*GLYCOLIPIDS OF HAMSTER FIBROBLASTS AND DERIVED MALIGNANT-TRANSFORMED CELL LINES**

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Sphingoglycolipids are, in general, cell-bound components representing a large variety of carbohydrate compositions and structures which are characteristic for a cell and can vary depending on the species and tissue of origin.[1-3] The glycolipids located on the surface of the cells may play a role in maintaining the "ektobiological" as well as the immunological characteristics of mammalian cells.[4]

A structural change of the "ektopolysaccharides" induced in bacteria by lysogeny and in spontaneous mutants has been well established.[5, 6] The question as to whether or not such a phenomenon would accompany the malignant transformation of mammalian cells is of interest for the understanding of the "ektobiological" characteristics of cancer cells. In view of the observations that an accumulation of a particular type of Le^a-active sphingoglycolipid ("tumor glycolipid")[7, 8] and a simultaneous deletion of A- and B-haptens[9-11] have been found in human cancer tissue, a structural change of the glycolipids or glycoproteins may well be expected to occur during the malignant transformation of mammalian cells.

A preliminary step to evaluate this possibility on an unequivocal basis would be the comparison of the glycolipid compositions of normal and malignant cells that have originated from the same source of cells. The present communication reports the chemical characterization and quantitation of the sphingoglycolipids present in normal hamster kidney fibroblasts (BHK 21-C13) and a malignant cell line derived from them by polyoma virus transformation. The results of such a comparison support the concept that the "incompleteness of the carbohydrate chain" in the sphingoglycolipids is one of the molecular events accompanying the malignant transformation of these cells.

Material and Methods.—Glycolipid samples used in this experiment were all prepared in this laboratory: ceramide galactoside and gangliosides were prepared from the human brain; ceramide lactoside, ceramide trihexoside, and globoside were prepared from human erythrocytes.[1] Hematoside was prepared from horse erythrocytes[12] and from human erythrocytes.[22]

Three hamster kidney cell lines used in this experiment were cloned in our laboratory from the hamster kidney cell line BHK-21 (clone 13). Clone 1, established in December 1966, shows good contact inhibition, and injection of 10^6 cells into 20-day-old hamsters did not give rise to a tumor after 1 month. Clone 2 was also established in December 1966, and showed less contact inhibition as compared to clone 1. Injection of 10^6 cells into hamsters gave rise to palpable tumors after 3 weeks. It is not clear whether the malignancy of this line was due to a spontaneous transformation or due to the selection of a malignant cell already present in the original population of clone 13 cells. However, since the cells of this line were never knowingly exposed to virus, they shall be referred to as the spontaneously transformed cell line. Clone 3 was obtained from clone 1 by treatment with polyoma virus and isolated in January 1967, by the selective agar assay

technique.[13] Injection of 10^6 clone 3 cells into hamsters gave rise to tumors after 1 week. The cells exhibit no contact inhibition. These cell lines were grown in modified Eagle's medium (2× amino acids and vitamins) supplemented with 10% horse serum.

Extraction, fractionation, and quantitative determination of glycolipids: Cells were harvested without trypsinization, centrifuged, washed twice with saline, and collected by centrifugation in a graduated conical centrifuge tube (2000 rpm, 5 min). The cell pellet was suspended in 3 times its volume of physiological saline and small aliquots (0.05–0.1 ml) were taken for the determination of protein content by the biuret reaction. Cell suspensions with equivalent biuret values were prepared. It was observed that the protein content was a direct function of the cell number. Cell suspensions containing 100 mg protein per 5 ml were used usually for the subsequent extractions. Such a cell suspension was mixed with 20 vol of chloroform–methanol (2:1) and blended in a Waring Blendor for 2 min, and filtered with suction (Extr. I). The residue on the filter was then extracted with chloroform–methanol (1:2) as described above and filtered (Extr. II). Extr. II was evaporated to dryness *in vacuo* and dissolved in Extr. I. Water ($^1/_6$ vol) was added and the mixture thoroughly shaken and centrifuged. The upper layer was removed and the lower layer was shaken twice with $^1/_3$ vol of 0.1% NaCl–methanol–chloroform (1:1:0.1). The upper layers were combined, concentrated to $^1/_{10}$ vol by distillation, dialyzed against distilled water for 2 days, lyophilized, and extracted with chloroform–methanol (2:1). Insoluble material was removed by centrifugation and the supernatant containing all gangliosides and 90% of hematoside was dried under nitrogen (Fr. 1). The Fr. 1 was dissolved in 0.2 ml of chloroform–methanol (2:1) and 20-μl portions were analyzed by thin-layer chromatography on silica gel H plates developed with chloroform–methanol–water (60:35:8) as the solvent. The principal glycolipid in this fraction, which was eventually characterized to be sialyllactosylceramide (hematoside), was separated by preparative thin-layer chromatography. Hematoside was quantitatively determined by the sialic acid content[14] after being separated on thin-layer chromatography followed by iodine stain and extraction of the spot.

The lower phase was distilled to dryness, dissolved in 0.1 *N* NaOH in methanol–water (9:1) and saponified at 37° for 6 hr. Two vol of chloroform and $^1/_2$ vol of water were added to the reaction mixture and the mixture shaken. The lower layer was again shaken with $^1/_3$ vol of water. The lower layer was evaporated under nitrogen and dried in a desiccator. The dried residue was dissolved in chloroform–methanol (9:1) and put onto a column of Florisil (0.9 × 5 cm) prepared in the same solvent. Elution was carried out with chloroform–methanol mixtures in the ratios: 8:2, 7:3, 6:4, 4:6, and 2:8. Each fraction was analyzed by thin-layer chromatography on silica gel H. Since the third fraction, eluted from the Florisil column with chloroform–methanol (6:4), contained only lactosylceramide, the quantitative determination of this glycolipid was carried out by analysis of the total hexoses of this fraction by cystein-sulfuric acid.[15] On the other hand, semiquantitative estimation of the glycolipids on thin-layer chromatography was performed by comparison of the intensities of the spots developed with orcinol-sulfuric acid with known quantities (3–30 μg) of standard glycolipids. This simple method was found to be highly dependable.

Characterization of glycolipids: The limited amounts of tissue culture cells available yielded only micromolar quantities of the glycolipids. Nevertheless, the main glycolipids have been sufficiently characterized on the bases of: (1) migration rates determined by thin-layer chromatography on silica gel H and on borate-impregnated silica gel H developed with chloroform–methanol–water, 60:35:8, 65:25:4, and chloroform–methanol–2.5 *N* ammonia, 60:35:8;[16] (2) sugar ratio analysis by gas-liquid chromatography of glycolipid fractions obtained from the thin-layer plates;[17] (3) identification of the oligosaccharides by thin-layer chromatography after osmium-periodate and alkaline degradation;[18] and (4) analysis of sphingosin by thin-layer chromatography.[19]

Cytoagglutination by wheat germ phytoagglutinin and its inhibition: Wheat germ phytoagglutinin was purified according to the method of Burger and Goldberg.[20] Agglutinability of different cell lines was tested according to Aub *et al.*[21] Cells were trypsinized

for 2 min and suspensions containing 10^6 cells per milliliter of phosphate-saline at pH 7.4 was used. Inhibition of the cytoagglutination was tested in the presence of "carrier lipid" as has been described earlier.[8]

Results.—Characterization of the glycolipids of hamster kidney fibroblast cells: The main glycolipid found in Fr. 1 of the three cell lines of hamster kidney fibroblasts was identified to be a *N*-acetylneuraminyllactosylceramide, based on the following findings: (1) the glycolipid had a R_f value identical to the "hematoside" of human erythrocytes[21] when subjected to thin-layer chromatography on silica gel H; (2) it contained equimolar quantities of glucose, galactose, and sialic acid; (3) hydrolysis in 0.1 *N* hydrochloric acid at 80° for one hour split the glycolipid into lactosylceramide and *N*-acetylneuraminic acid; and (4) degradation of the glycolipid as described by Hakomori[18] gave a spot which was coincident with *N*-acetylneuraminyl (2→3) galactosyl (1→4) glucose (product of General Biochemicals, Ohio) but not with *N*-glycolyl-neuraminyl (2→3) galactosyl (1→4) glucose.[18]

The hydrophobic glycolipids which are present in Fr. 2 were investigated after the glycerophosphatides had been eliminated by saponification.[23] Only one kind of glycolipid, which was identified as lactosylceramide, was found to be present. No detectable amounts of cerebroside, ceramide trihexoside, or globosides were found in any of the three cell lines. The identification of lactosylceramide was based on the liberation of lactose by the degradation previously described[18] and by the finding that the R_f values of the double spots given by this glycolipid on thin-layer chromatography were identical to those of lactosylceramide isolated from erythrocytes.[24]

Reciprocal relationship between the quantities of hematoside and lactosylceramide in normal and transformed cell lines: The quantities of hematoside and lactosylceramide present in a cell are found to show a reciprocal relationship depending on whether the cells are derived from the normal or one of the malignant cell lines (Fig. 1 and Table 1). The hematoside content was the highest in the normal fibroblast line (clone 1), followed by that in the spontaneously transformed line (clone 2), and the polyoma virus-transformed cell line (clone 3) contained only one fifth the quantity found in the normal fibroblasts. The quanti-

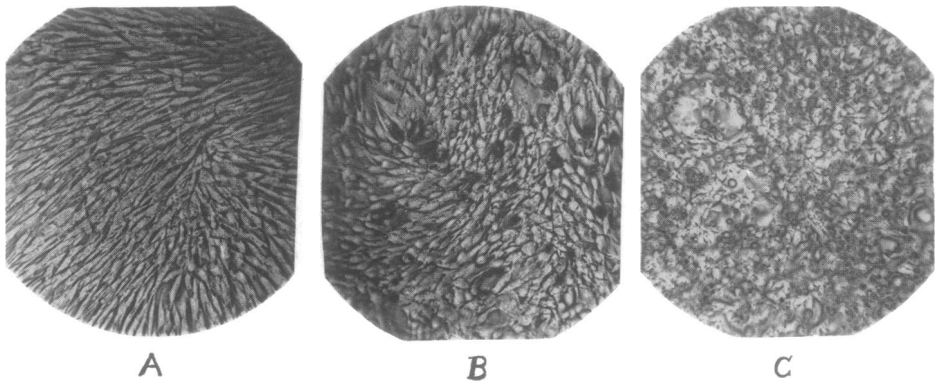

FIG. 1.—Microphotographs of three cell lines: (*A*) clone 1, (*B*) clone 2, and (*C*) clone 3.

TABLE 1. *The quantity of hematoside and lactosylceramide in normal and malignant-transformed fibroblasts.**

	Hematoside	Lactosylceramide
	(μg per 100 mg protein)	
Normal C13 fibroblasts (clone 1)	475	13
Spontaneously transformed cells (clone 2)	310	117
Polyoma virus-transformed cells (clone 3)	105	125

* Mean value of duplicate experiment.

ties of lactosylceramide were found to have a reversed relationship; the level in normal fibroblasts was only one tenth that found in the virus-transformed line and the level of the clone 2 cell line was almost the same as that of the malignant cell line.

Agglutinability of normal and malignant-transformed cells by wheat germ phytoagglutinin and the presence of a specific glycolipid in the malignant cells which is capable of inhibiting the malignant cytoagglutination: The malignant cytoagglutination evoked by wheat germ phytoagglutinin was found to be parallel with the malignancy of the cells (Table 2). In addition to hematoside and lactosylcera-

TABLE 2. *Agglutinability of normal and malignant-transformed fibroblasts.**

Purified wheat germ phytoagglutinin added (μg/0.1 ml)	Normal C13 cells (clone 1)	Spontaneously transformed C13 cells (clone 2)	Polyoma virus-transformed cells (clone 3)
20	0	1+	3+
10	0	1+	3+
5	0	0	2+
2	0	0	2+
1	0	0	1+
0.6	0	0	0

* Wheat germ phytoagglutinin (0.1 ml) was mixed with 0.1 ml of cell suspension that contains 10^5 cells (10^6 cells/ml). Read according to Aub *et al.*[21]

mide, Fr. 1 contained a small number of gangliosides or higher glycolipids in much smaller quantities. The ganglioside fraction of the malignant cell line inhibited the malignant cytoagglutination specifically (Table 3). The finding of Burger and Goldberg[20] that *N*-acetylglucosamine is the sole monosaccharide inhibitor of this agglutination system has been confirmed.

The ganglioside fraction of the normal cell line was found to have no inhibitory activity. However, oxidation of the normal ganglioside fraction in aqueous solution with 0.01 *M* sodium metaperiodate for one hour, followed by sodium borohydride treatment and weak acid hydrolysis (Smith degradation[25]) yielded a product which was found to be capable of inhibiting the malignant cytoagglutination (see Table 3). Control experiments with globoside and "tumor glycolipid" indicated that only the nonreducing sugar was degraded under the same condition. Simple acid hydrolysis did not cause any activity changes in the normal gangliosides.

Discussion.—The glycolipids of fibroblasts have not been investigated previously. It has become clear through this study that the main glycolipid of normal hamster fibroblasts is a "hematoside" type of glycolipid,[1, 12] containing *N*-

TABLE 3. *Inhibition of malignant cytoagglutination by the ganglioside fraction of normal and malignant-transformed fibroblasts and by some other glycolipids.**

Glycolipids	Minimum quantity (in μg) of glycolipid required for 100% inhibition of malignant cytoagglutination
Fr. 1 of normal C13 fibroblast (clone 1)	>100
Fr. 1 of spontaneously transformed C13 fibroblasts (clone 2)	12.5
Fr. 1 of malignant-transformed cell (clone 3)	6
Gangliosides of Fr. 1 of malignant cells, obtained on thin-layer chromatography	3
Fr. 1 of normal fibroblasts, treated by 0.1 *N* HCl, 80°, 1 hr	>100
Fr. 1 of normal fibroblasts, product of the Smith degradation	12.5
Human brain ganglioside plus lactosylceramide	>100
Trihexosylceramide (human erythrocytes)	>100
Globoside (human erythrocytes)	>100
Hematoside (human erythrocytes)	>100

* Determined in the presence of "carrier lipid" according to Hakomori *et al.*[8]

acetylneuraminic acid. The same glycolipid has been known to be a component of human spleen.[26] Lactosylceramide and a small number of gangliosides were found to be minor components. This pattern of glycolipid composition is much simpler than that of erythrocytes or various organs, since no detectable amounts of cerebrosides, ceramide trihexosides, globosides, or sulfatides were found as components.

It is of interest that the hematoside content of fibroblasts was greatly diminished, while that of lactosylceramide, a precursor of the hematoside, was apparently increased after the fibroblasts had been transformed into malignant cells by polyoma virus (Fig. 2). The cell line (clone 2) whose malignant property was intermediate between the normal and the virus-transformed line had an intermediate pattern of glycolipid composition.

A fairly good correlation was found between the quantity of hematoside and the contact-inhibitory properties of the three lines of hamster kindey fibroblasts studied. The quantity of lactosylceramide present was in reciprocal proportion to the hematoside. In other words, the more contact-inhibited cells contained more glycolipids with completed carbohydrate chains and a smaller quantity of precursor glycolipid, and vice versa. The larger quantity of lactosylceramide and the smaller quantity of hematoside in the noncontact-inhibited malignant cells could be the result of either incomplete synthesis of hematoside, possibly due to an impaired sialyl transfer mechanism, or enhanced neuraminidase activity. These results contrast to the earlier finding that sialic acids on cell surface of malignant cells were higher than that of normal cells.[27] Sialic acids bound to lipid is a small portion of total sialic acid, and the true mechanism which determines the quantity of hematoside and lactosylceramide may be more complex than is simply predicted. Whatever the mechanism is, this finding suggests the occurrence of "incomplete carbohydrate chains" in the glycolipid of malignant cells. The large increase in the concentration of lactosylceramide, following malignant transformation, may be related to the finding by Rapport *et al.*[28] that lactosylceramide ("cytolipin H") is a tumor-specific hapten in many

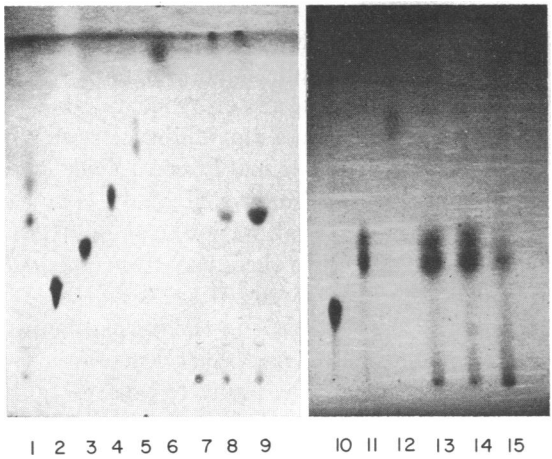

FIG. 2.—Thin-layer chromatography of glycolipid of normal fibroblasts and its malignant-transformed cell lines.

(*1*) Hematoside fraction of horse erythrocytes; (*2*) human brain gangliosides; (*3*) globoside of human erythrocytes; (*4*) ceramide trihexoside of human erythrocytes; (*5*) ceramide dihexoside (CL) of human erythrocytes; (*6*) cerebrocide; (*7*) Fr. 1 of clone 3; (*8*) Fr. 1 of clone 2; (*9*) Fr. 1 of clone 1; (*10*) ceramide trihexoside; (*11*) ceramide lactoside (CL); (*12*) cerebroside; (*13*) CL fraction of clone 3; (*14*) CL fraction of clone 2; (*15*) CL fraction of clone 1.

The quantity of the starting cells was identical as 100 mg protein/5 ml; Fr. 1 was dissolved in 0.2 ml, and 20 μl was applied. CL fraction was dissolved in 0.2 ml, and 20 μl was applied.

cases of human tumor. The parallelism in the quantity of hematoside and the contact-inhibitory property of the fibroblast may suggest a certain role for the hematoside in maintaining the "ektobiological" characteristics of the normal fibroblasts. However, one has to be careful in drawing such a conclusion since other glycolipids were also altered by malignant transformation. No evidence was furnished, at the present time, that hematoside and lactosylceramide are located on the cell surfaces.

The agglutinability of the three cell lines by the purified wheat germ phytoagglutinin showed a surprisingly good parallelism between their contact inhibitory properties. The ganglioside fraction of malignant cells could indeed inhibit the malignant cell agglutination but the ganglioside fraction of normal cells was without activity. This again indicates that some structural change of the gangliosides may occur concomitant to the malignant transformation of cells and the appearance of an abnormal ganglioside may closely relate to the change in property of the cell surface. The characterization of such abnormal gangliosides is now in progress.

The conversion of the normal gangliosides with no inhibitory activity to a material which is capable of inhibiting the malignant cytoagglutination by the Smith degradation suggests that the reactive group is masked in the normal ganglioside by a periodate-susceptible group (either galactose or fucose). The removal of such a masking group from the normal ganglioside can transform the normal ganglioside to an abnormal ganglioside which is capable of inhibiting the malignant cytoagglutination. This again suggests the occurrence of an incom-

plete carbohydrate chain in the glycolipids of malignant cells. This concept is compatible with the earlier results showing the deletion of blood group A- and B-haptens and the simultaneous accumulation of Le^a and H-glycolipids in human adenocarcinoma.[8, 9, 29] Such a "deletion" or "incompletion" of carbohydrate chains must be a result of either a defect or an inhibition of enzymes participating in metabolism of sugars which are presumably controlled by regulatory genes. Galactose-epimerase "choke" in different malignant cells as illustrated by Kalckar[30, 31] is such an example. The alteration in the pattern of sugar metabolism and function of cells in relation to the structural remodeling of glycolipids and glycoproteins are undoubtedly a key to open a secret box of malignancy.

Summary.—The main glycolipid present in the normal hamster kidney fibroblasts (BHK-21,C13) was identified to be *N*-acetyl-neuraminyllactosylceramide (hematoside), the quantity of which was found to be greatly diminished in the malignant-transformed cells. The second glycolipid, which was characterized to be lactosylceramide, increased in the malignant-transformed cells. A reciprocal relationship between the quantities of hematoside and lactosylceramide present has been noticed to exist depending on the biological properties of the cells.

The cytoagglutination evoked by purified wheat germ agglutinin, characteristic for malignant cells, was specifically inhibited by the ganglioside fraction of malignant cells but not by the same fraction of normal fibroblasts.

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