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Absence of a Specific Ganglioside Galactosyltransferase in Mouse Cells Transformed by Murine Sarcoma Virus

(RNA tumor virus/transformation)

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ABSTRACT The ganglioside composition of a nonproducer subclone (derived from BALB/c 3T3 mouse-embryo cells) transformed by Kirsten murine sarcoma virus was drastically altered compared to the nontransformed parental clone. The transformed clone was unable to synthesize the mono- and disialogangliosides, G_{M1} and G_{D1a}, due to the complete absence of a specific galactosyltransferase. The lack of this enzyme activity was established by sensitive radiochemical and enzymological techniques *in vivo* and *in vitro*.

Murine sarcoma virus (MSV) transforms cells in culture in the absence of virus replication (2). The nonproductively transformed cells show a striking alteration in cell morphology associated with the loss of normal growth controls. The cells form piled-up colonies, grow in soft agar, and produce tumors *in vivo* (2, 3). In addition, the transformants contain a sarcoma-associated cell-surface antigen (4) and show an increased agglutinability by certain plant lectins (5). However, biochemical alterations in the cell surface of MSV-transformed cells have not been described.

Glycolipids are constituents of the cell membrane that have been observed to change in a variety of virus-transformed cells (6). One class of glycolipids, the sialic acid-containing gangliosides, was found to be consistently altered in mouse cells transformed by DNA oncogenic viruses. This change was shown to be due to a block in ganglioside biosynthesis, namely, reduced activity of the enzyme hematoside: UDPGalNAc *N*-acetylgalactosaminyltransferase (7, 8). In the present studies, we have compared the composition and metabolism of gangliosides in an established clonal line of mouse-embryo cells, BALB/3T3, with that in a nonproducer subclone transformed by the Kirsten (Ki) strain of MSV.

MATERIALS AND METHODS

Cell Lines and Cell Culture. Cells were grown in Dulbecco's modification of Eagle's medium supplemented with 10% Colorado calf serum. The BALB/3T3 cell line is an established line of highly contact-inhibited, nontumorigenic mouse cells (9). A KiMSV-transformed nonproducer subclone, K BALB/3T3, of the A31 clone of BALB/3T3 has been described (10). Glycolipids were radioactively labeled by growing the cells for 24 hr with 1 μCi of D-glucosamine (54 Ci/mol) per ml of medium. Cells were harvested by mechanical scraping as described elsewhere (8).

Glycolipid Analysis. Glycolipids were extracted from cells as they reached confluency according to described procedures (11). Gangliosides were separated by thin-layer chromatography, with a solution of chloroform–methanol–0.25% aqueous $CaCl_2$ (60:35:8, v/v/v) (12) as the developing solvent. The individual gangliosides were detected with resorcinol spray and quantitated by densitometry (8, 13). Radioactivity was determined by scanning the thin-layer plates with a Varian Aerograph thin-layer scanner.

Glycosyltransferase Assays. The activity of G_{M3}:UDP-GalNac *N*-acetylgalactosaminyltransferase and G_{M2}:UDPGal galactosyltransferase was measured in whole cell homogenates essentially as described (14). The conditions for determining the activity of the latter enzyme were modified slightly as indicated in order to obtain maximal activity with the BALB/c cell lines used in the present investigation. Briefly, the cells were harvested, lysed by freeze-thawing four times in 4 volumes of 0.25 M sucrose solution containing 0.1% 2-mercaptoethanol, and dispersed by hand homogenization. The cellular extracts were incubated with the appropriate glycolipid acceptor and [^{14}C]sugar nucleotide donor and buffer, cation, and detergent. The reactions were stopped by addition of 20-volumes of chloroform–methanol solution (2:1, v/v). Radioactive products were separated from the labeled substrate by passing the dissolved material over a small (1.0 g) column of Sephadex G-25 superfine grade; the eluted radioactive ganglioside products were quantitated by liquid scintillation spectroscopy (14).

RESULTS

Ganglioside Composition of RNA Virus-Transformed BALB/3T3 Cells. There was a striking qualitative difference in the ganglioside composition of the parent BALB/3T3 Cl A31 cells and in the K-BALB/3T3 transformed derivative line (Fig. 1). The control cells contained gangliosides G_{M3}, G_{M2}, G_{M1}, and G_{D1a}, while the transformed cells contained primarily G_{M2}. In the KiMSV-transformed cells, G_{M1} and G_{D1a} were

Abbreviations: KiMSV, Kirsten strain of murine sarcoma virus; Gal, galactose; GalNAc, *N*-acetylgalactosamine. Gangliosides are designated by the symbols proposed by Svennerholm (ref. 1): G_{M3}, *N*-acetylneuraminylgalactosylglucosylceramide; G_{M2}, *N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide; G_{M1}, galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide; G_{D1a}, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide.

virtually absent, and the G_{M2} content was increased (Table 1). When the two types of cells were grown in the presence of [^{14}C] glucosamine, which is a good precursor of gangliosides under these conditions, most of the radioactivity in the cultured BALB/3T3 cells was found in G_{D1a} whereas over 95% of the label was associated with G_{M2} in the transformed cells. Although the gangliosides from BALB/3T3 cells had been identified previously by migration with authentic ganglioside standards in several chromatography systems and by gas–liquid chromatography of the trimethyl silyl derivatives of the sugar moieties (15), the identity of G_{D1a} was further substantiated in the present investigation by treatment of the extracted gangliosides with bacterial neuraminidase (Sigma). This enzyme removes only the terminal molecule of sialic acid from G_{D1a} and G_{M3} and has no effect on G_{M1} and G_{M2}. As expected, the G_{D1a} from the control cells was converted to G_{M1}, and the G_{M2} from both cell types was unaffected. Examination of the neutral glycolipids from these cells by thin-layer chromatography indicated that both cell lines contained similar amounts of glucosylceramide, lactosylceramide, trihexosylceramide, and globoside.

Galactosyltransferase Activity. The absence of G_{M1} and G_{D1a} and the increased G_{M2} content in the K-BALB/3T3 subclone suggested an attenuation of G_{M2}:UDPGal galactosyltransferase activity in those cells. The optimal pH for measuring the activity of this enzyme in BALB/c cells is 5.3. The apparent Michaelis constants for G_{M2} and UDPGal were 6 μM and 136 μM, respectively. Saturating concentrations of 150 μM and 750 μM were, therefore, used. A variety of detergents was examined, and a mixture of Tween 80 and Triton CF-54 was found to be the best. Under these conditions, the enzyme assay was linear for at least 2 hr and up to 200 μg of cell protein per incubation. The specific activity of galactosyltransferase could also be increased 2 to 3-fold if the lysed cells were centrifuged at 600 $\times$ *g* for 12 min and the crude particulate fraction was resuspended and assayed. Even with these optimizing assay conditions, there was no detectable G_{M2}:UDPGal galactosyltransferase activity in the KiMSV-transformed cells whereas it was easily demonstrable in the parent BALB/3T3 cells (Table 2). G_{M3}:-

TABLE 1. *Ganglioside composition and distribution of radioactivity in gangliosides of normal and KiMSV-transformed BALB/3T3 mouse cells grown in the presence of* [^{14}C]*glucosamine*

	Cell line			
	BALB/3T3		K-BALB/3T3	
Ganglioside	nmol of sialic acid	cpm	nmol of sialic acid	cpm
G_{M3}	0.46	45	0.28	0
G_{M2}	1.24	2,216	1.83	1,741
G_{M1}	0.05	326	<0.01	23
G_{D1a}	0.94	7,958	0.03	68
Total	2.69	10,581	2.14	1,832

The gangliosides were extracted, separated by thin-layer chromatography, visualized, and quantitated. The values are expressed per mg of protein in the respective cell samples and represent the mean of three separate experiments with each cell type.

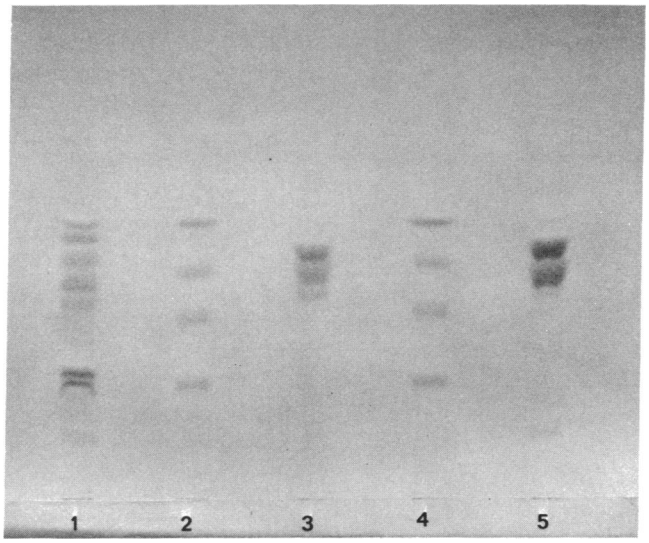

FIG. 1. Thin-layer chromatogram of gangliosides isolated from normal and KiMSV-transformed BALB/3T3 cells. Glycolipids were extracted from freshly harvested cells, the extracts were partitioned, and aliquots of the dialyzed upper phases equivalent to 5 mg of cellular protein were chromatographed on thin-layer Silica gel G plates. The gangliosides were detected by resorcinol spray. (*1*) Gangliosides from BALB/3T3 control cells. (*2* and *4*) Ganglioside standards from *top* to *bottom:* G_{M3}, G_{M2}, G_{M1}, and G_{D1a}. (*3* and *5*) Gangliosides from two batches of K-BALB/3T3 transformed cells.

UDPGalNAc *N*-acetylgalactosaminyltransferase was unaffected by transformation. The silalyltransferase, which catalyzes the formation of G_{D1a} from G_{M1}, was also unchanged (data not presented because the latter assays were not performed with saturating quantities of substrates).

Identification of Product of G_{M2}:UDPGal Galactosyltransferase. In order to substantiate the nature of the ganglioside produced in the galactosyltransferase reaction, the assay

TABLE 2. *Glycosyltransferase activities in BALB/3T3 and KiMSV-transformed BALB/3T3 cells*

	N-Acetylgalactosaminyltransferase*	Galactosyltransferase†	
Cell line	Whole homogenate	Whole homogenate	Resuspended particles
	nmol per mg of protein per hr‡		
BALB/3T3	2.51	0.25	0.58
K-BALB/3T3	2.58	<0.015	<0.015

* The incubation mixtures contained 50 nmol of G_{M3}, 10 nmol of UDP[^{14}C]GalNAc, 200 μg of Nonidet P40 detergent (Shell Oil Co.), 2.5 μmol of cacodylate buffer (pH 7.0), 1 μmol of $MnCl_2$, and 75 μg of protein in a final volume of 0.05 ml.

† The incubation mixtures contained 7.5 nmol of G_{M2}, 37.5 nmol of UDP[^{14}C]Gal, 100 μg of Tween 80 (Nutritional Biochemicals Corp.), 50 μg of Triton CF54 (Rohm and Haas), 2.5 μmol of cacodylate buffer (pH 5.3), 1 μmol of $MnCl_2$, and 200 μg of protein in a final volume of 0.05 ml.

‡ Activity is corrected for incorporation into endogenous acceptors. The values represent the mean of three separate experiments with each cell type.

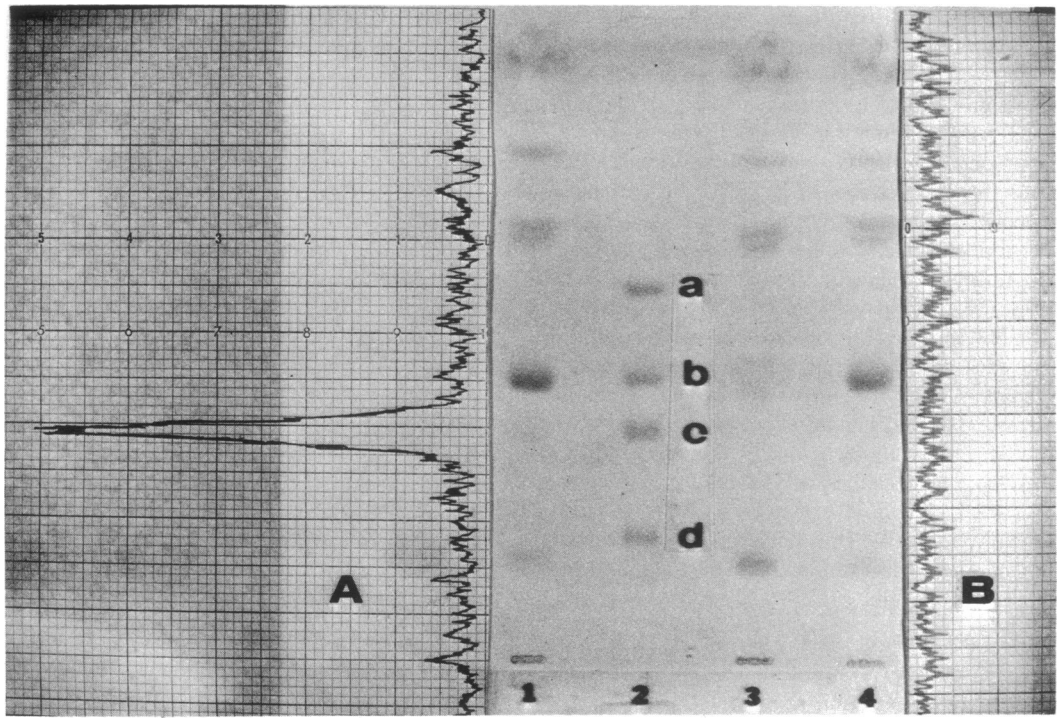

FIG. 2. Identification of the radioactive product of the G_{M2}:UDPGal galactosyltransferase reaction. Whole homogenates of BALB/3T3 and K-BALB/3T3 cells were assayed for galactosyltransferase activity. Each assay contained 0.35 μCi of UDP [^{14}C]Gal and 200 μg of cell protein. After the samples were incubated for 2 hr at 37°, 1 ml of chloroform–methanol solution (2:1, v/v) was added to each of two incubation vessels and the suspensions were applied to a 1 × 2.5-cm column of Sephadex G-25 superfine grade previously equilibrated with a solution of chloroform–methanol–water (60:30:4.5, v/v/v). The columns were eluted with 4 ml of the same solvent and the effluents were combined and concentrated under a stream of N_2. The samples were applied to a thin-layer chromatogram and analyzed. (*1*) Product from incubation of BALB/3T3 cells with added G_{M2} as acceptor. (*2*) Ganglioside standards: *a* = G_{M3}; *b* = G_{M2}; *c* = G_{M1}; and *d* = G_{D1a}. (*3*) Product from incubation of BALB/3T3 cells without added G_{M2}. (*4*) Product from incubation of K-BALB/3T3 cells with G_{M2}. (*A*) Radioactive scan of *1*; (*B*) radioactive scan of *4*.

procedure was conducted in duplicate vessels with UDP [^{14}C]Gal with increased specific activity. The effluents from the Sephadex columns were combined and evaporated under a stream of N_2 and the radioactive product was isolated by thin-layer chromatography. The results of this experiment are shown in Fig. 2. The only radioactive ganglioside produced (lane *1*) migrated with authentic G_{M1}. A small amount of labeled G_{M1} was formed in the control cell extracts incubated without added G_{M2} due to the presence of a small amount of endogenous acceptor (lane *3*, radioactive scan not shown). When extracts from K-BALB/3T3 cells were used, no radioactive G_{M1} was produced with or without exogenous G_{M2}. These results provide strong confirmation of the absence of G_{M2}:UDPGal galactosyltransferase activity in the K-BALB/3T3 cells.

DISCUSSION

We report a specific change in the ganglioside composition of BALB/3T3 cells transformed with the Kirsten strain of murine sarcoma virus. The transformed cells were no longer able to synthesize the more complex gangliosides G_{M1} and G_{D1a} due to loss of a specific galactosyltransferase required for the formation of these components. Based on the results of several sensitive radiochemical techniques, there was no detectable G_{M2}:UDP Gal galactosyltransferase activity in the transformed cells.

The effect of transformation by KiMSV on glycolipid metabolism in these cells appears to be highly specific. There was no reduction of *N*-acetylgalactosaminyltransferase activity, the preceding enzymatic step in the ganglioside biosynthetic pathway (16). Similarly, there was no apparent change in the activity of the sialyltransferase, which catalyzes the synthetic reaction subsequent to the formation of G_{M1}, or in the synthesis of any of the neutral sphingoglycolipids. Since the transformed cell line was a subclone of a clonal line of BALB/3T3 and the transformed cells were nonproductively transformed (no detectable virus production), the alteration in ganglioside metabolism appears to be directly related to changes associated with transformation.

A consistent decrease in *N*-acetylgalactosaminyltransferase activity has been observed in mouse cells transformed by DNA oncogenic viruses (see refs. 6 and 25; but also refs. 17 and 18) and more recently in Swiss 3T3 cells transformed by the Moloney isolate of MSV (19). Changes in the glycolipid composition of hamster fibroblasts and chick-embryo cells after transformation by DNA and RNA tumorigenic viruses have been reported (20–22) as well as changes in the glycosyltransferases involved in their synthesis (23, 24). These previous observations together with the present report indicate that changes in cellular glycolipids after viral transformation are very complex and may be influenced by both the type of cells involved and the viruses used. A block in the biosyn-

thesis of ganglioside G_{M1} also appears to occur in other transformed cell systems. These include SV40-transformed human fibroblasts (ref. 20; P. H. Fishman, unpublished), and spontaneously transformed mouse-embryo cells (25). More recently this same change has been observed by us in subclones of A31 cells after chemical and x-irradiation-induced transformation. These latter findings suggest that there may be common biochemical responses by cells to various transforming agents and that the observed defect in ganglioside biosynthesis may be an important aspect of the transformation process.

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