CYTOLOGY, GROWTH CHARACTERISTICS AND CELLULAR ALTERATIONS FOLLOWING SV40-INDUCED TRANSFORMATION OF HUMAN FOETAL BRAIN CELLS DERIVED FROM A Gm2 GANGLIOSIDOSIS AND CONTROL

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SUMMARY

Cell cultures were derived from the cerebra of a control and a Gm2 gangliosidosis (Tay-Sachs disease (TSD)) foetus. Both cell lines were identified as astrocytic, based on the ultrastructural demonstration of glial fibres. The control culture exhibited morphological differentiation when exposed to dibutyryl cAMP, a finding which was not observed with the TSD cells. The TSD culture demonstrated the pathobiological features of the disease, which included the absence of hexosaminidase A, increased concentration of Gm2 ganglioside and the detection of membranous cytoplasmic bodies by electron microscopy.

Control and TSD cells were exposed to SV40 virus, resulting ultimately in the isolation of transformants which differed from the parental cell types in morphology, growth rate and greatly accelerated cell death. Both control and TSD cell lines have been in propagation for over 200 subcultures and the transformants were identified as astrocytic, based on the retention of characteristic glial fibres. The control culture demonstrated a chromosome range of 34-65, with a mean of 47. In contrast, the TSD transformants exhibited a range of 50-107 and a mean of 74. Transformed lines retained their parental hexosaminidase isoenzyme profiles; Hex A and B in control, and Hex B in TSD cells. Membranous cytoplasmic bodies persisted in the TSD line. Neither line could be induced to differentiate after exposure to cAMP. Additionally, they had a population doubling time of under 85 h and failed to release infectious virus particles.

Significant alterations in the total quantity and distribution profile of gangliosides were noted following viral transformation. A large increase in the percentage of Gm3 and a more modest increase in Gm2 were detected. In contrast, transformed lines were characterized by substantial reduction in the percentage of glucosamine-containing Gm2 and polysialoganglioside. Additionally, cultures exhibited a characteristic reduction in ganglioside content after transformation.

The in vitro transformation of human brain cells has resulted in the derivation of permanent astrocytic lines which are, by virtue of their rapid growth rate and long-term survival, uniquely suited and adapted to the large scale in vitro production of substantial quantities of cells required for extensive biochemical study. Significantly, those characteristics which are unique to the Gm2 gangliosidosis storage disease have been retained in a permanent model CNS cell line.

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INTRODUCTION

Although in vitro transformation of cells by Simian virus (SV40) has been widely documented, reports on the successful transformation of human cells of CNS origin have been limited. Shein (1967) described the transformation of human foetal astrocytes, but these cells did not recover from the crisis period (Girardi, Jensen & Koprowski, 1975) and none survived after 39 passages. Santoli et al. (1975) studied adult brain tissue derived from a case of progressive multifocal leukoencephalopathy transformed by SV40 virus. They reported that the cells entered crisis before 10 subcultures, and indicated that several of their lines apparently recovered from crisis and entered a period of growth and proliferation. However, no evidence was presented for a permanently transformed CNS cell line. We know of no other report in the literature describing a human cell line of CNS origin which has undergone successful long-term propagation after recovering from crisis.

In this communication, we present evidence for the transformation of cells from human foetal cerebrum and the subsequent establishment of permanent cell lines which have survived longterm propagation. One line was derived from normal foetal cerebrum (NFB-16), another, TSFB-20, was derived from a prenatally diagnosed Gm2 gangliosidosis (Tay-Sachs disease (TSD)) and exhibited an absence of hexosaminidase A (Hex A), elevated Gm2 ganglioside, and membranous cytoplasmic bodies (MCBs) which are characteristic features of this disease (Schneck, 1975).

The morphology of these cell lines is characterized by light and electron microscopy. Growth rate alterations in the various stages of cell line evolution from the pretransformed state to a stable post-crisis transformed line are described. Additionally, the cell lines are defined in terms of chromosome number, isoenzyme profile, ganglioside distribution and differentiation with cyclic AMP. The data demonstrate permanent cell lines of CNS origin possessing novel biological and biochemical characteristics, which testify to viral-induced alterations in cellular function relating to growth control, morphology, karyological make-up and ganglioside synthesis.

MATERIALS AND METHODS

Establishment and maintenance of cell cultures

The normal foetal brain cell line (NFB-16) was established as a monolayer culture from the trypsin-dispersed cerebrum of a 16-week male foetus. Tissue was collected aseptically, washed in Hanks' balanced salt solution (HBSS) and cleansed of meninges and large blood vessels. The tissue was minced into small fragments and then treated with 0.1% trypsin in HBSS at 37 °C for 10 min until it was soft enough to disperse with aspiration using a 10-ml pipette. Foetal bovine serum (FBS) (10%) was added to the dispersed cells to stop the action of trypsin, and was followed by 3 washes with HBSS. The dispersed cells were planted in T-30 tissue culture flasks (Falcon) and maintained in Eagle's minimum essential medium (MEM) supplemented with 10% FBS, non-essential amino acids, penicillin 100 units/ml, streptomycin 100 μg/ml and Fungizone 1.25 μg/ml (Amsterdam & Brooks, 1975).

The culture of Tay-Sachs disease foetal cerebrum (TSFB-20) was derived from a 20-week female foetus. Since only a small amount of tissue was available, the culture was established by explant techniques. Brain tissue was washed, minced into 1-mm fragments and planted in
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T-30 flasks containing 2 ml of medium (Amsterdam & Brooks, 1975). Culture conditions were as for NFB-16.

African Green monkey kidney cells (BSC-1) were maintained in complete MEM containing 5% FBS. They were fed on alternate days and were subcultured at 1:4 or 1:8 split ratios upon reaching confluence.

Cell transformation

SV40 virus (strain J436) was obtained from The National Institute of Allergy and Infectious Disease (Bethesda, Md.). Virus was titred by CPE endpoint determination for multiple replicate serial dilutions of virus on monolayers of BSC-1 cells in multiwell plates (6 x 4 Linbro).

The NFB-16 cells were exposed to SV40 at the seventh tissue culture passage, when the cells were in a logarithmic growth stage. In contrast, TSFB-20 were exposed during the 16th subculture when the cells were in a non-proliferative growth phase. Virus, suspended in HBSS (0.5 ml containing 10⁴ TCIDs₆₀/T-30 flask) was adsorbed to the cell monolayer for 1 h at 37 °C. Thenceforth, cultures were incubated at 37 °C, and examined daily for the presence of morphologically transformed cell foci.

Transformation-induced changes in the growth rate of all cell lines were measured as a function of population doubling time (PDT), which is the interval required for a cell inoculum to double. This interval was calculated for a cell population from the split ratio at subculture and the time needed to regenerate an equivalent cell population.

Electron microscopy

Cell specimens were harvested for electron microscopy by scraping from T-30 flasks into PBS using a Teflon spatula. The cells were then fixed in 3% glutaraldehyde for 1 h and postfixed in 1% osmium for 1 h at room temperature. Fixation was followed by dehydration and embedding in epoxy resin. Ultrathin sections were cut with a diamond knife and the sections were stained with uranyl acetate and lead hydroxide. Specimens were viewed with RCA EMU 3E and 3G electron microscopes.

Karyological analysis

Chromosome spreads were made from cells of post-crisis transformed lines. Subconfluent cultures were treated with colcemid (0.6 μg/ml) for 4–6 h. Cells were removed from the plastic flask with trypsin : EDTA, washed in phosphate-buffered saline (PBS), and then suspended in hypotonic (0.075 M) KCl for 12 min. The cells were centrifuged (50 g) and the pellet fixed by 2 changes of methanol : acetic acid (4:1). Several drops of this mixture were transferred to a cold, wet slide and ignited. Chromosome spreads were stained with Giemsa stain; metaphase spreads were photographed and chromosome counts determined for a minimum of 50 metaphase spreads.

Hexosaminidase assays

Hexosaminidase isoenzyme analysis of cultured cells was done by acrylamide gel electrophoresis as reported by Perle & Saifer (1975). Results are expressed as percentages of Hex A and B activity in the sample, and represent the average enzyme values over long-term cultivation of the cells.

Ganglioside analysis

The details of the procedures used for the extraction, identification and quantitation of gangliosides have been described in a previous publication (Hoffman, Amsterdam & Schneck, 1976). Briefly, the chloroform–methanol crude lipid extract was partitioned, and the ganglioside fraction was subjected to alkaline methanolysis, then separated from salt on a Sephadex G-25 column. TLC analysis of the gangliosides from the pre- and post-transformed cells was carried out on Silica gel G plates developed in chloroform : methanol : ammonium hydroxide, and then identified with resorcinol for densitometric quantitation. With the gangliosides
obtained from the large quantities of transformed cells, a second preparative plate for GLC analysis was run. The gangliosides from the preparative plate were subjected to methanolysis. Fatty acid methyl esters were extracted with hexane and the methyl glycosides were analysed as their trimethylsilyl derivatives on a 3% SE-30 column. Gangliosides were expressed as percentage sialic acid residues, which were determined by the method of Svennerholm (1957).

RESULTS

Cell growth pre- and post-transformation

Initially, the growth of NFB-16 was characterized by a layer of large astrocyte-like (A) cells (Fig. 1A) which adhered directly to the substratum. This monolayer was covered by a network of small spongioblast-like (S) cells which exhibited a high nuclear/cytoplasmic ratio with one to four characteristic thin cytoplasmic processes. The S cells were never observed to adhere to the substratum directly, but required a supporting layer of A cells for attachment and proliferation. The number of S cells decreased with advancing duration in culture, but some were observed until the cell line went through senescence. Treatment of NFB-16 with 0.5 mM dibutyryl cAMP (Prasad & Kumar, 1973) at the 29th subculture resulted in the extension by the cells of elongated cytoplasmic processes within 24 h (Fig. 1B, c). Population doubling time (in h) of pretransformed NFB-16, as a function of number of days in culture, is presented in Fig. 2.

After infection of NFB-16 with SV40 virus, the S cells lysed and were eliminated from the culture, leaving a residual population composed solely of A cells. Three weeks post-infection, several transformed clones exhibiting altered morphology and growth rate were noted among the surviving cells. Within 10 days, these transformed clones overgrew the original population and became the predominant cell type. Many mitotic figures were noted and final density approached $3.3 \times 10^5$ cells/cm$^2$. The rapid growth rate of NFB-16/SV40 is evidenced by a PDT ranging from 25 to 65 h (Fig. 2) compared to a PDT of about 215 h just prior to the onset of viral infection. This pre-crisis stage of transformation lasted for 4 months and was followed by a crisis period of 90 days, during which the rate of growth decreased (population doubling time ranged from 135 to 300 h). Crisis was characterized by the appearance of large numbers of necrotic cells which detached from the substratum, but which were replaced by mitosis among the surviving cells. Equilibrium was established between mitosis and cell death, resulting in almost no net increase in the cell population.

The recovery stage was manifest by a rapid increase in growth rate and a concomitant reduction in the number of necrotic cells and floating debris. During recovery the population doubling time ranged from 55 to 110 h. A stable post-crisis period followed recovery and was characterized by a further reduction in PDT to a range of 35 to 72 h. To date, the cells have been in continuous culture for over 250 passages and are characterized as small and epithelial-like with prominent nucleoli (Fig. 1D). Cells tend to adhere to each other in culture and grow to a final density of $3 \times 10^5$ cells/cm$^2$. Unlike their pretransformed counterparts these cells did not demonstrate differentiation when treated with cyclic AMP.
Fig. 1. (A) Pretransformed NFB-16 showing A (a) and S (s) cell types; ×280.
(B) Pretransformed NFB-16 cells demonstrating cytoplasmic extensions following treatment with cAMP; ×95.
(C) Pretransformed NFB-16 cells demonstrating cytoplasmic extensions following treatment with cAMP; ×280.
(D) Transformed NFB-16/SV40 cells in post-crisis; ×280.
(E) Pretransformed TSFB-20 cells; ×95.
(F) Transformed TSFB-20/SV40 cells in post-crisis; ×280.
In contrast to the control culture, the TSFB-20 line exhibited only the A cell type, which appeared similar to those observed with NFB-16. No spongioblasts were noted, and the reason for this probably relates to the method of initial establishment of the culture. It has been our experience that explant cultures usually do not show a spongioblast population, contrasting to the rich spongioblast network observed with trypsin-dispersed brain cell cultures.

Fig. 2. Population doubling times of NFB-16 and NFB-16/SV40 following transformation (T).

The A cell morphology was maintained by TSFB-20 for a period of 6 months, at which time several proliferating clones of bipolar cells showing oriented growth and autoagglutination appeared (Fig. 1E). This cell type was isolated and grown in pure culture for 6 additional passages, before the cells entered a stationary phase. When TSFB-20 was challenged with cAMP, no differentiation was noted. The population
doubling times of TSFB-20 are presented in Fig. 3. It shows the reduction of PDT to under 350 h after the appearance of the bipolar cell types prior to transformation.

Several days after SV40 infection of TSFB-20, viral cytopathogenic effect (CPE) was noted. The culture slowly but progressively degenerated for approximately 30 days and many dead or necrotic cells were evident. This signalled the beginning of the crisis period. The first 65 days of crisis were characterized by a PDT averaging 310 h and were followed by a more prolonged late crisis state which lasted for about 140 days, during which the PDT was 681 h (Fig. 3). The crisis period was followed by recovery which lasted for 140 days, during which a decrease in necrotic cells and floating debris was noted, along with a concomitant decrease in PDT to a range of 110 to 150 h. The cell line then entered, and is presently in, a stable post-crisis period.
during which the PDT ranges from 40 to 75 h. This cell line has been passaged over 200 times.

The Tay-Sachs foetal-brain transformed cells (TSFB-20/SV40) are characterized as small epithelial-like cells which are similar to NFB-16/SV40 in appearance but tend to pack less densely and show less cell overlap (Fig. 1f). The maximum cell density averaged 1.3 x 10^5/cm^2.

Post-crisis NFB-16/SV40 and TSFB-20/SV40 cultures were assayed for T-antigen by the immunofluorescence technique of Pope & Rowe (1964). Transformed cells proved to be T-antigen positive, whereas non-transformed diploid controls did not demonstrate the presence of the T-antigen. Supernatant media from the same transformed cells produced no detectable CPE when plated on BSC-1 monolayers. We conclude that no infectious SV40 virus is spontaneously released from these permanent cell lines. This finding is in agreement with other reported information concerning failure of transformed cells to release infectious Simian virus after the crisis period (Girardi et al. 1965).

Electron microscopy

Prior to SV40 transformation, ultrastructural observation of the normal cerebrum line (NFB-16) revealed the presence of large cells which contain pleomorphic nuclei and abundant glial filaments (Fig. 4A). These features typify astrocytic cells. Similarly, the TSD brain cell line (TSFB-20) exhibited pleomorphic A cells containing numerous glial filaments and occasional membranous cytoplasmic bodies (MCBs) which are characteristically observed in CNS cells of Tay-Sachs disease (Fig. 4B) (Adachi, Schneck & Volk, 1974).

After viral transformation, post-crisis cells of the normal cerebrum line (NFB-16/SV40) still displayed the pleomorphic nuclei and glial filaments (Fig. 4C). Likewise, the post-crisis transformed TSD line (TSFB-20/SV40) showed A cells containing a pale nucleus, numerous glial filaments, dilated cisternae of the endoplasmic reticulum and characteristic MCBs (Fig. 4D).

Chromosome frequency distribution

The NFB-16/SV40 and TSFB-20/SV40 cells exhibited an aneuploid chromosome complement; however, the range and distribution frequency differed greatly between the 2 cell lines. A significant difference in the mean chromosome number was also noted. Cell line NFB-16/SV40 showed a chromosome range of 34-63 with a mean chromosome number of 47. This contrasts with TSFB-20/SV40 which had a range of 50-107 while demonstrating a mean chromosome number of 74. A comparison of the chromosome frequency distribution between the two post-crisis cell lines is shown in Table 1.

Enzyme assay

The pretransformed NFB-16 cell line had an enzyme profile showing an average of 59% hexosaminidase A activity and 41% hexosaminidase B activity. After transformation, the average Hex A activity of NFB-16/SV40 was reduced to 49% and
Fig. 4. (a) Electron micrograph of pretransformed NFB-F6 cells. Arrows point to glial fibres; ×12600. (b) Electron micrograph of pretransformed TSFB-20 cells. Arrow points to glial fibres. m, membranous cytoplasmic body; ×29100. (c) Electron micrograph of transformed NFB-16/SV40 cells, post-crisis. Arrow points to glial fibres; ×12600. (d) Electron micrograph of transformed TSFB-20/SV40 cells. Arrow points to glial fibres. m, membranous cytoplasmic body; ×21000.
there was a corresponding increase in Hex B. Both non-transformed and post-crisis transformed TSD brain cell lines express 100% Hex B (no detectable Hex A) which is the typical TSD enzyme pattern.

Table 1. Chromosome frequency distribution of transformed foetal brain cells

<table>
<thead>
<tr>
<th>Chromosome range</th>
<th>NFB-16/SV40</th>
<th></th>
<th>TSFB-20/SV-40</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of cells</td>
<td>%</td>
<td>No. of cells</td>
<td>%</td>
</tr>
<tr>
<td>30–39</td>
<td>4</td>
<td>7.4%</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>40–49</td>
<td>33</td>
<td>61.1%</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>50–59</td>
<td>16</td>
<td>29.6%</td>
<td>2</td>
<td>3.7%</td>
</tr>
<tr>
<td>60–69</td>
<td>1</td>
<td>1.8%</td>
<td>11</td>
<td>20.7%</td>
</tr>
<tr>
<td>70–79</td>
<td>0</td>
<td>—</td>
<td>20</td>
<td>37.7%</td>
</tr>
<tr>
<td>80–89</td>
<td>0</td>
<td>—</td>
<td>17</td>
<td>32.0%</td>
</tr>
<tr>
<td>90–99</td>
<td>0</td>
<td>—</td>
<td>2</td>
<td>3.7%</td>
</tr>
<tr>
<td>100–109</td>
<td>0</td>
<td>—</td>
<td>1</td>
<td>1.8%</td>
</tr>
<tr>
<td>Total</td>
<td>54</td>
<td>99.9%</td>
<td>53</td>
<td>99.6%</td>
</tr>
</tbody>
</table>

Table 2. Ganglioside* distribution of cultured foetal brain cells as determined by thin-layer chromatography

<table>
<thead>
<tr>
<th>Ganglioside</th>
<th>Normal foetal cerebrum</th>
<th>TSD foetal cerebrum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NFB-16/SV40</td>
<td>TSFB-20/SV-40</td>
</tr>
<tr>
<td>Gm3</td>
<td>17.8%</td>
<td>13.9%</td>
</tr>
<tr>
<td>Gm2</td>
<td>16.3%</td>
<td>24.2%</td>
</tr>
<tr>
<td>GCGgt</td>
<td>47.4%</td>
<td>23.0%</td>
</tr>
<tr>
<td>Polysialoganglioside</td>
<td>18.5%</td>
<td>37.7%</td>
</tr>
<tr>
<td>Total sialic acid</td>
<td>0.050</td>
<td>0.078</td>
</tr>
</tbody>
</table>

* As percentage total sialic acid.
† Glucosamine-containing Gm2.
‡ As percentage dry weight.

Ganglioside analysis

The ganglioside distribution, expressed as percentage sialic acid, in pre- and post-transformed cells is presented in Table 2.

The distribution of Gm3 ganglioside was markedly altered by transformation. The pre-transformed control brain cell line NFB-16 had 17.8% Gm3. After viral transformation, the proportion of Gm3 increased to 55.1%. The TSD cell line TSFB-20 contained 13.9% of ganglioside as Gm3, which increased to 29.4% after transformation. A more modest increase in Gm2 was noted after transformation. The percentage of Gm2 in control cells increased from 16.3 to 24.2% after transformation, while the Gm2 in TSD brain cells increased from 25.4 to 28.2%. The total of Gm3 for transformed TSD cells was calculated to be almost twice that found in the transformed control cells due to the greater overall ganglioside concentration of the TSFB-20...
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transformed cell line. Significant reduction in the percentage of higher polysialo-ganglioside was noted after transformation with both control and TSD cells. A transformation-dependent reduction was also noted in the percentage of a novel glucosamine-containing ganglioside in both cell lines. The total sialic acid expressed as percentage of dry weight showed a substantial reduction in control and TSD cell lines after transformation.

DISCUSSION

The in vitro transformation of fibroblasts by SV40 (Koprowski et al. 1962) has proved to be a valuable probe for studying cellular alterations involving loss of growth control. Additionally, cell transformation is a technique which facilitates the derivation of permanent lines from diploid cells, with the capacity for limitless growth. The increased proliferative potential of the transformants is of significant importance considering the difficulties involved in generating adequate quantities of brain cells for study (Wroblewska et al. 1975). Providing that the transformants can be recovered from the crisis stage (Girardi et al. 1975), transformation provides a means by which cells with specific biological markers such as abnormal storage products and enzyme defects can be released from growth restraints, permitting the specific trait to be fixed in a cell population with the potential for rapid and long-term proliferation (O'Brien et al. 1971; Schneck, Amsterdam & Volk, 1974).

As detailed above, we have successfully established 2 post-crisis transformed CNS cell lines which have survived over 200 subcultures. The ultrastructural demonstration of glial fibres in the parental cells, and their persistence after transformation, has enabled us to confirm the astrocytic origin of the derived permanent cell lines. Although Shein (1967) reported the retention of gliofibrils after viral transformation of human foetal brain cells, no permanent lines were derived from that work, since the culture did not survive crisis. We believe that our study represents the first report of an experimental model of a human Gm₂ gangliosidosis and control culture of astrocytic origin, permanently transformed by SV40.

The comparative study of the TSD transformed cells and the control culture shows that despite the retention of the biological and biochemical markers associated with the glycolipid storage disorder, specifically the absence of Hex A, increased Gm₂ ganglioside concentration and membranous cytoplasmic bodies, the TSD line shared common characteristics with the transformed control cells. Both lines exhibited almost identical cell morphology, the absence of cAMP-mediated differentiation (Prasad & Kumar, 1973), failure to release infectious virus particles (Girardi et al. 1975) and a PDT of under 85 h. Similar biochemical alterations in the ganglioside distribution were also evident. There was a significant increase in the percentage of Gm₂ in both TSD and control cultures following transformation, as well as smaller increases in Gm₂. In contrast, both lines exhibited a substantial decrease in the percentage of glucosamine-containing Gm₂ (Schneck, Hoffman, Brooks & Amsterdam, in preparation) and polysialoganglioside. A reduction in total ganglioside content, as evidenced by a decrease in sialic acid, was also characteristic of both transformed lines. The
predominance of $G_m3$ and the decrease in sialic acid after transformation has been reported for other cell systems (Brady & Mora, 1970) and has been attributed to an enzymic block in ganglioside synthesis in the transformants (Cumar et al. 1970; Brady, Fishman & Mora, 1973).

Several variations were noted between the TSD and control transformed cells. The control culture displayed a pre-crisis proliferative stage which was not evident with the TSD line, since these cells entered crisis immediately following transformation. Additionally, the duration of crisis was greatly extended with the TSD culture. These variations in virus-mediated cell modulations may be due to the difference in in vitro age of the cells. The TSD culture was transformed when in a senescent condition while the control culture was established following SV40 infection of a vigorous cell line. The most notable variation between cell lines was in karyological make-up. The average chromosome number for the TSD cells was 74, contrasted to 47 in the control cultures, with very little overlap in the range. Despite the wide variation in total DNA content between the lines, the similarities in other aspects of virus-mediated alterations were evident.

This study has resulted in the establishment of permanent CNS cell lines endowed with the vigour and long-term growth potential necessary to facilitate the in-vitro production of the massive quantities of cells required for thorough biochemical analysis. In addition, the unique characteristics of a $G_m2$ ganglioside storage disease have been retained in a permanent model cell line.

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