Neutral glycolipids and gangliosides of concanavalin A-selected SV3T3 revertant cells and of normal and SV40-transformed Balb/c 3T3 cells

Gabriele MUGNAI, Rossana COPPINI, Donatella TOMBACCINI, Anna FALLANI and Salvatore RUGGIERI

Institute of General Pathology, University of Florence, Florence, Italy

(Received 19 November 1980/Accepted 27 November 1980)

The structural analysis of neutral glycolipids and gangliosides of the SV40-transformed Balb/c3T3 cells (SV3T3 cells) and concanavalin A-selected SV3T3 revertant cells, both compared with untransformed Balb/c3T3 cells, has shown: (i) a content of neutral glycolipids in revertant cells near to that found in the untransformed parental cells; (ii) a similar decrease of the higher gangliosides in transformed and revertant cells; (iii) a content of ganglioside G_{M3} in revertant cells much higher than that found in both SV3T3 and untransformed Balb/c3T3 cells. The possible role of ganglioside G_{M3} in growth control is discussed.

Although a decrease in the more complex glycolipids (neutral glycolipids and gangliosides) frequently accompanies malignant transformation (Brady & Fishman, 1974; Hakomori, 1975; Critchley, 1979), the relevance of this molecular alteration to loss of growth control is still uncertain (Critchley, 1979). To clarify this question, glycolipid studies have been extended to variants of transformed cells with biological properties similar to those of the untransformed parental cells, so-called revertant cells (Mora *et al.*, 1971; Critchley & Macpherson, 1973; Den *et al.*, 1974). The presence in revertant cells of a glycolipid composition similar to that of untransformed cells could substantiate the relevance of the altered glycolipid pattern of malignant cells to the biological properties of these cells. However, although Mora *et al.* (1971) reported a complete reversion of ganglioside changes in the 5-fluorodeoxyuridine-revertants of SV40-transformed Swiss 3T3 cells, only a partial restoration to a normal pattern was found in the gangliosides of 5-fluorodeoxyuridine-revertants of polyoma virus-transformed Swiss 3T3 cells (Mora *et al.*, 1971). Moreover the revertants isolated from dimethylnitrosamine- and polyoma-virus-transformed hamster cells exhibited the same block in ganglioside biosynthesis as found in transformed cells (Den

et al., 1974). Furthermore, the 5-fluorodeoxyuridine-revertants of hamster sarcoma virus-transformed NIL₂ cells did not regain synthesis of the more complex neutral glycolipids (Critchley & Macpherson, 1973).

In view of the foregoing ambiguous results, we decided to study the structure of neutral glycolipids and gangliosides of concanavalin A-selected SV3T3 revertant cells (Culp & Black, 1972) together with that of Balb/c3T3 cells and their SV40-transformants (SV3T3 cells). Revertant cells used in the present study exhibit growth properties and molecular characteristics very similar to those of the untransformed parental cells, although they contain SV40-genetic information (Culp & Black, 1972; Schultz & Culp, 1973; Roblin *et al.*, 1975).

Materials and methods

Cells and culture conditions

The cells under study were the Balb/c3T3 cells (clone A31), SV3T3 cells (clone SVT2) and SV3T3 revertant cells (clones 81 and 84) kindly supplied by Dr. P. H. Black (M.G.H., Harvard Medical School, Boston, MA, U.S.A.). Cells were grown in our laboratory under the conditions described previously (Ruggieri *et al.*, 1979). Throughout several experiments, SV3T3 revertant cells maintained a large polygonal morphology and showed no foci of rereversion and no tendency of transformation into small spindle-shaped cells.

Analysis of neutral glycolipids and gangliosides

Cells were detached from the substratum at

Abbreviations used for gangliosides follow the nomenclature system of Svennerholm (IUPAC-IUB Commission on Biochemical Nomenclature, 1977): G_{M3} , II³NeuAc-LacCer; G_{M2} , II³NeuAc-GgOse₃Cer; G_{M1} , II³NeuAc-GgOse₄Cer; G_{D1a} , IV³NeuAc,II³NeuAc-GgOse₄Cer.

confluence by using 0.02% EGTA solution in phosphate-buffered saline (Dulbecco & Vogt, 1954), washed twice in phosphate-buffered saline and then sonicated (Labsonic sonicator, model 1510; Braun, Melsungen, Germany). Lipids were extracted from the sonicated cell suspensions by the method of Suzuki (1965) and fractionated on a Sephadex G-25 column (250 mm $\times$ 10 mm) with the elution system of Siakotos & Rouser (1965). By using this procedure, neutral glycolipids were eluted in the first fraction together with neutral lipids and phospholipids, whereas gangliosides were eluted in the second and third fractions.

Neutral glycolipids were separated from phospholipids and neutral lipids by Florisil column chromatography by the method of Saito & Hakomori (1971). The isolated neutral glycolipids were fractionated on 0.25 mm silica-gel H pre-coated plates (Merck A.G., Darmstadt, Germany) with chloroform/methanol/water (65:25:4, by vol). By using this procedure, neutral glycolipids of the three cell lines under investigation were separated into two diphenylamine-positive fractions (Skipski, 1975). The gangliosides were chromatographed with two runs in chloroform/methanol/conc. NH_3 /water (60:35:1:7, by vol.) on 0.25 mm pre-coated silica gel H plates activated at 100°C for 3 h immediately before use. Under these conditions gangliosides of the three cell lines were fractionated into four resorcinol-positive fractions (Skipski, 1975), each migrating as a doublet.

Structural analysis of sugar moieties of neutral glycolipid and ganglioside fractions of Balb/c3T3, SV3T3 and SV3T3 revertant cells

In experiments devoted to structural analyses of neutral glycolipids and gangliosides, the chromatograms were detected by exposure to I_2 vapour and the various fractions were eluted with 8 ml of chloroform/methanol/water/pyridine (20:28:6:1, by vol.) from the adsorbent scraped into small columns. Since we found that 95% of sialic acid of ganglioside G_{M1} and G_{D1a} standards, as determined colorimetrically (Miettinen & Takki-Luukkainen, 1959), was eluted from silica gel by this solvent mixture, we assumed that a comparable percentage of cellular glycolipids was recovered from the adsorbent. Neutral glycolipid and ganglioside fractions were submitted to acid-catalysed methanolysis with 1 ml of 0.5 M-HCl in dry methanol at 82°C for 18 h, together with a known amount of inositol. After neutralization with Ag_2CO_3 , all samples were treated with acetic anhydride (0.1 ml) for 18 h at room temperature. After adding 0.05 ml of water, the mixtures were extracted with hexane to remove fatty acid methyl esters. The water/methanolic mixtures were then transferred into small reactor vials and dried under N_2 . Methyl glycosides were converted

into trimethylsilyl derivatives with 50 μl of pyridine/hexamethyldisilazane/chlorotrimethylsilane (5:2:1, by vol.) and analysed by g.l.c. in glass columns (0.2 cm $\times$ 180 cm) packed with 3% OV17 on Supelcoport (80–100 mesh; Supelco, Bellefonte, PA, U.S.A.) and mounted in a Perkin–Elmer chromatograph model 3920 equipped with hydrogen flame detectors. Analyses were performed at a programmed temperature ranging from 120 to 230°C (2°C/min) with a carrier gas flow rate of 20 ml/min. Peak areas were measured with a Perkin–Elmer Sigma 10 integrator. Gas-chromatographic data were used to calculate molar ratios of the methyl glycoside groups, which were used to assign specific structures to the various glycolipid fractions. The concentrations of individual neutral glycolipids and gangliosides were evaluated on the basis of the quantitative gas-chromatographic analysis of their methyl glucoside group in the presence of inositol as an internal standard. Losses of methyl glycosides during analytical procedures were corrected by using factors derived from the carbohydrate analysis of known amounts of a pure standard of ganglioside G_{M1} that was submitted to the same procedure as the cellular glycolipids.

Ganglioside standards

Pure ganglioside G_{M1} was kindly donated by Dr. G. Tettamanti, University of Milan, Italy. Ganglioside G_{D1a} was isolated by preparative t.l.c. starting from a ganglioside mixture prepared in our laboratory from rat brain by the method of Suzuki (1965).

Protein determination

The protein content of the sonicated cell suspension was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Results and discussion

The gas-chromatographic analysis of carbohydrate groups of the two neutral glycolipid fractions isolated by t.l.c. showed molar ratios that correspond to glucosylceramide and lactosylceramide. The molar ratios of the carbohydrate groups of the four ganglioside fractions were found to be compatible with the structure of gangliosides G_{M3} , G_{M2} , G_{M1} and G_{D1a} respectively.

The concentrations of total and individual neutral glycolipids and gangliosides of Balb/c3T3, SV3T3 and SV3T3 revertant cells are reported in Table 1. Balb/c3T3 cells contained glucosylceramide as the major neutral glycolipid, whereas lactosylceramide was present only in very small amounts. A prevalence of glucosylceramide over lactosylceramide has previously been noted in the Balb/c3T3 cells studied by Rosenfelder *et al.* (1977) as well as in the

Table 1. *Neutral glycolipid and ganglioside content of Balb/c3T3, SV3T3 and concanavalin A-selected SV3T3 revertant cells*

The values (means $\pm$ S.E.M.), expressed as nmol of glycolipid glucose/mg of cellular protein,* are derived from the quantitative gas-chromatographic analysis of the methyl glucosides of individual neutral glycolipids and gangliosides. The numbers of different experiments is indicated in parentheses. Abbreviations used: GL1a, glucosylceramide; GL2a, lactosylceramide.

		Neutral glycolipids				Gangliosides				
		Total†	GL1a	GL2a		Total†	G _{M3} ‡	G _{M2}	G _{M1}	G _{D1a}
Balb/c3T3	(7)	3.73 $\pm$ 0.76	3.43 $\pm$ 0.72	0.30 $\pm$ 0.06	(8)	3.51 $\pm$ 0.23	0.46 $\pm$ 0.05	0.77 $\pm$ 0.11	0.72 $\pm$ 0.05	1.56 $\pm$ 0.17
SV3T3	(8)	1.21 $\pm$ 0.28	1.01 $\pm$ 0.25	0.20 $\pm$ 0.05	(13)	1.76 $\pm$ 0.20	0.73 $\pm$ 0.10	0.30 $\pm$ 0.04	0.26 $\pm$ 0.03	0.46 $\pm$ 0.06
SV3T3 revertant§ (8)		2.55 $\pm$ 0.45	2.21 $\pm$ 0.42	0.35 $\pm$ 0.06	(6)	3.57 $\pm$ 0.33	2.47 $\pm$ 0.31	0.32 $\pm$ 0.06	0.29 $\pm$ 0.04	0.49 $\pm$ 0.09

* Protein content per 10⁶ cells averaged 280 μ g, 240 μ g and 480 μ g in Balb/c3T3, SV3T3 and SV3T3 revertant cells respectively.

† In each separate experiment, the sum of individual glycolipids was measured. 'Total' refers to means $\pm$ S.E.M. for these sums.

‡ Ganglioside G_{M3} values also include amounts of the ganglioside occasionally eluted in the first fraction of the Siakotos & Rouser (1965) chromatographic system.

§ Variations between clones 81 and 84 of SV3T3 revertant cells were negligible and therefore means were calculated by combining data for the two clones.

Swiss 3T3 cells studied by Dawson *et al.* (1972). Compared with Balb/c3T3 cells, SV3T3 cells showed a decrease in neutral glycolipids, which is accounted for by a lower content of glycosylceramide ($P < 0.01$). In SV3T3 revertant cells the neutral glycolipid content tended to revert toward that of Balb/c 3T3, SV3T3 or SV3T3 revertant cells. In Balb/c3T3 cells, ganglioside G_{D1a} was the most prevalent ganglioside, followed by gangliosides G_{M1}, G_{M2} and G_{M3}, a pattern similar to that reported in Balb/c3T3 cells by Fishman *et al.* (1976). In comparison with Balb/c3T3 cells, SV3T3 cells showed a decrease of total ganglioside content that was due to the significantly lower concentration of gangliosides G_{M2}, G_{M1} and G_{D1a} ($P < 0.001$). There was no significant difference in the content of ganglioside G_{M3} between SV3T3 and Balb/c3T3 cells. A decrease in the higher gangliosides was reported in other virally transformed cell systems (Mora *et al.*, 1969, 1971; Fishman *et al.*, 1974, 1976) as well as in cells transformed by chemical carcinogens (Coleman *et al.*, 1975; Langenbach & Kennedy, 1978) and X-rays (Coleman *et al.*, 1975). In SV3T3 revertant cells, the content of total gangliosides reverted to that of untransformed Balb/c3T3 cells. Analysis of individual gangliosides revealed that reversion of total ganglioside content in SV3T3 revertant cells was accounted for by an accumulation of ganglioside G_{M3}, whereas the concentrations of gangliosides G_{M2}, G_{M1} and G_{D1a} had the same low values as observed in SV3T3 cells. The decrease in the higher gangliosides in SV3T3 revertant cells indicates that the concanavalin A-selected SV3T3 revertant cells were not reverted with respect to the enzymic activities responsible for

the synthesis of higher gangliosides. In this regard, concanavalin A-selected SV3T3 revertant cells behave similarly to the revertants of polyoma-virus- and dimethylnitrosamine-transformed hamster cells, which fail to revert to the normal pattern expected for higher-ganglioside synthesis (Den *et al.*, 1974). However, they differ from 5-fluorodeoxyuridine-revertants isolated from SV40-transformed Swiss 3T3 cells, which exhibit a normal pattern of the higher gangliosides (Mora *et al.*, 1971). The accumulation of ganglioside G_{M3} probably reflects the block of the synthesis of the more complex homologues. An accumulation of ganglioside G_{M3} may have been prevented in SV3T3 cells by a decreased availability of simple glycolipid precursors, as suggested by the low concentrations of neutral glycolipids in these cells. The very high concentration of ganglioside G_{M3} in SV3T3 revertant cells, paralleling a normal phenotype, could suggest a possible role for ganglioside G_{M3} in growth control. In this regard it should be mentioned that when ganglioside G_{M3} was added exogenously to normal and SV40-transformed Swiss 3T3 cells, it decreased their growth rate (Keenan *et al.*, 1975). Moreover, Hakomori *et al.* (1977) found a restoration of normal ganglioside G_{M3} concentration in chick-embryo fibroblasts transformed by temperature-sensitive mutants of avian sarcoma virus that had regained a normal phenotype after being shifted to a non-permissive temperature.

In conclusion, the results of the present investigation show that low concentrations of higher gangliosides are compatible with normal growth properties in concanavalin A-selected SV3T3 revertant cells. Ganglioside G_{M3}, which was found to

accumulate in these cells, may replace the more complex homologues in their possible function in growth control.

We thank Professor Alberto Fonnesu, Chairman of the Institute of General Pathology, for his interest in this work. This investigation was supported by a research grant from Consiglio Nazionale delle Ricerche (Contract no. 78.0286396, Progetto Finalizzato del CNR 'Controllo della Crescita Neoplastica').

References

- Brady, R. O. & Fishman, P. H. (1974) *Biochim. Biophys. Acta* **355**, 121–148
- Coleman, P. L., Fishman, P. H., Brady, R. O. & Todaro, G. J. (1975) *J. Biol. Chem.* **250**, 55–60
- Critchley, D. R. (1979) in *Surfaces of Normal and Malignant Cells* (Hynes, R. O., ed.), pp. 63–101, John Wiley and Sons, Chichester
- Critchley, D. R. & Macpherson, I. (1973) *Biochim. Biophys. Acta* **296**, 145–159
- Culp, L. A. & Black, P. H. (1972) *J. Virol.* **9**, 611–620
- Dawson, G., Matalon, R. & Dorfman, A. (1972) *J. Biol. Chem.* **247**, 5944–5950
- Den, H., Sela, B. A., Roseman, S. & Sachs, L. (1974) *J. Biol. Chem.* **249**, 659–661
- Dulbecco, R. & Vogt, M. (1954) *J. Exp. Med.* **99**, 167–182
- Fishman, P. H., Brady, R. O., Bradley, R. M., Aaronson, S. A. & Todaro, G. J. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 298–301
- Fishman, P. H., Brady, R. O. & Aaronson, S. A. (1976) *Biochemistry* **15**, 201–208
- Hakomori, S. (1975) *Biochim. Biophys. Acta* **417**, 55–89
- Hakomori, S., Wyke, J. A. & Vogt, P. K. (1977) *Virology* **76**, 485–493
- IUPAC-IUB Commission on Biochemical Nomenclature (1977) *Eur. J. Biochem.* **79**, 11–21
- Keenan, T. W., Schmid, E., Franke, W. W. & Wiegandt, H. (1975) *Exp. Cell Res.* **92**, 259–280
- Langenbach, R. & Kennedy, S. (1978) *Exp. Cell Res.* **112**, 361–372
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Miettinen, T. & Takki-Luukkainen, I. T. (1959) *Acta Chem. Scand.* **13**, 856–858
- Mora, P. T., Brady, R. O., Bradley, R. M. & MacFarland, V. W. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **63**, 1290–1296
- Mora, P. T., Cumar, F. A. & Brady, R. O. (1971) *Virology* **46**, 60–72
- Roblin, R., Albert, S. O., Gelb, N. A. & Black, P. H. (1975) *Biochemistry* **14**, 347–357
- Rosenfelder, G., Young, W. W. & Hakomori, S. (1977) *Cancer Res.* **37**, 1333–1339
- Ruggieri, S., Roblin, R. & Black, P. H. (1979) *J. Lipid Res.* **20**, 772–783
- Saito, T. & Hakomori, S. (1971) *J. Lipid Res.* **12**, 257–259
- Schultz, A. R. & Culp, L. A. (1973) *Exp. Cell Res.* **81**, 95–103
- Siakotos, A. N. & Rouser, G. (1965) *J. Am. Oil Chem. Soc.* **42**, 913–919
- Skipiski, V. P. (1975) *Methods Enzymol.* **35**, 396–425
- Suzuki, K. (1965) *J. Neurochem.* **12**, 629–638