GANGLIOSIDES IN DNA VIRUS-TRANSFORMED AND SPONTANEOUSLY TRANSFORMED TUMORIGENIC MOUSE CELL LINES

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Abstract.—In mouse cell lines transformed by SV40 virus, a decrease was observed in the higher ganglioside homologues disialo-ceramidetetrahexoside and monosialo-ceramidetetrahexoside. Such change was observed only in cells which carry the virus genome, and it correlated with increased saturation density in tissue culture and with rejection in immunologically competent syngeneic host. This indicates that the change is one of the virus-regulated functions, and it is postulated that it relates to the rejection of the virus-transformed cells.

Introduction.—The glycolipid components of cell membranes are of interest when studying acquisition of malignant potential by cells. Of special interest are the gangliosides, which contain sialic acid groups, specific carbohydrate sequences in an oligosaccharide chain, and hydrophobic lipid residues. These groups, especially if present on the cell surface, may influence, respectively, the electronegative surface charge, the antigenic property, and the adhesiveness of the cell. These properties, in turn, could influence the mutual control of division of cells when in contact with each other.

To assure reproducibility and to allow more clear-cut interpretation, the cells of choice are established cell lines in tissue culture. A mouse fibroblast cell line, the 3T3 cells established by Todaro and Green[1] originally from a random-bred Swiss mouse embryo, is very sensitive to contact inhibition of cell division in tissue culture.[2, 3] These cells lose contact inhibition when transformed by the viruses polyoma and SV40.[4] With the use of 3T3 mouse cells and also other noninbred hamster and human fibroblast cell lines, recent analyses[5, 6] indicated a decrease in the content of one of the gangliosides (hematoside) after viral-induced transformation in tissue culture.

We decided to investigate the changes of the various gangliosides in normal and DNA tumor virus-transformed variants of established cell lines and to relate possible changes not only to differences in contact inhibition of division of cells in tissue culture but also to tumorigenic potential when tested in animals. The latter became possible with the development of cell lines from inbred mouse strains which, after transformation in tissue culture with SV40 virus, induce tumors in syngeneic mice, but only when the mice were rendered immunologically incompetent.[7, 8] Since normal mouse cell lines after prolonged cultivation in tissue culture,[9] especially under crowded condition,[8] often transform "spontaneously" to tumorigenic cells as tested in immunologically competent syngeneic host,[10] our study was extended to compare gangliosides in normal, spontaneously transformed and virus-transformed cells.

Materials and Methods.—Cells: Two types of cell lines were employed. The first type (3T3 and SVPY 3T3) was donated by Dr. G. J. Todaro; the second type, the inbred AL/N cell lines, was given by Dr. K. K. Takemoto.

3T3 (clone 421) is a continuous aneuploid mouse embryo fibroblast cell line of Todaro and Green,[1] from randomly bred Swiss mouse.

SVPY 3T3 (clone 11) includes the above 3T3 cells transformed by both polyoma and SV40 viruses.[4] The cells do not release infectious virus; however, they contain both SV40 and polyoma T antigens.[4]

N AL/N is a "normal" established cell line from inbred mouse strain AL/N embryos,[11] an epithelial-like cell line[7] (Takemoto, passage 25) which in early passages exhibited contact inhibition of growth and no tumorigenicity.[7] (However, see below).

T AL/N are N AL/N cells after passage 43 "spontaneously" transformed in Dr. Takemoto's laboratory. The cells produce tumor in syngeneic mice.[10] This cell line was tested and carried for 28 additional passages.

SVS AL/N cells are N AL/N cells transformed in tissue culture by Takemoto *et al.*, using a small plaque-forming variant of SV40 virus.[7] The cells contain SV40-specific T antigen and transplantation antigen, do not release virus, and produce tumors only when inoculated into irradiated or thymectomized syngeneic mice.[7] The cells were carried for 25 additional passages.

T AL/NT, a tumor which was induced in this laboratory by TAL/N cells in a normal syngeneic mouse, was passed back into tissue culture and carried for ten passages.

MES is mouse embryo secondary culture from Balb/c mice. These are mixed cells containing mainly fibroblast types and are used as a control to exclude possible artifacts by tissue culturing.

Growth conditions: Various growth conditions were employed because the different cell lines grow optimally in somewhat different media. The 3T3 and SVPY 3T3 lines were grown as described previously[3] under Dulbecco-Vogt modification of Eagle's medium, supplemented with 10% fetal calf serum, penicillin, and streptomycin, with added mycostatin. Mouse embryo secondary cells and T AL/N or SVS AL/N lines in experiments 1 and 3 (see Table 3) were grown in Eagle's medium containing 1× amino acids and vitamins, 10% fetal calf serum and penicillin, streptomycin, and mycostatin. In experiment 4 (Table 3), all the AL/N cells were grown in Eagle's medium with 10% added fetal calf serum and fortified by 2× amino acids and vitamins, and also containing penicillin, streptomycin, mycostatin, achromycin, and kanamycin. The fortification of Eagle's medium was found helpful for the growth of the N AL/N cells.

Cells were grown in Falcon plastic Petri dishes in a humidified incubator at 37° containing 5% CO_2. Medium was changed every 2 days. For the chemical determinations, all cells were harvested just before reaching monolayer confluency to ensure similar physiological (growth) stage. For each analysis, cells from about 100 plates were used, representing about 10^8 cells, or 0.5–1 ml wet packed cell volume. In one experiment (expt. 3, Table 3) T AL/N cells were also grown at 37° in glass roller tubes to near-confluency in order to test the possible effect of the substratum.

In certain experiments cells were kept beyond confluency in the plastic Petri dishes to determine maximum attainable cell density. The medium was changed every 2 days. In these experiments (Table 2), the medium contained 10% calf serum and was fortified by 2× amino acids and vitamins and contained antibiotics, as in experiment 4, Table 3 (see above).

In the normal cultivation of both 3T3 and N AL/N cells, care was exercised to avoid growth in crowded conditions when plating cells for future generations in culture, since such conditions are known to increase spontaneous tumorigenic transformation by selection of non-contact-inhibited variants.[8]

Harvesting conditions: The medium was removed, and the cells were washed *in situ* with saline and then removed by gentle scraping with a rubber policeman in the various solutions of harvest as indicated in Table 3. Solutions of 0.5% (0.013 *M*) EDTA, saline alone, and saline containing 10^{-3} *M* EDTA were evaluated. These concentrations of

EDTA are generally used to detach cells from the substratum. However, the presence of EDTA had to be evaluated, since it is known to affect electrophoretic mobility and adhesiveness of certain cells.[12]

The cells were collected by centrifugation, resuspended and washed with saline three times, and stored frozen at −70° under minimum amount of saline.

Glycolipid analysis: The washed, harvested cells were suspended in 3 vol of 0.1 *M* KCl, and 20 vol of chloroform–methanol 2:1 (v/v) were added. The mixtures were heated for 15 min at 50°. Denatured protein was removed by centrifugation and the residue was extracted with chloroform–methanol 1:2 by using a volume equivalent to one half of that for the extraction with chloroform–methanol 2:1. The lipids were partitioned and quantitatively recovered according to the method of Folch *et al.*[13] The contents of the upper (aqueous) phase were dialyzed overnight against distilled water. The retentate was taken to dryness by lyophilization. The organic solvents in the lower phase were evaporated under a stream of nitrogen. This residue was taken up in a small volume of methanolic NaOH and saponified according to the technique of Kishimoto *et al.*[14] The solution was neutralized with *N*-acetic acid, and sufficient chloroform–methanol 2:1 was added to produce a clear solution. Two phases were produced by adding $^1/_5$ vol of water. The aqueous phase was decanted and the lower phase was shaken with $^1/_5$ vol of "theoretical upper phase water."[13] The sphingolipids in the aqueous and organic phases were separated by thin-layer chromatography on glass plates coated with silica gel G which were activated by heating for 1 hr at 110° just before using. Aliquots of each phase were applied to two separate plates. The following solvent systems were employed: chloroform–methanol–2.5 *N* NH_4OH, 60:35:8 (v/v/v), and chloroform–methanol–water, 65:25:4 (v/v/v). Authentic sphingolipid standards were run simultaneously in parallel. Sialic acid-containing sphingolipids (gangliosides) were visualized by spraying the plates with resorcinol reagent.[15] The resorcinol-positive areas of the chromatograms were individually scraped from the plates and transferred to small centrifuge tubes. The colored product was extracted with gentle warming into 0.35 ml of butylacetate–butanol, 85:15 (v/v). The suspensions were centrifuged, and the absorbancy of the supernatant solution was measured at 580 and 450 nm. With this procedure, recovery of standard hematoside and GM_1 (for nomenclature see Table 1) applied to the thin chromatograms ranged between 79 and 90% (mean 84%). The values in Table 3 are not corrected for losses which occurred in the colorimetric extraction procedure. The data reported in Table 3 indicate the sum of hematoside present in both phases.

Hematoside was prepared from beef erythrocytes by Dr. A. K. Percy. Other ganglioside standards were generously supplied by Dr. E. H. Kolodny.

Results.—Table 2 compares tumorigenicity *in vivo* and saturation density in tissue culture of the AL/N cell lines investigated. The cell line T AL/NT, which was passed through the syngeneic mouse once as a tumor, produced tumor rapidly when a relatively low number of cells were inoculated into an immunologically competent syngeneic mouse. The T AL/N cell line, which is a "spontaneous" transformant carried in tissue culture, induced tumor with a somewhat

TABLE 1. *Structure of gangliosides.*

Name	Structure	Designation*
(1) Hematoside	*N*-Acetylneuraminylgalactosylglucosylceramide	GM_3
(2) Tay-Sachs ganglioside	*N*-Acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide	GM_2
(3) Monosialoganglioside	Galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide	GM_1
(4) Disialoganglioside	*N*-Acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide	GD_{1a}

* The symbols proposed by Svennerholm[16] are used in this communication.

TABLE 2. *Saturation density of AL/N cell lines in tissue cultue and tumorigenicity in vivo.*

Cell lines*	Saturation density† (cells/cm² × 10⁵)	Tumorigenicity in Immunologically Competent Syngeneic Host: Cells inoculated (no.)	No. tumors/ no. animals	Latent time‡ (weeks)
N AL/N	1.0	10^6	3/5	9
SVS AL/N	3.0	10^7	0/40	24
T AL/N	1.7	3×10^5	20/20	2–3
		10^5	9/10	3–4
		10^4	3/5	6–8
		10^3	0/5	8
T AL/NT	1.2	10^6	5/5	2
		10^5	4/5	2
		10^4	5/5	2
		10^3	1/5	3
		Tumorigenicity in Irradiated Syngeneic Host§		
SVS AL/N	3.0	10^6	4/5	2
		10^5	0/5	4

* See *Materials and Methods.*

† The maximum cell number attainable from densely growing cultures on plastic Falcon Petri dishes, after the medium (in all of these experiments as in expt. 4, Table 3 containing 2× amino acids and vitamins and 10% fetal calf serum; see *Materials and Methods*) was changed every 2 days. Each value represents average from 7–11 independent experiments.

‡ Time when large, palpable tumor (~0.5-cm diameter) is first observed at the site of the inoculation. The tumors grow rapidly and usually cause death in 2 more weeks. In cases when no tumor is observed, the weeks of observation are reported in this column.

§ SVS AL/N cells are tumorigenic only when injected into syngeneic mice made immunologically incompetent.[7] In these experiments mice were irradiated with 300 r before inoculation with the cells.

longer latent time. The "normal" cell line N AL/N did not induce observable tumor in three weeks at the dose of 10^5 cells/mouse (data not shown), although tumors started to appear after nine weeks' latent time. Apparently, this cell line also became weakly tumorigenic during culturing.

It is significant that in immunologically competent syngeneic host the least tumorigenic cell line was the SV40 virus-transformed SVS AL/N line. These cells were shown to contain tumor transplantation rejection antigen, as tested on mice first immunized with SV40 virus,[7] and are probably rejected in the immunocompetent syngeneic host because of this antigen, even when inoculated at high dose (10^7 cell), unless the host is rendered immunologically incompetent such as by radiation.[7, 8] In irradiated mice, 10^6 cells induced tumor in two weeks in the majority of the animals (Table 2).

The SVS AL/N cells attained the highest saturation density in tissue culture—about three times that of the N AL/N cells. This is qualitatively similar to the well-known phenomenon that the DNA tumor virus-transformed cell lines attain higher saturation density.[4, 8] We also consistently found a relatively lower saturation density of the tumorigenic T AL/NT and T AL/N cells when compared to the SVS AL/N cells (Table 2).

As shown in Table 3, hematoside (GM_3) was found to be the major sialic acid containing lipid in the various cell lines which we examined in these investigations. This observation agrees with the findings of Hakomori and Murakami.[5] In the conventional partitioning procedure employed in our experiments, hematoside was almost equally distributed between the upper (aqueous) and lower

(organic) phases. The other gangliosides were quantitatively recovered in the aqueous phase.

In contrast to the reports of Hakomori and Murakami[5] and Hakomori *et al.*,[6] in the cell lines that we investigated we did not find a marked or consistent decrease of hematoside (GM_3) in virally transformed cells; in fact, in experiments 1 and 2 there seemed to be a small increase of hematoside in these cell cultures, and also in experiment 4 the lowest hematoside content was found in the "normal" cell line.

There was, however, a dramatic and consistent decrease in the higher ganglioside homologues, disialo-ceramidetetrahexoside (GD_{1a}) and monosialo-ceramidetetrahexoside (GM_1) in the virally transformed cells (Table 3 and Fig. 1). There also was a consistent over-all net decrease in total ganglioside based on the protein content in the virally transformed cells. Similar results were obtained when the cells were harvested with 0.5% EDTA, with saline containing 0.001 *M* EDTA, or with saline alone and when the cells were grown on either plastic or glass surface, and when the cells were grown out from an early or late passage (Table 3). Based on protein, a slightly lower total ganglioside content was attained in all analyses in experiment 4, when the growth medium was fortified with twice the normal amino acid and vitamin content.

We did not observe chromatographically any consistent or significant alteration of the non-sialic acid-containing sphingolipids in the virally transformed cells.

TABLE 3. *Distribution of gangliosides in control and virally or spontaneously transformed cells.*

Expts.	Cell type	Solution of harvest	Gangliosides GD_{1a}	GM_1	GM_2	GM_3	Total
			(nmoles/mg of protein)				
1	T AL/N	0.5% EDTA	3.8	1.6	1.2	4.0	10.6
	SVS AL/N	0.5% EDTA	0.5	0.6	1.8	5.2	8.1
2	3T3	Saline	2.4	2.6	1.8	4.0	10.8
	3T3	Saline, 0.001 *M* EDTA	2.8	2.8	2.4	4.8	12.8
	SVPY 3T3	Saline	0.6	0.8	0.4	4.8	6.6
	SVPY 3T3	Saline, 0.001 *M* EDTA	0.6	0.6	0.8	5.3	7.3
3	MES	Saline	3.6	1.6	0.8	4.8	10.8
	MES	Saline, 0.001 *M* EDTA	3.4	1.6	0.8	5.2	11.0
	T AL/N	Saline, 0.001 *M* EDTA	3.1	2.7	1.8	4.8	12.4
	T AL/N	Saline	2.9	1.8	2.2	5.0	11.9
	T AL/N (glass*)	Saline, 0.001 *M* EDTA	2.8	1.5	2.0	4.9	11.2
	SVS AL/N	Saline, 0.001 *M* EDTA	0.2	0.5	0.9	4.7	6.3
	SVS AL/N	Saline	0.3	0.4	0.7	4.4	5.8
4	N AL/N	Saline	2.2	1.4	0.4	2.5	6.5
	SVS AL/N	Saline	0.25	0.4	0.8	3.0	4.45
	T AL/N	Saline	2.5	1.8	1.0	3.8	8.7
	T AL/NT	Saline	3.8	2.8	0.0	3.1	9.7

* Grown on glass surface.

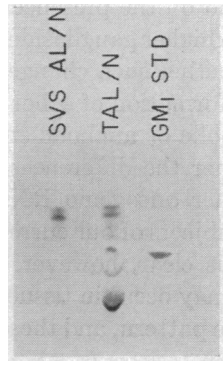

FIG. 1.—Thin-layer chromatogram of dialyzed upper-phase glycolipids. Portions of the upper (aqueous) phases obtained by extracting SVS AL/N cells (*left lane*), T AL/N cells (*center*), and monosialo-ceramide-tetrahexoside (GM_1) standard (*right lane*) were chromatographed on a silica gel G plate and developed with chloroform–methanol–2.5 *N* NH_4OH (60:35:8) (v/v/v). The gangliosides were detected by resorcinol reagent.[15] Equivalent aliquots of cell extracts were plated, based on the protein content of the harvested cells. Although the slowest migrating ganglioside (identified in other experiments as GD_{1a}) appears darkest in this figure, it should be kept in mind that this compound has two resorcinol-reactive sialic acid groups in its molecule, and approximately only 50% of the hematoside is recovered in the aqueous phase.

Discussion.—In virally transformed cells the reduction of the highest ganglioside homologue disialo-ceramidetetrahexoside (GD_{1a}) is the most pronounced, followed by monosialo-ceramidetetrahexoside (GM_1) and by the total ganglioside content. This pattern of reduction was consistently observed in cells transformed by and carrying[17] the viral genome and did not occur in cells not transformed by the virus, irrespective of the cell type, growth medium, saturation densities of the cells in tissue culture, their tumorigenicity *in vivo* in immunocompetent syngeneic host (Table 2), and of the method of collection of cells (Table 3). The spontaneously transformed cells T AL/N and T AL/NT, while highly tumorigenic in normal immunocompetent syngeneic mice, did not show decrease in higher ganglioside homologues.

The virally transformed cells grow to higher saturation density in tissue culture.[2, 4] Saturation density of cultured cells depends on a heritable property of the cell line investigated, such as in stable cell lines established by employing selection pressures during cell cultivation, e.g., consistently allowing extensive cell contact in cultivation through many cell generations or conversely minimizing such contact (for the latest exploitation of this technique, see ref. 8). It also depends on the constitution of medium such as the serum concentration or on the frequency of medium change, etc. With the latter conditions kept constant, the saturation densities of cell lines reported in Table 2 were reproducible within about 50 per cent maximum deviation, and the average values reported reflect consistent and meaningful relative differences under the experimental conditions given. The AL/N cell lines (including the N AL/N cells) were not cultivated under as consistently and rigorously controlled conditions with respect to cell contact as those recently used by Aaronson and Todaro.[8] This might explain, in part, our lack of clear-cut correlation with saturation density and tumorigenicity.

The SV40 virus-transformed SVS AL/N cell line carries specific tumor transplantation antigen(s).[7] It is likely that the presence of this antigen(s) facilitates the rejection of these cells when inoculated into immunocompetent mice.[7, 8]

Our result suggests that, in the SV40 virus-transformed mouse cell lines investigated, the cell surface and transplantation-rejection antigenic changes which

depend on the presence of the viral genome are related to the observed decrease in the higher ganglioside homologues.

Whether such changes are of broader occurrence in other DNA virus-induced transformation of other type of cells, whether these changes are consequences of catabolic or anabolic enzymatic changes in ganglioside synthesis or breakdown, whether the differences are detectable in isolated cell plasma membranes, and whether oncogenic RNA virus-transformed cells show such a change at all, are subjects of our current investigation.

It is clear, however, that spontaneous tumorigenic transformation of mouse cells may occur in tissue culture, which is not accompanied by shifts in the ganglioside pattern, and these cells are not rejected by the syngeneic host.

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