM_2
Cl_2TSV_5-R	2.60	2.20
Cl_2TSV_5-S	1.94	0.20
Cl_2TSV_5-RR	1.74	0.86

The gangliosides were isolated as described in Materials and Methods. They were separated by thin-layer chromatography on silica gel G. The plate was sprayed lightly with resorcinol spray reagent (19). It was heated at 150°C for 5 min after covering with a glass plate. As soon as color developed, the plate was removed from the oven, cooled, and the colored zones of GM_3 and GM_2 were scraped with a blade. The contents were transferred to tubes and the sialic acid determination was carried out with the resorcinol reagent (19). After the color reaction, the tubes were centrifuged and the optical density was determined at 580 and 470 nm. This method of estimation has been described by MacMillan and Wherret (26). The values are averages of determination in two samples of each type of cells.

Ganglioside Pattern of the Cells Lines

In Fig. 1, the ganglioside pattern of the four cell lines is shown. Three bands appear in the actinomycin D-resistant, actinomycin D-sensitive, and -resistant cells grown in the absence of actinomycin D, whereas BHK cells contain only one double band of a ganglioside. The neuraminic acid-hexosamine-hexose ratios of the three gangliosides isolated from actinomycin D-resistant cells after thin-layer chromatography were as follows: top band (GM_3, neuraminyl-galactosylglycosyl ceramide), 1:0:1.8; middle band (GM_2, *N*-acetylgalactosaminyl neuraminylgalactosylglycosyl ceramide), .0:0.85:1.7; bottom band (DSH, disialohematoside), 1:0:0.78. However, the identification of bottom band as DSH has to be regarded as only tentative since the amount of this ganglioside isolated was relatively small to make the identification accurate. These results are in agreement with those of Hakomori (9) who also demonstrated the presence of these three gangliosides in his hamster cell lines.

One of the major differences between actinomycin D-resistant and -sensitive cell lines and actinomycin D-resistant cells grown in the absence of actinomycin D is that the amounts of GM_2 are different (Table III) although they have exactly the same pattern (Fig. 1). On the other hand, BHK cells demonstrate a different pattern insofar as they show only GM_3 as the major ganglioside (Fig. 1). Actinomycin D-resistant cells contain a higher amount of GM_2 than actinomycin D-sensitive cells; but actinomycin D-resistant cells grown in the absence of actinomycin D show an intermediate amount for GM_2 (Table III).

Table IV shows the data on the activity of *N*-acetylgalactosamine: hematoside *N*-acetylgalactosaminyl transferase activity in actinomycin D-resistant and -sensitive cells and actinomycin D-resistant cells grown in the absence of actinomycin D. Actinomycin D-resistant cells have a much higher activity (threefold) than actinomycin D-sensitive cells, whereas the actinomycin D-resistant cells grown in the absence of actinomycin D show an intermediate level of activity between the two cell lines. In Fig. 2, it is clearly seen that the activity in one of the homogenates of actinomycin D-resistant cells is high enough so that the appearance of GM_2 can be observed on thin-layer chromatography of the gangliosides, whereas in one preparation of actinomycin D-sensitive cells it is so low that the GM_2 band does not appear in the chromatogram under identical conditions of incubation and isolation of the gangliosides from the two reaction mixtures.

DISCUSSION

It is well known that variant cells can be selected (33, 34) from a population of transformed cells that have lost transformed properties, but still contain viral genome. Variants can also be selected which exhibit certain "normal" and some "transformed" characteristics. For example, variants have been isolated that are tumorigenic but have growth properties of normal cells (35, 36), or those that have a saturation density characteristic of normal cells but a serum requirement of transformed cells (37). Although actinomycin D-resistant cells (Cl_2TSV_5-R) were originally isolated (15) to study the mechanism of the development of drug resistance, closer examination of their phenotypic properties shows that adaptation of the cells to actinomycin D accompanies cell membrane alteration which gives them many characteristics of normal cells. These cells grow slowly, exhibit contact inhibition at confluency, do not grow in agar, and are agglutinated by a concentration of wheat germ agglutinin higher

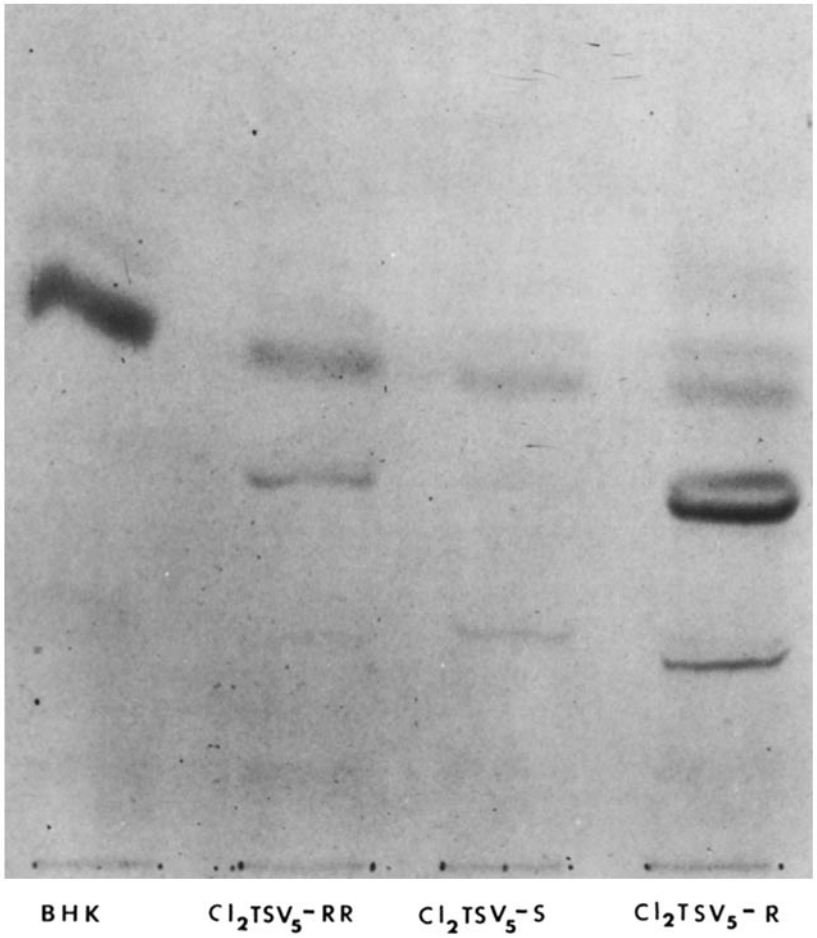

FIGURE 1 Ganglioside pattern of hamster cell lines. The figure is a photograph of a thin-layer chromatogram of ganglioside fractions isolated from the three cell lines. Details of isolation and chromatography are provided in Materials and Methods. Developing solvent was $CHCl_3$-MeOH-NH_4OH, 7N-H_2O (60:35:1:7, vol/vol/vol/vol) and the chromatogram was run in the ascending direction. The migration of the gangliosides was detected by spraying with resorcinol reagent (18). Slight upward migration of the gangliosides on the left-hand side of the photograph is due to slightly higher movement of the solvent front on that side. In each case the amount of ganglioside applied was equivalent to that obtained from about 30 mg of cell protein. In other experiments, ganglioside from hamster cells run along side with human brain ganglioside mixture showed that GM_3 and GM_2 bands of hamster cells correspond to GM_3 and GM_2 bands in human brain ganglioside mixture. The bottom band is tentatively identified as disialohematoside (see Results).

than that required for the agglutination of actinomycin D-sensitive parent cells (Cl_2TSV_5-S). They also show a higher content of glycolipid and glycoprotein-bound sialic acid compared to Cl_2TSV_5-S cells, in a manner similar to that reported for normal (3T3) or embryonic cells and counterpart DNA oncogenic virus-transformed cells (32). Cl_2TSV_5-R cells can, therefore, be described as a normal variant of transformed cells. The only transformed character they exhibit is the presence of viral genome which expresses T antigen; and that these cells develop tumor when they are injected into recipient animals at a cell concentration 25 times higher than is required for tumor development by actinomycin D-sensitive cells. However, it has been observed that tumor development by cultured cells is not the exclusive property of transformed cells alone

TABLE IV

UDP-N-Acetylgalactosamine: Hematoside N-Acetylgalactosaminyl Transferase Activity in Hamster Cell Lines

Exp. no.	Cell line	Enzyme activity (*N*-acetylgalactosamine incorporated)
		cpm/mg protein
1	Cl_2TSV_5-R	40,485
	Cl_2TSV_5-S	11,421
	Cl_2TSV_5-RR	19,872*
2	Cl_2TSV_5-R	56,083
	Cl_2TSV_5-S	8,838
	Cl_2TSV_5-RR	9,249*
3	Cl_2TSV_5-R	31,540‡
	Cl_2TSV_5-S	9,924‡

The composition of the reaction mixture and the measurement of incorporation of [^{14}C]UDP-N-acetylgalactosamine with hematoside is described in Materials and Methods.

* These differences are presumably due to the fact that Cl_2TSV_5-RR cells taken in exp. 2 were at a 62 passage in the absence of actinomycin D, compared to the same cells in passage 30 in exp. 1.

‡ Dog erythrocyte hematoside used as acceptor. The values are corrected for blanks obtained in the absence of added hematoside.

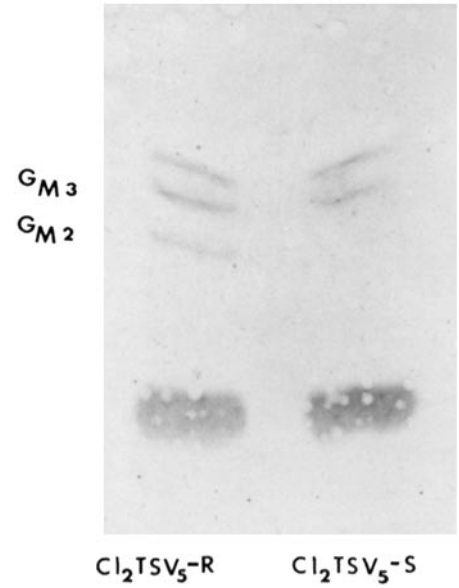

FIGURE 2 Photograph of a thin-layer chromatogram of gangliosides isolated from reaction mixtures containing Cl_2TSV_5-S (right) and Cl_2TSV_5-R (left) cells incubated for 3 h in the presence of UDP-*N*-acetylgalactosamine and BHK hematoside. The composition of the reaction mixture, the isolation of gangliosides, and conditions for the development of the chromatogram are given in Materials and Methods and in Fig. 1. The amount of protein in the extracts was 200 μg in each case.

since cultured normal cells are also tumorigenic when injected into recipients under appropriate conditions (3, 38). On the other hand, it should be noted that development of resistance to actinomycin D is not an essential requirement for a transformed cell to exhibit normal characteristics since normal cells are not resistant to actinomycin D; but when transformed cells do become resistant to actinomycin D, the adaptation results not only in the reversion of certain properties towards normal but also in more extensive alteration of the cell membrane so that the cells disallow the entry of actinomycin D as well as other antibiotics (39). The presence of actinomycin D in the medium also appears to disallow genetic drift of Cl_2TSV_5-R that is otherwise exhibited as spontaneous transformation in most normal embryonic cells when they are passaged over many generations. This genetic drift becomes explicit when cells (Cl_2TSV_5-R) are grown in the absence of actinomycin D over many generations, as seen by the phenotypic behavior of Cl_2TSV_5-RR cells.

In this investigation we have observed that at least one enzyme activity increases in response to previous gradual exposure to actinomycin D, and an alteration in the activity of this enzyme closely corresponds to alterations in the ganglioside amounts. Since gangliosides are known to be associated with cell membranes (5, 40, 41), it is possible that they may have a functional role in the transport of actinomycin D. However, it cannot be ruled out that cell surface glycoproteins are not involved in actinomycin D entry in the resistant cells. In fact, increase in protein-bound sialic acid and galactosamine would suggest that they could also be a determining factor for actinomycin D entry.

Using Pollack's flat revertants of SV40- and polyoma-transformed 3T3 cells, Mora et al. (12) have made observations similar to those described in this paper. However, flat revertants have been obtained by only two treatments of fluorodeoxyuridine, thus leaving open the possibility of clonal selection. Indeed, the ganglioside pattern of flat revertants is significantly different from that of

parent 3T3 cells to qualify them as clones which are phenotypically similar to the normal 3T3 cells, although they carry viral information in the form of T antigen. The discovery of the flat revertant in fact demonstrates the ability in certain cells to suppress viral information necessary for the expression of the transformed behavior.

In the case of actinomycin D-sensitive cells, clonal selection is also apparent, since their ganglioside pattern is more complex than that of parent BHK cells. Indeed, actinomycin D-sensitive cells are a clone derived from a SV40-induced hamster tumor. Therefore, we can limit our comparisons only to actinomycin D-sensitive and -resistant cells and actinomycin D-resistant cells grown in the absence of actinomycin D in which an identical pattern of gangliosides exists but only the amounts of these gangliosides differ. From the estimation of the GM_3 and GM_2 and the determination of *N*-acetylgalactosamine: hematoside *N*-acetylgalactosaminyl transferase activity, it is clear that the normal phenotypic behavior of cells is associated with an increase of bound galactosamine on the cell surface.

The phenotypically normal state of actinomycin D-resistant cells seems to be maintained by growth of these cells in the presence of actinomycin D. This behavior of actinomycin D resembles that of dibutyryl cyclic AMP (42) which maintains normal phenotypic behavior of transformed cells as long as it is present in the medium, and its exclusion from the medium causes the cells to revert back to their transformed state characteristics. This back reversion process is very much slower in the case of actinomycin D-resistant cells, indicating that certain secondary changes occur during the development of actinomycin D resistance which have to be diluted by successive passages in the absence of actinomycin D before back reversion can occur.

The present comparative work carried out with suitable cell lines clearly suggests that functioning of certain parts of the viral genome (excluding T antigen) is necessary for the maintenance of altered growth properties of virally transformed cells and is subject to modulation (expression or repression) and it parallels the expression or repression of a biochemical event, that is, the activity of an enzyme involved in cell ganglioside biosynthesis.

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