# Mammary Carcinoma: Enzymatic Block in Disialoganglioside Biosynthesis

Abstract. *The sialyl transferase of disialoganglioside formation is depressed in mammary tumors induced in the rat by 7,12-dimethylbenz*[a]*anthracene. Specific activities of other glycosyltransferases of the pathway ceramide to monosialoganglioside are unchanged or elevated so that the ganglioside $GM_1$ accumulates and higher gangliosides are depressed. These findings with a solid tumor are critical to an involvement of gangliosides in the cell-surface changes of tumorigenesis.*

Gangliosides, glycosphingolipids that contain sialic acid, are ubiquitous in mammalian tissues (*1*) and have been implicated in cellular recognition and adhesion (*2*). Altered ganglioside patterns have been demonstrated in chemically transformed and virus-transformed cell lines (*3*). A buildup of lower gangliosides at the expense of higher ganglioside homologs has been observed. In several lines of transformed cells of mice, Brady and his colleagues (*4*) found an accumulation of $G_{M3}$ that they attributed to depressed activity of the enzyme which transfers *N*-acetylgalactosamine from UDP-*N*-acetylgalactosamine to $G_{M3}$ with resultant formation of $G_{M2}$ (*5*).

If, as has been implied, these ganglioside changes are related to phenotypic manifestations in tumorigenesis, similar alterations should be manifested in solid tumors. It is of particular importance to demonstrate such alteration in solid tumors since, with cultured cells, it is difficult to separate effects of transformation from cloning effects. We now report an altered ganglioside pattern in rat mammary carcinomas; in this tumor $G_{M1}$ accumulates at the expense of disialogangliosides. This we attribute to depression of the activity of the enzyme that transfers NAN (*5*) from CMP-NAN to $G_{M1}$ with resultant formation of disialogangliosides.

Rat mammary carcinomas were induced by administering a single oral dose of 7,12-dimethylbenz[*a*]anthracene (12 mg dissolved in sesame oil) to female Holtzman rats weighing 150 to 170 g (*6*). Control rats received sesame oil without added carcinogen. Tumors ranging in diameter from 0.2 to 1 cm were collected, and analyses were conducted with combined tumors or control mammary tissue from at least eight rats.

Tissue was homogenized in distilled water for chemical analyses or in 0.32*M* sucrose containing 14 m*M* 2-mercaptoethanol for enzyme assays. Lipids were extracted with a mixture of chloroform and menthanol; gangliosides were recovered by partitioning, purified, and then analyzed by sialic acid assay and thin-layer chromatography (*7*). Methods for neutral lipid and phospholipid analyses have been described (*8*). Homogenates were analyzed for protein (*8*), total and bound sialic acid (*9*), and 5′-nucleotidase activity (*10*) (adenosine 5′-monophosphate substrate).

For enzyme determination, tumor and control homogenates were centrifuged at 1000*g* for 10 minutes to remove unbroken cells, nuclei, and debris. The supernatants containing the glycosyltransferase activities were centrifuged at 176,000*g* for 60 minutes, and the resulting supernatants and floating lipid plugs were removed and discarded. Pellets were resuspended in 0.32*M* sucrose containing 14 m*M* 2-mercaptoethanol and used as the enzyme source. Assay mixtures were patterned after those referenced (*11*). For synthesis of Cer-Glu and Cer-Glu-Gal, reactions were terminated by addition of five volumes of a mixture of chloroform and methanol (2:1, by volume); the lower chloroform phases were recovered and placed on thin-layer plates. The thin-layer plates were developed in a mixture of chloroform, methanol, and water (70 : 22 : 3, by volume), and the zones corresponding to authentic Cer-Glu and Cer-Glu-Gal were recovered and assayed by liquid scintillation counting. For other transferase assays, reactions were terminated by addition of one volume of methanol, and the reaction mixture was applied to Whatman 3 MM paper. Papers were developed (descending) overnight in 1 percent sodium tetraborate to separate the radioactive sugar nucleotides and their degradation products from glycolipids, which remained at the origin (*11*). The origins were cut out, eluted with 15 ml of a mixture of chloroform, methanol, and water (2 : 1 : 0.2, by volume), and the eluates were evaporated and the radioactivity was counted by liquid scintillation methods. Lipid acceptors were either prepared by standard methods in this laboratory or were gifts (*12*).

Table 1. Composition of rat mammary tissue and 7,12-dimethylbenz[*a*]-anthracene-induced rat mammary tumors.

| Constituent | Control | Tumor |
|---|---|---|
| Sialic acid* | | |
| Total | 19.6 | 48.6 |
| Bound | 18.2 | 40.5 |
| Ganglioside | 1.05 | 2.09 |
| 5′-Nucleotidase† | 7.0 | 11.6 |
| Total lipid‡ | 17.8 | 0.6 |
| Lipid phosphorus§ | 4.3 | 9.0 |
| Total sterol§ | 26.9 | 42.4 |

* Units are nanomoles per milligram of protein. † Units are micromoles of adenosine monophosphate hydrolyzed per hour per milligram of protein. ‡ Units are milligrams per milligram of protein. § Units are micrograms per milligram of protein.

As compared to control tissue, tumors contained at least double the amount of total sialic acid, bound sialic acid, and ganglioside sialic acid on a protein basis (Table 1). The 5′-nucleotidase activity of tumors was also elevated; we have consistently noted a parallelism between 5′-nucleotidase activity and ganglioside content of homogenates and plasma membrane fractions (*7*). The total lipid content of tumors was much lower than that of control tissue; however, elevated concentrations of phospholipid and sterols were encountered in tumors. Among individual lipid classes, triglyceride concentrations were depressed in tumors. Distribution patterns and fatty acid composition of individual phospholipids and neutral lipids other than triglycerides were similar for both control tissue and tumor (*13*).

The ganglioside composition of mammary tumors was less complex than that of control mammary tissue (Fig. 1). Whereas control tissue had several major gangliosides with the thin-layer mobilities of disialo- and trisialogangliosides, only one major component, which migrated with authentic $G_{M1}$, was present in tumors. As was determined by densitometry, the component with $G_{M1}$ mobility accounted for about 58 percent of the total ganglioside sialic acid in tumor and for only about 12 percent of the total in control tissue. Whereas the di- and trisialo types accounted for nearly 80 percent of the total ganglioside sialic acid in control tissue, they accounted for only about 32 percent of the total recovered from tumors.

Enzymatic activities for the glycosyltransferases involved in ganglioside biosynthesis are given in Table 2. The specific activities in the tumor of all enzymes involved in the synthesis of

monosialogangliosides starting with ceramide were nearly the same as or above the respective enzymatic activities observed with control tissue. Similarly the enzyme involved in transfer of NAN from CMP-NAN to $G_{M3}$ with resultant formation of the disialoganglioside $G_{D3}$ was greatly elevated in the tumor. The specific activity of the enzyme transferring NAN from CMP-NAN to $G_{M1}$ with resultant formation of $G_{D1a}$ or $G_{D1b}$ disialogangliosides was only 9 percent as active in tumors compared to control tissue. These results were obtained under conditions where product formation was proportional to incubation time and enzyme activity. Experiments where tumor and control fractions were mixed gave results for CMP-NAN: $G_{M1}$ sialyltransferase which agreed with values calculated from specific activity data. This indicates that the result obtained was not due to the presence of an inhibitor or neuraminidase in the tumor. Our results are in contrast to, but agree in principle with, observations from virus-transformed cells of mouse liver, where the enzyme UDP-GalNAC : $G_{M3}$ *N*-acetylgalactosaminyltransferase is reduced (*4*). In these virus-transformed cells, levels of CMP-NAN : $G_{M1}$ sialyltransferase are normal (*4*).

Our results show, for the first time, a block in synthesis of higher gangliosides in a solid tumor. Other workers have found altered ganglioside patterns in solid tumors (*14*). While total gangliosides are elevated in mammary tumors, the net effect of this enzymatic aberration is the accumulation of high levels of ganglioside $G_{M1}$. The high specific activity of the enzyme CMP-NAN : $G_{M3}$ sialyltransferase imparts to the tumor the ability to synthesize $G_{D3}$ disialoganglioside. However, the low levels of components migrating with other disialogangliosides in the mammary tumor (Fig. 1) suggests that further glycosylation of $G_{D3}$ is not an important pathway in vivo. Kaufman *et al.* (*15*) obtained kinetic evidence suggesting that in brain different sialyl transferases are involved in synthesis of $G_{D1a}$ and $G_{D3}$. We have observed that in rat liver, these two transferase activities were localized in different subcellular fractions (*16*).

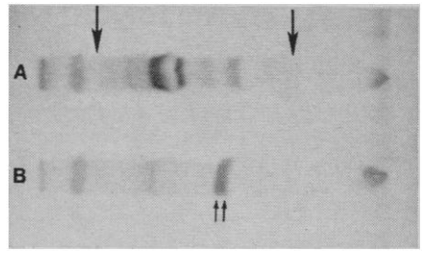


Fig. 1. Thin-layer chromatographic separation of ganglioside fractions from control rat mammary tissue (a) and rat mammary tumor (b). Components between single arrows gave positive reactions with resorcinol spray. The major ganglioside from tumor (double arrows) migrated with human brain $G_{M1}$. Plate coated with a 500-$\mu$m layer of silica gel G was developed in a mixture of chloroform, methanol, 28 percent ammonia and water (60 : 35 : 7 : 4, by volume) and sprayed with resorcinol reagent. The origin is on the left.

This biochemical aberration in ganglioside biosynthesis is potentially important in view of the known role of carbohydrate residues in imparting specificity to surface membranes (*2*). There is no assurance that this modification in ganglioside accumulation is specifically associated with plasma membrane. It had long been assumed that gangliosides were specifically localized in plasma membrane; however, we have recently found that, although gangliosides are enriched in surface membranes, they are also present in subcellular endomembranes (*7*). We have also found that several of the glycosyltransferases involved in ganglioside biosynthesis, including CMP-NAN : $G_{M1}$ sialyltransferase, are localized in rat liver Golgi apparatus (*16*). Thus, although the cellular localization of the elevated levels of $G_{M3}$ and of the enzymatic aberration in the mammary tumor remains to be elucidated, it seems likely that the Golgi apparatus will be involved.

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Table 2. Ganglioside biosynthetic enzymes in rat mammary tissue and in dimethylbenzanthracene induced rat mammary tumors. Incubation mixtures contained the following components in final volumes of 0.1 ml. For synthesis of ceramide monohexoside (CMH) and ceramide dihexoside (CDH): bicine-HCl (*p*H 6.8), 20 $\mu$mole; $MnCl_2$, 0.5 $\mu$mole; UDP-Glu (for CMH), 0.1 $\mu$mole or UDP-Gal (for CDH), 0.1 $\mu$mole; a mixture of Cutscum and Triton X-100 (2 : 1), 600 $\mu$g; ceramide (for CMH), 0.2 $\mu$mole or Cer-Glu (for CDH), 0.1 $\mu$mole. For the synthesis of $GM_2$ : cacodylate hydrochloride (*p*H 7.3), 15 $\mu$mole; $MnCl_2$, 2.5 $\mu$mole; UDP-GalNAC, 0.0025 $\mu$mole; Triton X-100, 300 $\mu$g; $G_{M3}$, 0.05 $\mu$mole. For the synthesis of $G_{M1}$: cacodylate hydrochloride (*p*H 7.3), 15 $\mu$mole; $MnCl_2$, 2.5 $\mu$mole; UDP-Gal, 0.1 $\mu$mole; a mixture of Tween 80 and Triton CF-54 (1 : 2), 600 $\mu$g; $G_{M2}$, 0.05 $\mu$mole. For the synthesis of $G_{M3}$, $G_{D3}$, and $G_{D1a}$ or $G_{D1b}$ cacodylate hydrochloride (*p*H 6.35), 15 $\mu$mole; $MgCl_2$, 1 $\mu$mole: CMP-NAN, 0.05 $\mu$mole; a mixture of Tween 80 and Triton CF-54 (1 : 2), 600 $\mu$g; acceptor (CDH for $G_{M2}$; $G_{M3}$ for $G_{D3}$; $G_{M1}$ for $G_{D1a}$ or $G_{D1b}$) 0.05 $\mu$mole. All reaction mixtures contained 50 $\mu$l of enzyme source (0.464 mg of protein). Controls without added acceptor were incubated simultaneously, and the values obtained were subtracted to obtain specific activities with exogenous acceptor. Incubations were for 3 hours at 37°C. Specific activity values are picomoles of carbohydrate incorporated per hour per milligram of protein, except for the conversion of $G_{M3} \rightarrow G_{M2}$, where specific activity is CPM *N*-acetylgalactosamine incorporated per hour per milligram of protein.

| Reaction | Specific activity | | |
|---|---|---|---|
| | Control | Tumor | Percent of control |
| Cer + UDP-Glu→CMH | 16.1 | 33.4 | 205.6 |
| CMH + UDP-Gal→CDH | 44.7 | 35.8 | 80.1 |
| CDH + CMP-NAN→$G_{M3}$ | 110.7 | 122.6 | 110.7 |
| $G_{M3}$ + UDP-GalNAC→$G_{M2}$ | 23.0 | 137.9 | 599.6 |
| $G_{M2}$ + UDP-Gal→$G_{M1}$ | 51.4 | 113.2 | 220.2 |
| $G_{M3}$ + CMP-NAN→$G_{D3}$ | 243.3 | 1630.6 | 670.2 |
| $G_{M1}$ + CMP-NAN→$G_{D1a}$ or $G_{D1b}$ | 130.8 | 12.8 | 9.0 |

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5. Abbreviations: Cer, ceramide (*N*-acylsphingosine); Glu, glucose; Gal, galactose; GalNAC, *N*-acetyl-galactosamine; NAN, *N*-acetylneuraminic acid; CMP, cytidine 5′-monophosphate; UDP, uridine 5′-diphosphate. Symbols proposed by L. Svennerholm [*J. Neurochem.* **10**, 613 (1963)] are used for abbreviation of gangliosides as follows: $G_{M3}$, Cer-Glu-Gal-NAN; $G_{M2}$, Cer-Glu-Gal-(NAN)-GalNAC; $G_{M1}$, Cer-Glu-Gal-(NAN)-GalNAC-Gal; $G_{D1a}$, Cer-Glu-Gal-(NAN-NAN)-GalNAC-Gal; $G_{D1b}$, Cer-Glu-Gal-(NAN)-GalNAC-Gal-NAN; $G_{D3}$, Cer-Glu-Gal-NAN-NAN.
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## Early Social Responses in Gallus: A Functional Analysis

Abstract. *Early socialization leads to the establishment of species awareness, or imprinting, in many birds and mammals.* Gallus *chicks normally develop species-specific preferences prior to exposure to a parent, but early exposure is necessary to maintain these preferences. Descriptive rather than functional analyses have led to apparent disagreement about early socialization behavior; from an ecological viewpoint, such behavior is both adaptive and predictable.*

The process of early social bonding between parent and precocial offspring during an early sensitive period was termed "imprinting" by Lorenz (*1*) and remains a central feature of ethology. Imprinting per se is usually not studied, but approach, following, stay-near tendencies, and vocalizations are used to infer that socialization with the stimulus object is taking place. Contradictory results of such studies are the rule rather than the exception (*2*), and Klopfer (*3*) has compared imprinting with a Cheshire cat. Domestic chicks are often said to be especially poor subjects for imprinting studies because of their great response variability.

Laboratory and field studies conducted within an ecological framework demonstrate that early socialization processes in domestic chicks are highly consistent, highly adaptive, and very similar to those observed in wild jungle fowl. I show that *Gallus* chicks do not imprint to a parent in the sense of establishing a preference, but early exposure to key features of the parent is necessary to maintain preferences developed prior to exposure. To be effective, such exposure must be within the "sensitive period" that lasts for about 4 days in *Gallus* chicks (*4*). Chicks respond selectively to the parent at hatching, but early deprivation or exposure to inappropriate stimuli impairs subsequent responsiveness to parental stimuli. These results are consistent with the hypothesis that selective early experience can have behavioral as well as anatomical and physiological consequences (*5*). Lorenz (*1*) envisioned imprinting as a process by which "a sort of consciousness of species" is established in offspring that do not have "innate perceptory patterns," or preferences; I extend imprinting to include maintenance or consolidation of preferences developed prior to exposure to an imprinting stimulus.

In the first study, domestic chicks were individually placed in a circular apparatus 120 cm in diameter with a central core that housed a kymograph motor with a revolving arm. Objects suspended from the arm revolved between inner and outer walls about 38 to 40 times during each 20-minute session and were visible to subjects; an implanted speaker could provide auditory stimuli from the object. A speaker suspended from the arm outside the outer cloth wall could furnish auditory stimuli independent of visual cues. The number of revolutions (each 3 m) followed during training, the predominant type of vocalization, and postural changes were recorded. In addition, each chick was classified into a functional category (*6*) of contact-seek, contact-maintain, or contact-avoid on the basis of overall performance during an exposure session.

Chicks were individually exposed to a continuous tape recording of the clucking of a broody hen (A), to a multicolored ball 20 cm in diameter (V), or to the ball with the recorded clucking (AV) for 20 minutes per day on days 1 to 5 after hatching (first exposure at 24 ± 6 hours). In order to determine if exposure led to relatively stable preferences, chicks were individually given both simultaneous and sequential choice tests on day 12 (most investigators test for "stable" preferences after only 24 hours of isolation). Between exposure sessions, they were housed in 30-cm$^3$ heated wooden boxes and furnished with food and water.

Results were clear and consistent. All chicks exposed to clucking from an unseen speaker followed the sound almost constantly during days 1 to 5 after hatching (Fig. 1). They constantly uttered localization (or "distress") calls and maintained the head-up posture associated with contact-seeking. Naturalistic studies on jungle and domestic fowl (*7*) show that chicks separated visually, but not auditorily, from their mother exhibit the same "lost chick" phenotype seen in the A birds of this study. Such conditions cause both parent and offspring to contact-seek. On day 12, after a week of isolation, 38 of the 39 survivors exhibited clear positive responses toward the cluck, but not if the recording was played at faster or slower than normal speed. They responded negatively toward silent objects.

Chicks exposed to the clucking model (AV) followed less strongly than did those exposed to clucking alone (Fig. 1). Their general posture and vocalizations suggested little distress (the social unit of hen and chick is intact). Again, the parallel between chicks in the AV situation in the laboratory and naturalistic behavior of undisturbed jungle fowl or domestic chicks foraging near the parent was striking. In the circular apparatus, a chick could learn that it maintained auditory and visual contact most of the time without constantly following. Many of the chicks would follow the model for part of a revolution, stop and peck at the floor or walls, then orient toward or approach the model, uttering "pleasure peeps" as the model approached from the other direction. From a functional point of view, AV chicks behaved as if they were with the mother. Chicks with the mother normally feed, preen, explore, and exhibit other behaviors that are usually inhibited when the social unit is disrupted. Tests on day 12 showed that 65 of 80 survivors responded posi-