its natural habitat. These compounds could thus represent reliable cues signaling the termination of a high-quality food supply.

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Concomitant Elevations in Serum Sialyltransferase Activity and Sialic Acid Content in Rats with Metastasizing Mammary Tumors

Abstract. *Rats with transplantable spontaneously metastasizing mammary tumors have elevated levels of both serum sialoglycoconjugate and serum sialyltransferase activity compared with normal female rats or rats with various nonmetastasizing mammary tumors. A direct relationship was observed between the amount of serum protein–bound sialic acid and serum sialyltransferase activity in all rats studied. Serum sialyltransferase activity in rats with a representative metastasizing mammary tumor, SMT-2A, was also correlated with tumor age. Microsomes prepared from the SMT-2A tumor have a sixfold higher sialyltransferase activity than do microsomes prepared from the nonmetastasizing mammary tumor MT-W9B. Normal rat liver microsomes have the same level of activity as microsomes prepared from livers of animals with either SMT-2A or MT-W9B tumors. The data indicate that spontaneously metastasizing mammary tumor cells have an increased production and release, perhaps through cell surface shedding, of a sialyltransferase. It is suggested that this sialyltransferase may increase the serum half-life of certain tumor-specific circulating glycoconjugates by increasing the content of protein-bound sialic acid and may thereby play a role in the immune escape mechanism of metastasizing tumor cells.*

Cell surface glycoconjugates and glycosyltransferases have been implicated in controlling cell division and intercellular association in a variety of normal cell types. Alteration of such components may lead to loss of growth control (*1*). Increases in specific sialyltransferases have been observed in rat (*2*) and human (*3*) mammary tumor tissue as compared to normal breast tissue. Kim and Chatterjee observed that galactosyltransferase, another specific glycosyltransferase, is higher in spontaneously metastasizing rat mammary tumors than in nonmetastasizing tumors (*4*). These authors also found that nonmetastasizing mammary tumors have a thick ruthenium red–stainable glycocalyx surface coat, while metastasizing mammary tumor cells lack this stainable surface material. It has been suggested that this difference is due to increased turnover and shedding of membrane glycoconjugate rather than to lack of production of the material in the metastasizing tumors. Once shed, this material may provide the tumor with an immune escape mechanism by interfering with the immune response of the host to the tumor (*5*).

Besides containing surface glycoconjugates, mammary tumor cells may also contain surface glycosyltransferases. Bernacki (*6*) and Porter and Bernacki (*7*) reported biochemical and electron microscopic evidence for the presence of an ectosialyltransferase on the surface of murine leukemic cells. Thus, an increase in plasma membrane shedding might result in an increase in serum sialyltransferase activity. Elevated serum sialyltransferase activities have already been observed in animals with a variety of tumors, including mammary tumors (*8*), especially in women with malignant breast tumors (*9*). In this report we compare sialyltransferase (E.C. 2.4.99.1) activities in the serum, liver, and tumors of W/Fu rats with nonmetastatic and metastatic mammary tumors. We conclude that the increased serum sialyltransferase found in rats with metastasizing mammary tumors is provided by the tumor cells, probably through tumor plasma membrane shedding.

The induction of spontaneously metastasizing and nonmetastasizing mammary tumors by 3-methylcholanthrene and the biological and biochemical properties of the resulting induced tumors have been described (*5*). The representative non- or weakly immunogenic metastasizing mammary carcinoma (SMT-2A) and the nonmetastasizing, immunogenic mammary carcinoma (MT-W9B), both induced with 3-methylcholanthrene and maintained in the same strain of W/Fu female rats, were selected for comparative studies since they are similar in their degree of structural differentiation and growth rate.

At appropriate times, normally after the transplanted tumors have reached 1 to 2 cm in average diameter, or as otherwise stated, the rats were anesthetized with ether and the tissues (blood and liver or tumor or both) removed and placed on ice for later assessment of sialyltransferase activity. Blood was allowed to clot and serum was obtained by centrifugation. The serum was frozen at −20°C and later assayed for sialyltransferase activity by using desialylated (*10*) fetuin (GIBCO, Buffalo, N.Y.) or desialylated human α_1-acid glycoprotein as an accep-

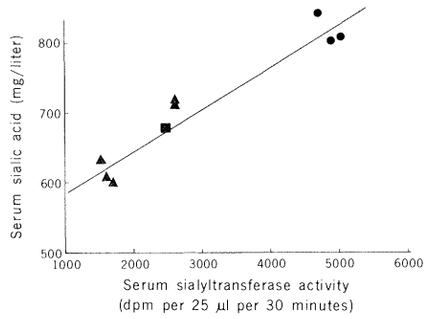

Fig. 1. Serum protein–bound sialic acid plotted against serum sialyltransferase activity in female rats with nonmetastasizing (▲) or metastasizing (●) mammary tumors and in controls (■). See Table 1.

tor. Fetuin, a fetal calf serum glycoprotein, has a number of different carbohydrate side chains that contain sialic acid (*11*) and may serve as an acceptor for a number of different sialyltransferases. It is assumed, however, that the majority of the sialic acid that is transferred to desialylated fetuin is covalently attached to the terminal galactose residues of the asparagine-linked carbohydrate side chains. The addition of sialic acid to desialylated α_1-acid glycoprotein occurs on a similar asparagine-linked carbohydrate side chain. Prolonged storage of serum, at either 4° or −20°C, was found to have little effect on sialyltransferase activity.

The serum sialyltransferase activity of rats with metastasizing mammary tumors was elevated above that of normal animals or animals with nonmetastasizing mammary tumors (Table 1 and Fig. 1). In general, animals with metastasizing STMT-058, DMBA-4, or SMT-2A tumors had exogenous serum sialyltransferase activities double those of rats with nonmetastasizing MT-W9B, MT-W9A, MT-66, or MT-100 tumors. A mean exogenous sialyltransferase activity of 2354 ± 676 (standard deviation) disintegrations per minute (dpm) per 25 μl per 30 minutes was calculated for rat serum obtained from animals with these nonmetastasizing tumors. This was similar to the control value of 2455 but significantly different ($P < .01$, Student t-test) from the mean value of 4942 ± 661 calculated for serum obtained from rats with metastasizing tumors. Animals with the DMBA-4R tumor, a metastasizing tumor that regresses spontaneously (Table 1), had normal levels of both serum sialyltransferase and sialic acid. (Serum was obtained from these animals after tumor regression was evident.)

Endogenous activities were low in all cases, although they were somewhat higher and more variable in serum obtained from animals with metastasizing tumors. A mean value of 55.4 ± 18.9 was obtained for endogenous serum sialyltransferase activity for rats with nonmetastasizing tumors. This was slightly higher than the control value (40) but significantly different ($P < .1$) from the endogenous serum activity for rats with metastasizing tumors (145 ± 83). Since desialylated serum glycoproteins are rapidly cleared from the serum by the liver (*12*) one would not expect nor did we measure any appreciable amount of endogenous sialyltransferase activity in normal serum (Table 1). Graphing total serum sialic acid against serum sialyltransferase showed a relatively linear relationship between the two parameters (Fig. 1). Whether they are directly related will be discussed later in light of Ashwell and co-workers' (*12*) findings of rapid clearance of desialylated plasma glycoproteins. It is also interesting to note (Fig. 1) that the differences in serum sialyltransferase activity between animals with metastasizing tumors and those with nonmetastasizing tumors are larger and more easily discernible than the differences in serum sialic acid content. A mean value of 674 ± 128 mg/liter was calculated for serum sialic acid in rats with nonmetastasizing tumors. This was similar to the control value of 678 and much lower than the mean value of 816 ± 135 calculated for rats with metastasizing tumors. Although the difference between the means was large, it was not statistically significant because of the large variation in estimating sialic acid. Therefore sialyltransferase assays appear to provide a more sensitive measure of the metastasizing capacity of tumor cells than do determinations of serum sialic acid content.

Table 1. Serum sialyltransferase and sialic acid content. Assays are described in (*17*). Metastasizing capacity is denoted by 0 (nonmetastasizing) or + (metastasizing), as determined by morphological examination of various host organs (*5*).

Tumor type	Metastasizing capacity	Serum sialyltransferase (dpm per 25 μl per 30 minutes)		Serum sialic acid (mg/liter)
		Endogenous	Exogenous	
Control		40	2455	678
MT-W9B	0	60	2610	717
MT-W9A	0	43	1521	630
MT-66	0	60	2660	710
MT-100	0	46	1662	605
DMBA-4R*	+	41	1699	595
STMT-058	+	143	5031	807
DMBA-4	+	80	4877	802
SMT-2A	+	279	4769	840

*This tumor is a subline of DMBA-4 and metastasizes equally well; but the tumor, including metastatic lesions, regresses spontaneously when the primary tumor becomes approximately 2 cm in diameter.

Table 2. Microsomal sialyltransferase activity (*20*).

Host state	Exogenous sialyltransferase (dpm per 25 μl per 30 minutes)		
	Tissue	Day 21	Day 42
Control	Liver	100*	100
Nonmetastasizing MT-W9B	Liver	86	77
	Tumor	16	8
Metastasizing SMT-2A	Liver	113	89
	Tumor	23	48

*Activity of normal rat liver microsomes was set as 100 percent and the activities of the other tissues were compared with it. Normal liver microsomes exhibited a specific activity of 70,031 dpm per milligram of protein per 30 minutes. Enzyme activity was enriched two- to fourfold in the microsomal fraction of both liver and tumor compared to the initial homogenates (data not shown).

To find out whether increased serum sialyltransferase activities were related to tumor age or size, blood samples were collected weekly from the orbital veins of three rats with MT-W9B tumors and three rats with SMT-2A tumors. Serums were assayed for both sialyltransferase activity and sialic acid content (Fig. 2, A and B). Both serum sialyltransferase and serum sialic acid began to increase in rats with the SMT-2A tumor during the third week after tumor transplantation. At this point the tumor began to establish metastatic colonies in regional lymph nodes and perhaps to increase its own capacity for producing sialyltransferase (Table 2) and sialylated glycoproteins. At 21 days MT-W9B was twice as large as SMT-2A (24.3 and 12 mm in mean diameter, respectively). At 42 days both primary tumors were about the same size (~ 34 mm), but by this time SMT-2A had metastasized widely and its total volume was two to three times greater than that of MT-W9B. As SMT-2A increased in size the serum sialyltransferase and sialic acid levels increased, and kept increasing until the animals died (~ 45 days). Little or no increase was observed in animals with nonmetastasizing mammary tumors during the same period.

Since the liver is the main source of serum glycoproteins and thus a potential source of serum sialyltransferase, we compared liver and tumor microsomal sialyltransferase activity in rats with MT-W9B and SMT-2A tumors. Microsomes from liver or tumor (or both) from normal animals and animals with SMT-2A or MT-W9B tumors were prepared as previously described for liver (*13*). Tissues were washed in ice-cold saline,

minced, and homogenized in three volumes of ice-cold 0.154*M* KCl by use of a Potter-Elvehjem homogenizer with a Teflon pestle. The resulting crude microsomal preparations containing Golgi and some plasma membrane were used as the enzyme source for sialyltransferase activity. The results in Table 2 show that liver microsomal sialyltransferase activity either remains the same or decreases in rats after tumor implantation, and becomes elevated over time only in the metastasizing mammary tumor, SMT-2A. The sialyltransferase activity of the metastasizing tumor more than doubled between 21 and 42 days after implantation, and was six times higher than that of the nonmetastasizing tumor at 42 days after implantation. Therefore we assume that the metastasizing tumor itself is the source of serum sialyltransferase apparent from the third week after tumor implantation.

Increases in serum sialic acid, due largely to α_1-acid glycoprotein, in inflammation and advanced cancer were documented some time ago (*14*). We have confirmed these observations with metastasizing mammary tumors in rats and have correlated the increase in serum sialic acid with an increase in serum sialyltransferase, originating, in all likelihood, from the tumor itself. We now speculate on the mechanism of release of such tumor sialyltransferases and their possible physiological function.

Sialyltransferases are normal components of Golgi membranes and are located within Golgi vesicles, since detergent treatment has been shown to enhance enzyme activity (*15*). Glycosylation of most proteins and lipids occurs within these vesicles. Then perhaps, as a result of vesicle fusion with plasma membranes in the course of membrane biogenesis or in the secretion of intracellular material, sialyltransferases and glycosylated proteins and lipids become incorporated into the cell's surface. We have obtained ultrastructural evidence for the existence of such enzymes and substrates on cell surfaces (*6, 7*), and it is conceivable that they are shed into the general circulation along with other cell surface antigenic fragments, which are released in large quantities from this metastasizing mammary tumor (*5*). It is believed that these fragments interfere with host's immune response to the tumor, enabling the tumor to spread (*4, 5*).

Elevated levels of cellular sialyltransferase may have a physiological role in lengthening the half-life of glycoconjugates shed from the surfaces of tumor cells by increasing their sialic acid content, which would result in decreased plasma clearance rates (*12*). Recently, Waxman *et al.* (*16*) found an increase in vitamin B_{12} binding protein and sialyltransferase in the serum of patients with hepatomas. They attributed these increases to hypersialylation and secretion of B_{12} binding protein and sialyltransferase by the hepatoma. The hypersialylated glycoprotein is slowly cleared from the plasma. Accordingly, sialylated antigens shed into the plasma from mammary tumor cell surfaces may play a role in the immunological escape mechanism for metastasizing tumor cells. Alternatively, the physiological role for elevated cellular or circulating sialyltransferases in tumorigenesis may be sialylation of extracellular substrates. This is less likely since cytidine monophosphate (CMP)–sialic acid was found to be rapidly hydrolyzed in serum and no free sialic acid was detectable in serum (*17*).

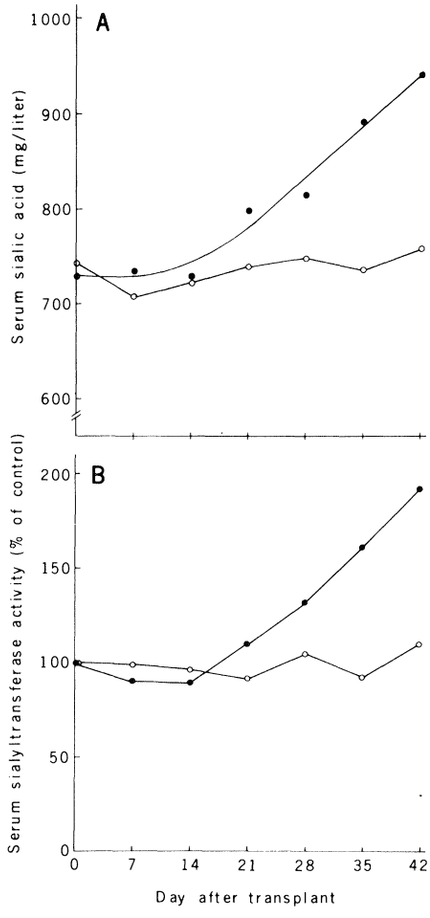

Fig. 2. (A) Serum sialic acid and (B) serum sialyltransferase activity after implantation of either metastasizing (•) or nonmetastasizing (○) mammary tumors. Each data point is the average of three assays performed in duplicate on individual blood samples drawn from three different animals.

Finally, it was observed that serum sialyltransferase activity and serum protein–bound sialic acid in rats are related. Increases in both parameters were observed in rats with spontaneously metastasizing mammary tumors but not in rats with nonmetastasizing mammary tumors. Metastasizing mammary tumors show an increased production of sialyltransferase and therefore are the most likely source of the increased serum sialyltransferase.

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(7:3). Breakdown of CMP-NANA to free NANA was greater than 30 percent in the absence of UTP and negligible in its presence. Addition of UTP may prevent breakdown by inhibiting CMP-NANA hydrolase [E. L. Kean and K. T. Bighouse, *J. Biol. Chem.* **249**, 7813 (1974)] and it may also enhance serum enzyme activity allosterically (*13*). Addition of 0.3 mg of desialylated human α_1-acid glycoprotein in place of desialylated fetuin resulted in relative enzyme activities slightly higher (~ 10 percent) than those found with fetuin (data not shown); otherwise the results were similar. No serum neuraminidase activity was detectable in any of the rat serums tested with a highly sensitive assay [R. J. Bernacki and H. B. Bosmann, *Eur. J. Biochem.* **34**, 425 (1973)], although substrate competition from endogenous serum sialoproteins cannot be entirely ruled out. The serum sialyltransferase assay systems were incubated for 30 minutes at 37°C and the reactions were terminated by addition of 2 ml of 1.0 percent phosphotungstic acid in 0.5*N* HCl. The insoluble material was extracted twice with 10 percent trichloroacetic acid and once with ethanol and ether (1:1). Radioactivity was determined by scintillation counting. All assays were performed in duplicate on at least two separate occasions. Serum sialic acid was determined in 50 μl of acid-hydrolyzed (0.1*N* H_2SO_4, 80°C, 1 hour) serum by the method of Aminoff (*18*). No free sialic acid was detected in serum. *N*-Acetylneuraminic acid (Sigma Chemical Co.) was used as a standard. Protein was determined in individual serum samples by the method of Lowry *et al.* (*19*) and very little variation in serum protein content was found in any of the rat serums (from control and tumor-bearing animals) used for these studies.

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20. Sialyltransferase activity was measured by using desialylated fetuin as an acceptor. Endogenous activity was subtracted from total activity (activity in the presence of 0.3 mg of desialylated fetuin) to obtain exogenous activity. Endogenous activity never exceeded 10 percent of total activity with liver microsomes but was higher and more variable with tumor tissue. The incubation medium consisted of 50 μl of microsomal enzyme suspension [0.1 to 0.5 mg of protein (*19*)]; 10 m*M* tris buffer, *p*H 7.0; 10 m*M* $MgCl_2$; 1 m*M* UTP; 0.3 mg of desialylated fetuin; and 0.5 μc of CMP-[4,5,6,7,8,9-^{14}C]sialic acid, 196 c/mole (Amersham/Searle). The incubation mixture was incubated for 30 minutes at 37°C and activity determined as described in (*17*). Duplicate samples were used. Liver and primary tumor free of necrotic tissue were pooled from three animals for each microsomal preparation.

21. Human α_1-acid glycoprotein used in these studies was provided by the National Fractionation Center of the American Red Cross with partial support from the National Institutes of Health [grant HE 1388 (HEM)]. We thank M. Hillman, N. Porter, and R. Izquierdo for their technical assistance. Supported in part by grants CA-19814, CA-15757, and CA-13038 and contract NO 1-CB-23864 from the National Cancer Institute and by American Cancer Society institutional grant IN-54-13.

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Visual Detection of Cryptic Prey by Blue Jays (*Cyanocitta cristata*)

Abstract. *Blue jays learned to respond differentially to the presence or absence of* Catocala *moths in slides. This detection of the moths by the jays was affected by the background upon which the moth was placed and its body orientation, thus providing an objective measure of crypticity. These procedures are useful for the study of visual detection of prey.*

The visual searching behavior of predators is affected by the density, crypticity, and oddity of prey (*1, 2*). However, the effects of these variables often have been difficult to isolate because of problems in controlling factors such as prey preferences, novelty, or the relative ease of capture of particular prey items (*3*). We have developed a new technique for the study of prey detection based on complex discrimination procedures, which provides excellent experimental control of these factors. Here we report data on the detection of cryptic, barklike *Catocala* (Noctuidae) moths by the blue jay (*Cyanocitta cristata*), an avian predator that commonly hunts them (*4*).

Catocala moths are active during the night, spending the daylight hours resting on appropriate substrates. Sargent (*4, 5*) has identified two aspects of the resting behavior of *Catocala* moths which probably affect their crypticity. The moths select resting substrates that match the reflectance of their forewings and adopt species-typical body orientations on the substrate by which they appear to align their disruptive markings with those of the substrate. However, there is little direct evidence that these behaviors affect a natural predator's ability to detect the moths (*6*).

The technique we have developed is based upon procedures used in the study of concept formation in pigeons (*7*). We first trained blue jays to respond differentially to the presence and absence of moths in projected images. After this task was learned the jays were tested with a wide variety of different slides. Their responses provided a measure of the effects of substrate choice and body orientation of the moths on the ability of the jays to detect the moths.

Six blue jays, obtained locally when 10 to 12 days old and hand-raised in the laboratory, were subjects. The birds were maintained at 80 percent of their free-feeding weight throughout the experiment. The apparatus was a modified Lehigh Valley Electronics pigeon chamber. A food magazine was located centrally on the intelligence panel, with an 11.4 by 7.5 cm stimulus key mounted to the left of the magazine and a round changeover (CO) key 2.54 cm in diameter mounted to the right. Slides could be projected upon the stimulus key by a programmable Kodak Carousel projector mounted behind the panel. Reinforcement consisted of halves of *Tenebrio* larvae delivered into the magazine by a Davis universal feeder mounted on top of the chamber. A wooden perch was located parallel to and in front of the intelligence panel.

Throughout the experiment positive slides contained the image of a moth and negative slides contained no moth. Each trial began with illumination of the CO key with red light. When the CO key was pecked once, an image was projected upon the stimulus key, and the display on the CO key changed to a white cross on a black background. If the projected slide was positive, ten pecks at the stimulus key (correct response) resulted in reinforcement. Reinforcement was followed by a 10-second intertrial interval (ITI) to allow ingestion of the reward before the next trial began. A peck at the CO key on positive trials (incorrect response) produced a 60-second ITI. On negative trials, the tenth peck at the stimulus key (incorrect response) was followed by a 60-second ITI, but a peck at the CO key (correct response) produced a 4-second ITI.

Slides were prepared in matched pairs, identical except for the presence or absence of a moth, by pinning a dead moth into position, taking a picture of the scene, then removing the moth and taking another picture. Three species of barklike moths were photographed: *Catocala cara* (dark brown forewings with a faint disruptive pattern of brown lines), *C. retecta* (gray forewings with a prominent disruptive pattern of brown lines), and *C. relicta* (white forewings with prominent stripes of black and gray). *Catocala retecta* and *C. cara* appear most cryptic on gray-brown bark and typically rest in a head-down position, while *C. relicta* appears most cryptic on white birch bark and rests head up. Three separate sets of matched pairs of slides were used. Set 1 consisted of 60 slides, taken indoors, of upright logs to which dead moths were pinned. Set 2 consisted of 60 slides taken outdoors in a lightly wooded area. Sets 1 and 2 were counterbalanced so that each species of moth appeared on both oak and white birch, at three subject-to-camera distances (0.6, 1.2, and 2.4 m). Set 3 consisted of 270 slides taken in a lightly wooded area and counterbalanced so that each species appeared once on oak, white birch, and a nonbark surface, in each of three orientations (head up, head down, and horizontal), at each of five distances (0.6, 1.2, 2.4, 3.6, and 4.8 m). In all sets, the quadrant of the slide in which the moth appeared varied randomly.