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Glycolipid-bound sialic acid in serum: Increased levels in mice and humans bearing mammary carcinomas

(ganglioside/blood/cancer/cancer detection)

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ABSTRACT **In mice bearing transplantable mammary carcinomas, serum levels of sialic acid-containing glycolipids were elevated 2.5-fold in pooled serum samples from which gangliosides were purified by column chromatography. A method is also described by which ganglioside content was estimated on as little as 1.0 ml of whole blood to permit studies with individual tumor-bearing mice and age- and litter-matched controls. Using this method, we observed similar elevations in ganglioside levels that were independent of age and sex of the animal and appeared in advance of palpable tumors. Following excision of the tumors, the glycolipid sialic acid values dropped below control levels and remained there. Serum sialic acid of the glycolipid fraction was elevated nearly 2-fold in human carcinoma patients and appeared to decline after surgery.**

Differences in the chemical makeup of the cell surface of normal and tumor cells have revealed that glycosylated macromolecules, glycoproteins and glycolipids, show consistent patterns of alteration (1). Warren, Glick, and their associates (2–5) and, more recently, others (6) have reported that tumor-characteristic sialofucosyl glycopeptides, present only in trace amounts in normal cells, are considerably elevated in transformed cells. Glycopeptide alterations have been demonstrated in tumor cells obtained from peripheral blood of patients with active leukemia (untreated patients with at least 80% leukemic cells in the white cell fraction) (7). Sialic acid-containing glycosphingolipids, termed gangliosides, which are associated with endo- and plasma membranes (8, 9), are also altered in the direction of increased amounts of sialic acid and a simplified pattern of distribution (10–13,¶). Glycolipids offer an experimental advantage when compared to glycoproteins in terms of extraction, quantification, and chemical identification (14, 15). Recent studies from our laboratory show that gangliosides and ganglioside biosynthetic enzymes are elevated not only in the tumors themselves but also in tissues surrounding the tumors.¶ Certainly different gangliosides are elevated in different types of tumors even from the same tissues of origin.¶ However, all contain sialic acid.

In this report, we show that tumor-bearing mice, an animal model, and human carcinoma patients have elevated levels of glycolipid-bound sialic acid in serum. Although we recognize that these increased levels of circulating glycolipids may be a secondary response to the tumor, indications are that the findings may provide the basis for a biochemical method for early detection of cancer.

MATERIALS AND METHODS

Experimental Animals and Tumors. The tumor used was a transplantable mammary carcinoma, designated ST II, which originated spontaneously in a retired breeding female of the mouse strain C_3H/HeJ. The tumor was propagated by subcutaneous implantation of tumor fragments into male or female syngeneic recipients. Age-matched controls of the same strain were injected with an equivalent amount of saline but received no tumor implant and were otherwise treated exactly as the principals. Tumor masses were examined for degree of vascularization and then were excised and weighed.

Determination of Gangliosides. Groups of 25 carcinoma-bearing mice were exsanguinated 60 days after implantation of tumor fragments. Sera were pooled following centrifugation. Gangliosides were extracted with chloroform/methanol and further purified by chromatography on DEAE-Sephadex and Unisil silicic acid (16). Sialic acid assays (17) were on purified gangliosides. By this method recovery of [6-^{3}H]galactose-*N*-acetylgalactosamine-(*N*-acetylneuraminic acid)-galactose-glucose-ceramide ([^{3}H]GM_1) added to samples averaged 96%.

Individual mice bearing tumors were exsanguinated at 3–90 days after implantation of tumors along with litter-matched control mice as described above. The sera (0.5–1 ml) obtained following centrifugation were extracted with chloroform/methanol (1:1 vol/vol) overnight. The extracts were filtered and the residue was extracted a second time with chloroform/methanol (2:1) overnight. The combined extracts were concentrated and adjusted to 3 ml. To precipitate sialic acid-containing lipo- and glycoproteins (not elevated in sera of tumor bearers), 100 μl of a 0.1% solution of tripotassium citrate (18) was added and the resulting precipitate was removed by centrifugation. The supernatant after citrate treatment was analyzed for sialic acid (17) using values calculated by the Warren (17) formula to correct for variation due to unspecific absorbance. With this method recovery of [^{3}H]GM_1 added to serum samples averaged 92%.

RESULTS

Animal model

The level of total gangliosides, determined on the basis of sialic acid, was elevated about 2.5 times above control values in sera of both male and female recipient mice carrying a transplantable mammary carcinoma of spontaneous origin (Table 1). Similar elevations were observed at 11, 21, and 35 days after transplantation; at 11 days palpable tumor masses were not detected. These increases were not reflected by whole serum sialic acid or by sialic acid content of precipitated lipoproteins and glycoproteins, which account for more than 90% of the sialic acid of the serum.

If sialic acid levels are to have diagnostic implications, it is essential that serum from individual animals be analyzed. Therefore, a simplified extraction and purification procedure was employed that permitted analyses on 0.5–1 ml of serum. When analyzed in this manner, all tumor-bearing mice had

¶ W. D. Merritt, T. W. Keenan, and D. J. Morré, unpublished data.

Table 1. Levels of ganglioside-bound sialic acid in pooled samples of sera from tumor-bearing mice 60 days after transplantation

Group	Sex	Sialic acid,* nmol/ml serum	Ratio, experimental/ control
Control	Male	14.7	
	Female	16.0	
Tumor-bearing	Male	32.6	2.21
	Female	42.4	2.65

* Gangliosides were extracted and purified by column chromatography (16) from pooled sera of 25 mice prior to determination of ganglioside sialic acid (17).

elevated serum levels of sialic acid when compared with age- and litter-matched controls (Table 2). The degree of elevation varied among individuals from 40 to 230%, but each was significantly different from controls at the 99% confidence interval. Near-quantitative extraction of gangliosides by the simplified method was shown from time-course extraction studies, recovery of known amounts of purified gangliosides, and comparisons with standard chloroform/methanol extraction procedures (compare Tables 1 and 2).

Determinations similar to those of Table 2 were conducted on the erythrocyte plug that formed after centrifugation of individual serum samples. This was carried out on the expectation that some proportion of the glycolipids of the serum might absorb to the erythrocyte membrane. There was, however, no increase in the level of glycolipid sialic acid of the erythrocytes that could be detected. Both control and tumor-bearing sera averaged 150 nmol/g of wet weight of red blood cells.

Time course studies showed that sialic acid levels were elevated after 10–15 days for a fast-growing tumor of spontaneous origin and after about 20 days for a slower-growing transplantable dimethylbenzanthracene-induced tumor. In both systems, tumor burdens of less than 0.5 g appeared capable of eliciting the ganglioside increase. On day 47, tumors were removed from eight mice and the animals were sacrificed 1, 2, 5, or 10 days after excision. Following excision, the serum levels of ganglioside sialic acid dropped from 50 ± 10 nmol/ml to 20 ± 5 nmol/ml and remained at this low level. Sham operation of control mice did not alter serum levels of ganglioside sialic acid.

Table 2. Lipid-soluble sialic acid in sera from individual male mice bearing transplantable mammary carcinomas

Degree of vascularization*	Tumor mass, g	Sialic acid, nmol/ml serum	Ratio, experimental/ control
Low	0.51	29	1.5
	0.98	44	2.2
	2.00	27	1.3
	3.20	34	1.7
	3.25	50	2.5
	5.20	61	3.0
High	5.10	35	1.7
	5.45	64	3.2
	6.00	31	1.5
	8.00	39	1.9
	8.80	43	2.1
	9.40	53	2.7

Gangliosides were determined on lipid extracts following precipitation of lipo- and glycoproteins by the citrate procedure for sera 8 weeks after transplantation. Experimental values are averages from duplicate assays on 0.5 ml of serum. Average control value (± SD) for four mice was 20 ± 4 nmol sialic acid per ml of serum. All individual experimental values were significantly different from controls at a 99% confidence interval.

* Subjectively judged from number and size of prominent blood vessels associated with the tumor mass.

Table 3. Lipid-soluble sialic acid in sera of human carcinoma patients

Sex	Tumor status	Individuals	Sialic acid, nmol/ml serum ± SD
Female	Normal*	10	16 ± 3
	Mammary carcinoma	7	49 ± 20
	After surgery†	6	31 ± 8
	Colonic carcinoma	5	49 ± 8
	After surgery†	3	39 ± 1
Male	Normal*	8	24 ± 4
	Colonic carcinoma	5	47 ± 9
	After surgery†	3	24 ± 9

* Control value for pooled transfusion blood (four different lots) was 20 ± 2 nmol sialic acid per ml of serum.

† 7–54 days.

Human studies

Data with human subjects have thus far been limited to the values summarized in Table 3. While these findings must be considered as preliminary, they are encouraging. Within the normal population, the variation was ±20%, which approximates the uncertainty of the extraction procedure coupled with that of the colorimetric method for determination of sialic acid. Sera from males yielded somewhat higher values than sera from females but the sample size is still too small to substantiate this trend. Values did not vary significantly with age of the individual; a slight downward trend was noted. All individuals were adults aged 21 to 71. As with the animal model, serum sialic acid of the glycolipid fraction was elevated nearly 2-fold in the carcinoma patients and appeared to decline after surgery. Other correlations, such as with size of primary tumor or degree of metastasis, must await analysis of a larger sample.

DISCUSSION

Because a large proportion of all cancer deaths might be preventable with early diagnosis, a priority objective of the National Cancer Program is the development of biochemical and/or immunological methods for early detection based on analysis of exfoliated cells or body fluids obtained from apparently normal individuals. Our findings indicate, for both an animal model and human carcinoma patients, elevated serum levels of sialic acid associated with tumor burden. The elevation occurs principally in the ganglioside fraction; the fractions analyzed contain virtually no protein. Neutral glycolipids would not be detected because they contain no sialic acid. Procedures for isolation, identification, and quantification of the individual gangliosides affected in different tumor-bearers remain to be applied.

An elevated and altered ganglioside pattern for pooled serum samples of Morris hepatoma-bearing rats (19) showed that the altered pattern in the serum corresponded to that of the tumor tissue. Our preliminary data for pooled sera from mice bearing

transplantable mammary carcinomas agree with these results.

The mechanism(s) causing alterations of serum glycolipid levels must be considered as unknown. Barkai and DiCesare (20) have shown that glycosphingolipids containing sialic acid (i.e., gangliosides) have relatively long retention times in blood plasma compared to asialo lipids (neutral glycolipids). Thus with the report of shedding of tumor antigens and membranes by tumor cells (21) it is possible that gangliosides might accumulate in the serum. Moskal *et al.* (22) have shown that tumorigenic clones of neuroblastoma cell lines derived from C-1300 tumors (23) have large elevations in sialyltransferase activities with several glycosphingolipid acceptors. Bernacki and Kim (24) observed concomitant elevations of both sialyltransferase activity with glycoprotein acceptors and total sialic acid content in serum samples from rats bearing transplanted, spontaneously metastasizing mammary tumors. Neoproteolipid S and neoproteolipid W are elevated in lipid extracts from whole serum and high density lipoproteins from tumor-bearing rats (25). The neoproteolipid S, at least, appears to contain gangliosides and may help to explain how gangliosides are carried in the blood (26). However, our findings indicate that an early response to a tumor or to tumor growth is an elevation of both the biosynthetic enzymes that produce gangliosides (up to 20-fold) and in the gangliosides themselves both in tumors and in surrounding tissues.¶ On the basis of these observations, a 2-fold elevation in circulating sialic acid-containing glycolipids is not unexpected.

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