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
(Cl8TSV5-S), its normal variant (Cl8TSV5-R) which are Cl8TSV5-S gradually adapted
to grow in the presence of 2 µg/ml actinomycin D and exhibit certain normal phenotypic
characteristics, and its back variant (Cl8TSV5-RR), which are Cl8TSV5-R cells grown in
the absence of actinomycin D for more than 60 passages and which exhibit greater phenoty-
pic similarity to Cl8TSV5-S cells, have been analyzed. All three cell lines contain N(ace-
tetylneuraminyl) galactosylglycosyl ceramide (hematoside, GM3), N-acetylgalactosaminyl
(N-acetylneuraminyl) galactosylglucosyl ceramide (GM2), and a higher ganglioside tenta-
tively identified as disialohematoside. However, Cl8TSV5-R have more GM2 than Cl8TSV5-S
whereas Cl8TSV5-RR contain an intermediate amount of GM2. The amount of GM2
is correlative with the activity of UDP-N-acetylgalactosamine: hematoside N-acetyl-
galactosaminyl transferase in the extract of the three cell lines and with their agglutination
by wheat germ agglutinin.

Alterations in the pattern of gangliosides of cul-
tured cells after transformation by oncogenic DNA
viruses has been shown by a number of investi-
gators (1-5). This is also true for chemically in-
duced hepatomas which exhibit a simplified pat-
tern 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 aug-
mentation in the activities of nucleoside diphos-
phate sugars:glycolipid glycosyltransferases on
cell-to-cell contact. Malignant cells are presumed
to lack this response and hence exhibit loss of con-
tact inhibition of growth and movement.

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

**B A B Y H A M S T E R K I D N E Y (B H K) C E L L S:**

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 Cl2TSV5-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 10⁶ cells/ml. The aggregation
was 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 appearance of palpable tumors during a 3 mo
period. The tumor dose 50% (TD50) was calculated
according to Reed and Muench (19).

Isolation of Gangliosides
Gangliosides were isolated according to Yoges-
waran 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 ac-
cording 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 migra-
tion of various ganglioside was detected by res-
corin 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 C12TSV5-R cells (2 ml packed cells), separa-
ting 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 CHCl₃-MeOH-H₂O (60: 30:4.5).
Sialic acid in each ganglioside was estimated
according to Aminoff (23) after hydrolysis of the
ganglioside in 0.1 N H₂SO₄ for 1 h at 80°C. After
sialic acid determination in an aliquot, the concen-
tration of H₂SO₄ 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 NaHCO₃, using the methods
described by Strominger et al. (24) with galactos-
amine as standard. Neutral sugars were determined
by anthrone reagent (25) after treatment of the
hydrolysate with Amberlite MB₅.
Gangliosides were estimated after their separation
on thin-layer plates as described by MacMillan and
Wherret (26). N-acetyleneuraminic 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-
acetyleneuraminylgalactosylglucosyl 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. [14C]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. Homogeniza-
tion 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; MnCl₂, 2.5 µmol; [1-14C]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 mix-
ture was 0.15 ml. The reaction mixture was incu-
bated for 3 h at 37°C. It was inactivated by the
addition of 2.4 ml of CHCl₃-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
CHCl₃-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 Chemi-
cals Inc., Uppsala, Sweden) and equilibrated with

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CHCl₃-MeOH-H₂O (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 erythrocyte 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 CHCl₃-MeOH (2:1), and filtered after overnight keeping in the cold. The precipitate was washed with 2 ml of CHCl₃-MeOH-H₂O (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 Cl₂TSV₅-S and Cl₂TSV₅-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 Cl₂TSV₅-S, Cl₂TSV₅-R, and Cl₂TSV₅-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 agglutinin 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 Cl₂TSV₅-R cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Saturation density (cell/cm² × 10⁴)</th>
<th>Tumorigenicity</th>
<th>Concentration of wheat germ agglutinin for 100% agglutination (µg/ml)</th>
<th>Plating efficiency in the absence of actinomycin D (%)</th>
<th>In the presence of actinomycin D (2 µg/ml) (%)</th>
<th>Colony efficiency in agar (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl₂TSV₅-S</td>
<td>1.17</td>
<td>10⁵</td>
<td>50</td>
<td>54</td>
<td>0</td>
<td>12.7</td>
</tr>
<tr>
<td>Cl₂TSV₅-R</td>
<td>0.47</td>
<td>2.5 × 10⁶</td>
<td>370</td>
<td>30</td>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td>Cl₂TSV₅-RR</td>
<td>1.18</td>
<td>ND</td>
<td>100</td>
<td>48</td>
<td>4</td>
<td>13.1</td>
</tr>
</tbody>
</table>

* Calculated at the end of the exponential growth phase after seeding 7 × 10⁴ 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 C12TSV5-RR cells have gained partially their sensitivity to actinomycin D as well as their ability to grow in agar (Table I). The resistant cells (C12TSV5-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 C12TSV5-RR is intermediate between the behavior of C12TSV5-R and C12TSV5-S cells.

The plating efficiency of C12TSV5-R is the same in the presence or absence of actinomycin D, whereas that of C12TSV5-S is 0 in the presence of actinomycin D and 54% in the absence of actinomycin D. The back variant (C12TSV5-RR) cells exhibit 49% plating efficiency in the absence and 4% in the presence of actinomycin D. The fact that plating efficiency of C12TSV5-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 C12TSV5-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 GM2, ganglioside-bound hexosamine can be estimated from the data presented for GM2 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>
<thead>
<tr>
<th>Type of cells</th>
<th>nmoles sialic acid/mg protein</th>
<th>nmoles hexosamine*/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lipid bound</td>
<td>Protein bound</td>
</tr>
<tr>
<td>BHK</td>
<td>4.0</td>
<td>31.4</td>
</tr>
<tr>
<td>C12TSV5-S</td>
<td>3.88</td>
<td>37.2</td>
</tr>
<tr>
<td>C12TSV5-R</td>
<td>5.82</td>
<td>60.0</td>
</tr>
<tr>
<td>C12TSV5-RR</td>
<td>4.5</td>
<td>45.0</td>
</tr>
</tbody>
</table>

* Calculated with α-galactosamine as standard.

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.
TABLE III

Distribution of GM3 and GM2 in Hamster Cell Lines

<table>
<thead>
<tr>
<th>Type of cells</th>
<th>GM3</th>
<th>GM2</th>
</tr>
</thead>
<tbody>
<tr>
<td>C12TSV5-R</td>
<td>2.60</td>
<td>2.20</td>
</tr>
<tr>
<td>C12TSV5-S</td>
<td>1.94</td>
<td>0.20</td>
</tr>
<tr>
<td>C12TSV5-RR</td>
<td>1.74</td>
<td>0.86</td>
</tr>
</tbody>
</table>

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 GM3 and GM2 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 (GM3, neuraminyl-galactosylglycosyl ceramide), 1:0:1.8; middle band (GM2, 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 GM2 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 GM3 as the major ganglioside (Fig. 1). Actinomycin D-resistant cells contain a higher amount of GM2 than actinomycin D-sensitive cells; but actinomycin D-resistant cells grown in the absence of actinomycin D show an intermediate amount for GM2 (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 GM2 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 GM2 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 (C12TSV5-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 CHCl₃-MeOH-NH₄OH, 7N-H₂O (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 GM₃ and GM₂ bands of hamster cells correspond to GM₃ and GM₂ bands in human brain ganglioside mixture. The bottom band is tentatively identified as disialohematoside (see Results).
TABLE IV

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

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Cell line</th>
<th>Enzyme activity (N-acetylgalactosamine incorporated)</th>
<th>cpm/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CI2TSV5-R</td>
<td>40,485</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CI2TSV5-S</td>
<td>11,421</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CI2TSV5-RR</td>
<td>19,872*</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>CI2TSV5-R</td>
<td>56,083</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CI2TSV5-S</td>
<td>8,838</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CI2TSV5-RR</td>
<td>9,249*</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>CI2TSV5-R</td>
<td>31,540‡</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CI2TSV5-S</td>
<td>9,924‡</td>
<td></td>
</tr>
</tbody>
</table>

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

* These differences are presumably due to the fact that CI2TSV5-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.

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 CI2TSV5-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 (CI2TSV5-R) are grown in the absence of actinomycin D over many generations, as seen by the phenotypic behavior of CI2TSV5-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 GM3 and GM2 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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