Comparison of Metastatic and Non-metastatic Transplantable Tumors from the Jaw Region of the Rat

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Introduction

The ability of a primary tumor to metastasize is frequently critical to the clinical expression of malignancy. In order for a tumor to metastasize, cells must detach from the primary tumor, be transported via the blood or lymphatic systems, and must reattach in another organ. Poste and Nicolson (15) have reviewed evidence that the detachment-reattachment process is due to changes in the cell surface.

In an effort to clarify these changes, we have initiated studies in which metastatic and non-metastatic transplantable tumor lines of similar origins are compared. In this report, two tumors from the jaw region of the rat, one non-metastatic and the other metastatic, are partially characterized in terms of light and electron microscopy and gross morphological characteristics, as well as ganglioside and neutral glycosphingolipid composition. While the metastatic tumor line has increased levels of total gangliosides, there is a marked decrease in the more complex gangliosides as compared to the non-metastatic line.

Materials and Methods

The tumor designated JT_2TS_f was isolated initially from soft tissues in the area of the articulation of the maxilla and mandible of a male inbred rat of the CDF strain (Harlan Industries, Indianapolis) which had been fed a diet containing the liver carcinogen 2-acetylaminofluorene as described (12). The second tumor, WJT, believed to be of spontaneous origin, was isolated initially from the same anatomical region of a 1.5 year old male albino rat of the same strain fed a standard laboratory diet. The tumor lines were maintained by subcutaneous implanation of approximately 10^6 tumor cells into syngeneic recipients (7).

At the time of sacrifice, portions of each tumor were fixed in 10% buffered formalin, embedded in paraplast and stained with hemotoxylin and eosin for light microscopy. For electron microscopy, portions were fixed overnight in cold 1% O_sO_4 in 0.05 M phosphate buffer, pH 7.2, rinsed in the same buffer, dehydrated in a graded series of acetone, embedded in Epon, sectioned and examined and photographed with a Philips EM/200.

For biochemical analyses, the tumors were minced and homogenized in four volumes of distilled water with a Polytron tissue homogenizer (Kinematica, Lucerne, Switzerland). An aliquot of the homogenate was used for protein determination according to the procedure of Lowry (10) with bovine serum albumin as standard. Gangliosides and neutral glycosphingolipids were isolated according to a modification of the method of Ledeen (9). The homogenates were extracted with ten volumes of freshly distilled chloroform-methanol (1:1, v/v) overnight at 4°C with stirring. The extract was filtered over glass and the residue re-extracted with ten volumes of chloroform-methanol (2:1, v/v) overnight at 4°C. The resulting

extract was filtered over glass and the combined filtrates were dried with a rotary evaporator. The dried sample was solubilized in 100 ml of solvent A (chloroform-methanol-water, 30:60:8, v/v) and applied to a DEAE-Sephadex A-25 column (acetate form) (9). After washing with solvent A, gangliosides were eluted with solvent B (chloroform-methanol-0.8 M sodium acetate, 30:60:8, v/v). The eluate was evaporated, saponified, dialyzed, lyophylized, solubilized in 10 ml distilled methanol and filtered over glass. Forty ml of chloroform was added to the final filtrate to adjust the solvent to a 4:1 ratio of chloroform to methanol. This mixture was applied to a Unisil column (Clarkson) and the purified gangliosides were eluted as described (9). Total ganglioside sialic acid was measured according to Warren (19) and 50 nmoles of ganglioside sialic acid of each sample were applied to heat activated silica gel 60 F-254 thin layer chromatography plates (E. Merck, Darmstadt, Germany). The plates were developed twice in chloroform-methanol-ammonium hydroxide-water (60:35:7.3, v/v). To allow for even passage of solvent, spotted plates were pre-run through the sample zone several times before the beginning of the separation. Visualization was with resorcinol-HCl (17). Brain gangliosides served as standards. For quantitation, plates were scanned with a densitometer (Photovolt).

Netural glycosphingolipids were purified from the solvent A fractions of the DEAE-Sephadex columns. The solvent was evaporated and the residue resuspended in 5 ml of chloroform and applied to a Unisil column. The column was washed with chloroform and ethyl acetate to remove neutral lipids and interfering pigments, respectively. Crude neutral glycosphingolipids were eluted with acetone-methanol (9:1, v/v) and dried. Following mild alkaline methanolysis and the addition of chloroform, the samples were washed twice with methanol-water (1:1, v/v) and once with methanol-9% potassium chloride (1:1, v/v). The lower phase was dissolved in chloroform and half the sample was spotted on a thin layer chromatography plate as for gangliosides. The plates were developed in chloroform-methanol-water (70:22:3, v/v) and neutral glycosphingolipids visualized by spraying the plates with 50% sulfuric acid and heating (18).

Results

Primary tumors of either the non-metastatic or metastatic lines were white in color, ovoid in shape and well circumscribed (Figure 1). The non-metastatic JT_2TS_f tumor illustrated is representative of those used in the study and was removed after approximately 1 month of growth at a rate of 0.17 cm/day (Table 1). The representative metastatic primary tumor, WJT, was removed after approximately 3 months.

Lung specimens taken from the tumor-bearing rats revealed metastatic foci only with the WJT line (Figure 2). In 47 transfers, the metastatic incidence of the WJT line has been a remarkable 100%. It has metastasized to the kidneys, diaphragm, lungs, thymus, lymphatics and the wall of the pleural cavity. In contrast, the JT_2TS_f line has not metastasized in 54 transfers.

Upon histological examination, both the metastatic WJT and the non-metastatic JT_2TS_f showed morphological features characteristic of squamous cell carcinomas (Figure 3). The principal difference was a tendency for the metastatic line to appear more homogeneous and less stratified than the non-metastatic line. Ultrastructurally, the two tumor lines were similar (Figures 4 to 6). Both had a poorly developed Golgi apparatus, sparse rough endoplasmic reticulum, bizarre mitochondrial forms, irregularly shaped nuclei and abundant lysosomes. In

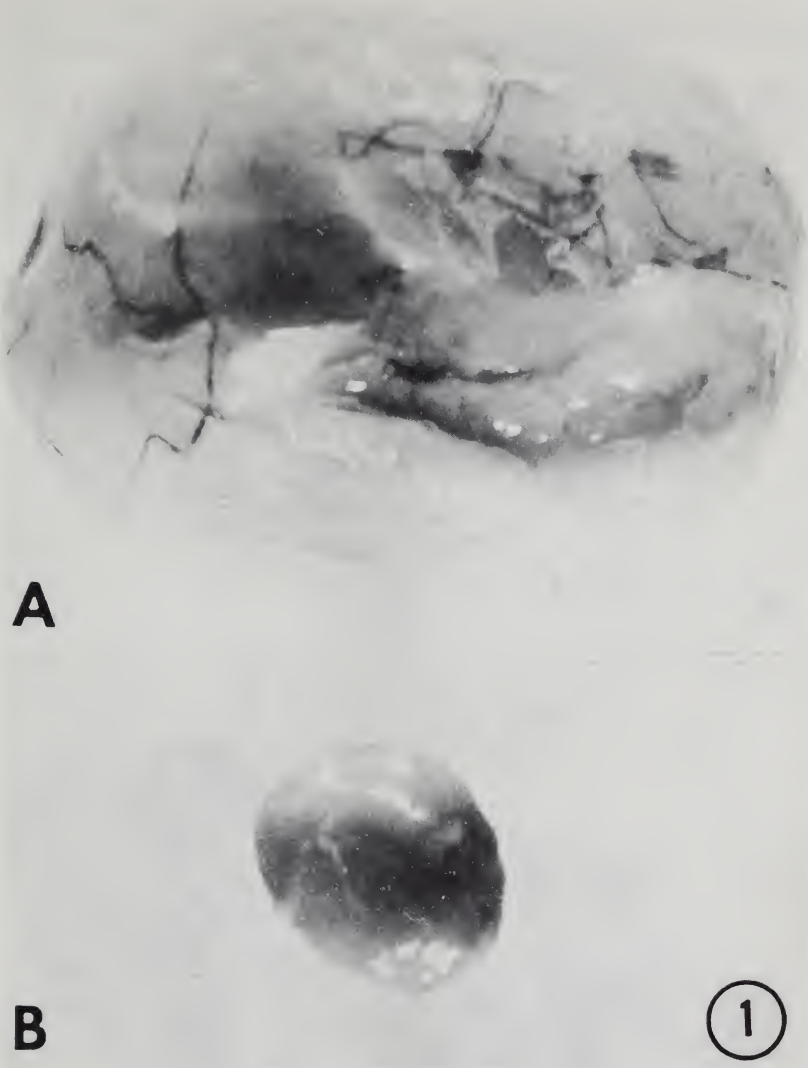

FIGURE 1. *Gross morphology of the primary tumors. A. Non-metastatic, fast growing JT_2TS_f after 1 month of growth. B. Metastatic, slow growing WJT after 2 months of growth. Both were white in color, ovoid in shape and well circumscribed. X 2.*

TABLE 1. *Growth rates comparing the non-metastatic (NM), JT_2TS_f, tumor line and the metastatic (M), WJT, tumor line*

	Growth (cm/day)	
Tumor Line	**Mean ± S.D.**	**Range**
JT_2TS_f (NM)[a]	0.17 ± 0.04	0.11-0.22
WJT (M)[b]	0.017 ± 0.005	0.01-0.025

[a]Twelve passages over approximately 1 yr
[b]Six passages over approximately 1.5 yr

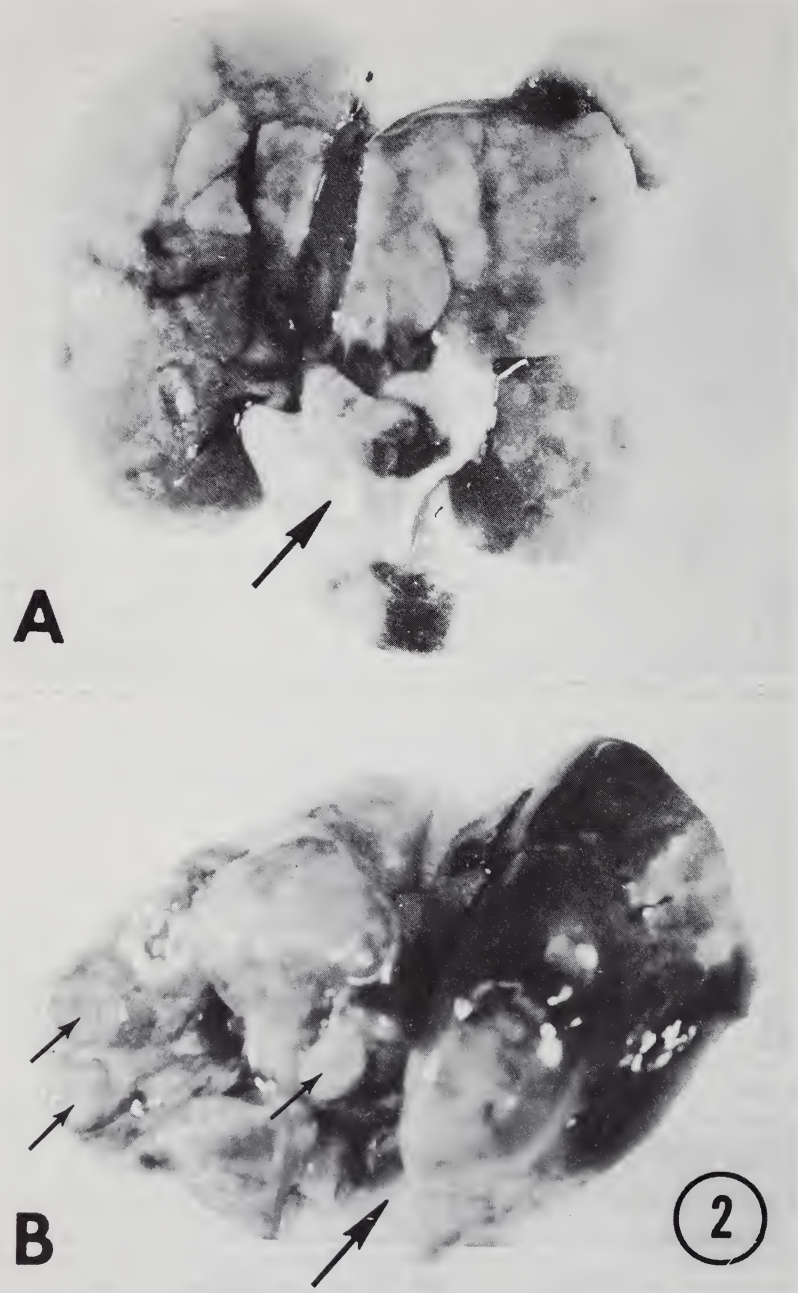

FIGURE 2. *Lung specimens from tumor bearing rats. A. Lung from JT_2TS_f. B. Lung from WJT. Small arrows indicate metastatic foci. Large arrow denotes bronchus. X 2.*

general, the Golgi apparatus appeared to be less well developed in the metastatic line whereas lysosomes appeared to be more abundant.

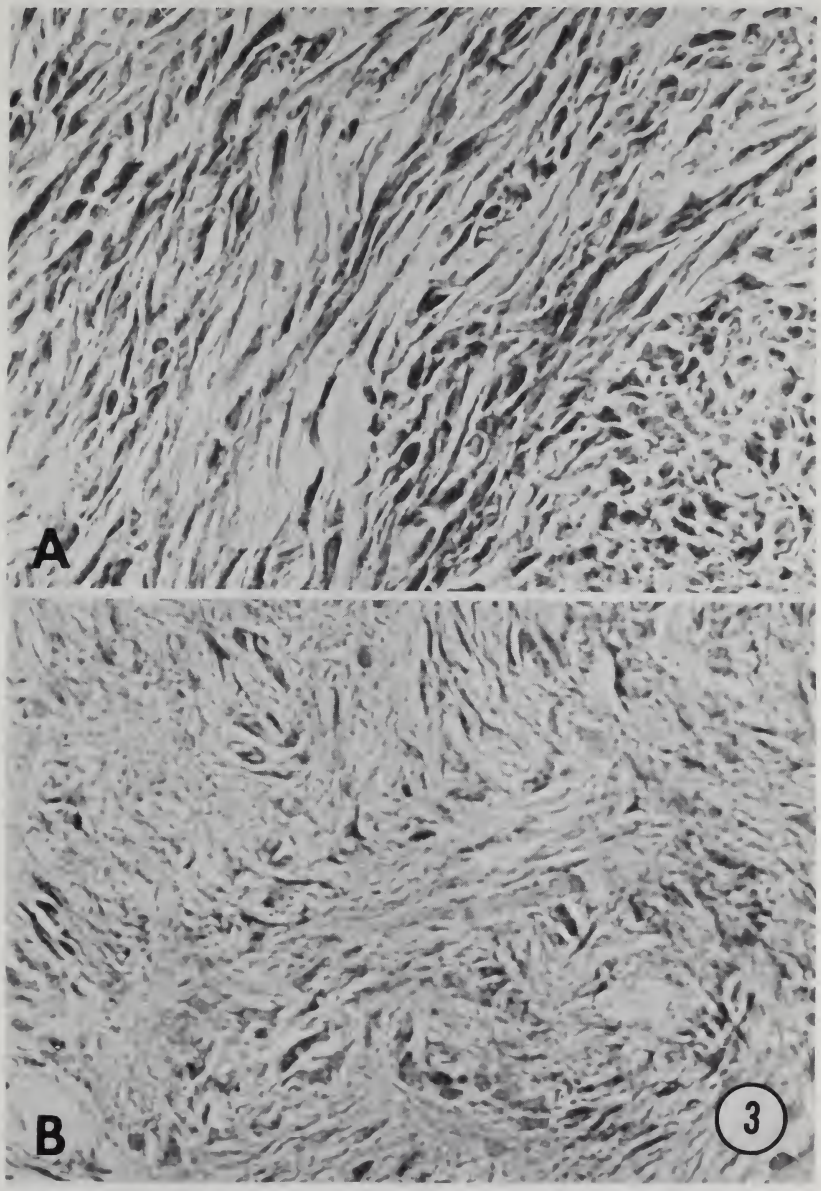

FIGURE 3. *Light micrographs demonstrating the appearance of the transplantable squamous cell carcinomas. A. Non-metastatic JT_2TS_f B. Metastatic WJT. Generally they were similar in appearance except that cells of the WJT line appeared more homogeneous and less stratified than those of the JT_2TS_f line. H & E stain. X 500.*

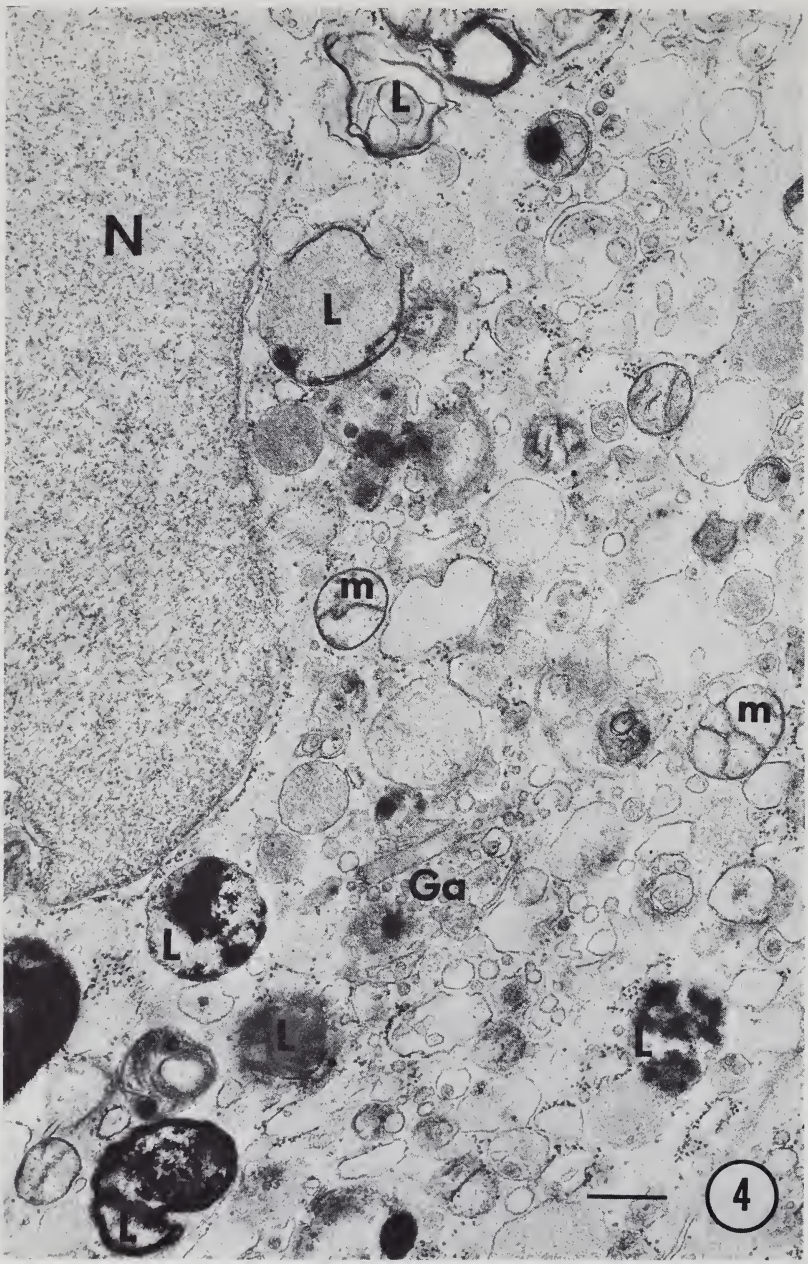

FIGURE 4. *Electron micrograph of the WJT, metastatic tumor line at low magnification. Note the paucity of rough endoplasmic reticulum, the presence of unusual mitochondrial forms (m) and numerous lysosomes (L). The Golgi apparatus (Ga) was poorly developed. N = nucleus. Bar = 0.5μ.*

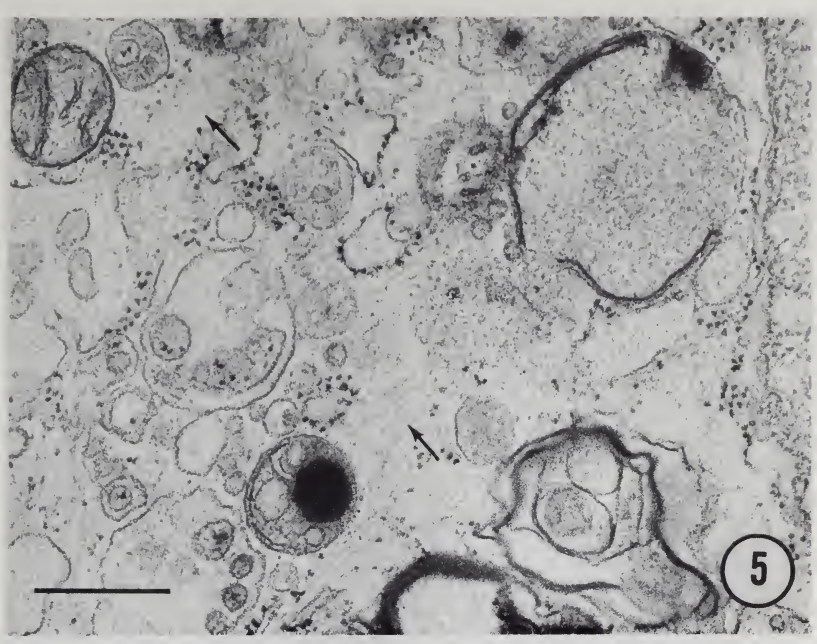

FIGURE 5. *Electron micrograph of WJT metastatic tumor line at higher magnification to reveal numerous filaments in the cytoplasm (arrows). Bar = 0.5μ.*

Total ganglioside sialic acid was approximately 3 times greater in the metastatic, slow-growing WJT than in the faster-growing, non-metastatic JT_2TS_f (Table 2). Thin layer chromatography revealed a large number of individual ganglioside species (Figure 8) some of which co-migrated with standards and others which did not. Densitometry traces of lanes corresponding to the same amounts of applied ganglioside sialic acid confirm what is observed visually from the plates in that ganglioside bands corresponding to the higher gangliosides G_{D1b} and G_{T1b} were much reduced or absent in extracts prepared from tumors of the metastatic line (Figure 9). Additionally, the metastatic line contained at least one

TABLE 2. *Ganglioside sialic acid and total protein comparing the non-metastatic, JT_2TS_f, and metastatic, WJT, tumor lines*

Tumor Designation	Wet Weight (g)	Protein (mg)	Protein (mg/g wet wt)	Ganglioside Sialic Acid (nmoles)	Ganglioside Sialic Acid (nmoles/mg protein)
$JT_2TS_fI^a$	27	2500	93	1420	568
$JT_2TS_fII^a$	31	3144	101	1020	310
$JT_2TS_fIII^{a,b}$ (nec)	19	520	27	160	320
WJT^c	3	120	40	170	1417

[a]Results from 3 different animals
[b]Necrotic tumor
[c]Results from pooled tumors

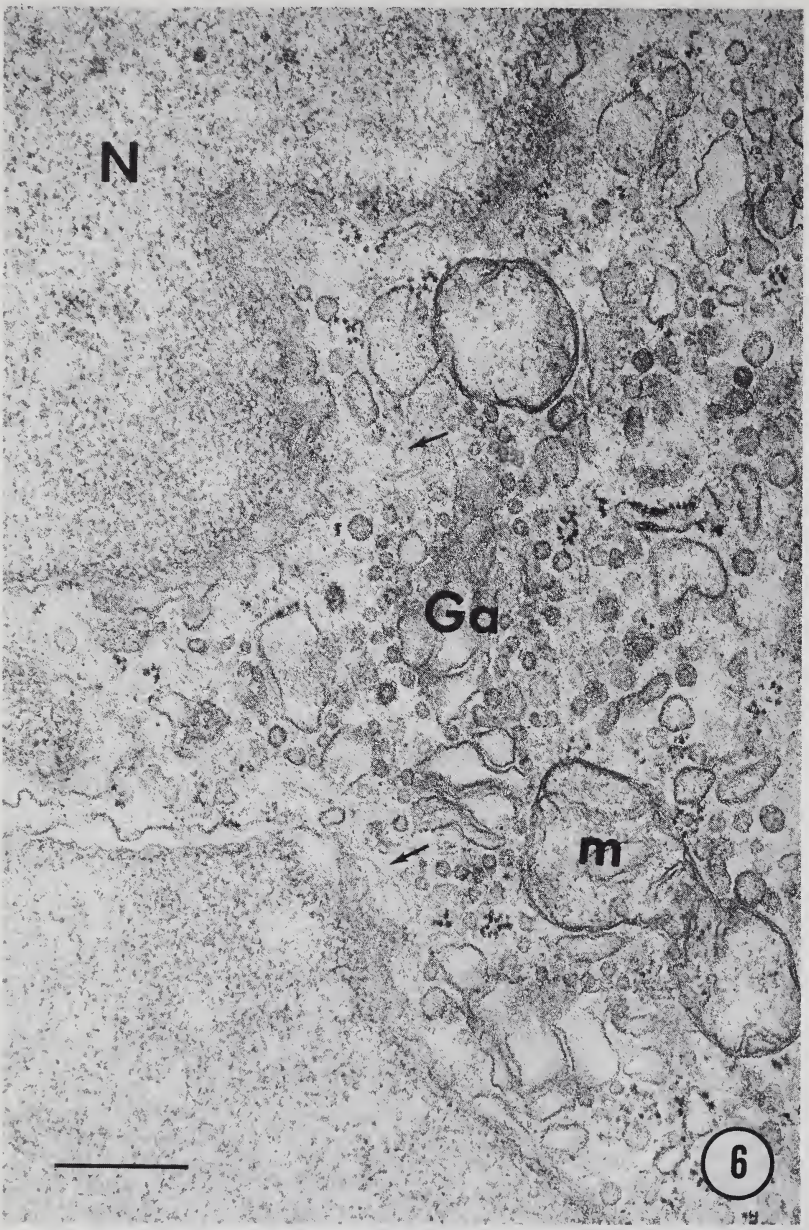

FIGURE 6. *Electron micrograph of JT_2TS_f for comparison. The nucleus (N) is highly lobed and irregular, lysosomes are less abundant and the Golgi apparatus (Ga) is better developed. Cytoplasmic filaments (arrows) are present. M = mitochondria. Bar = 0.5μ.*

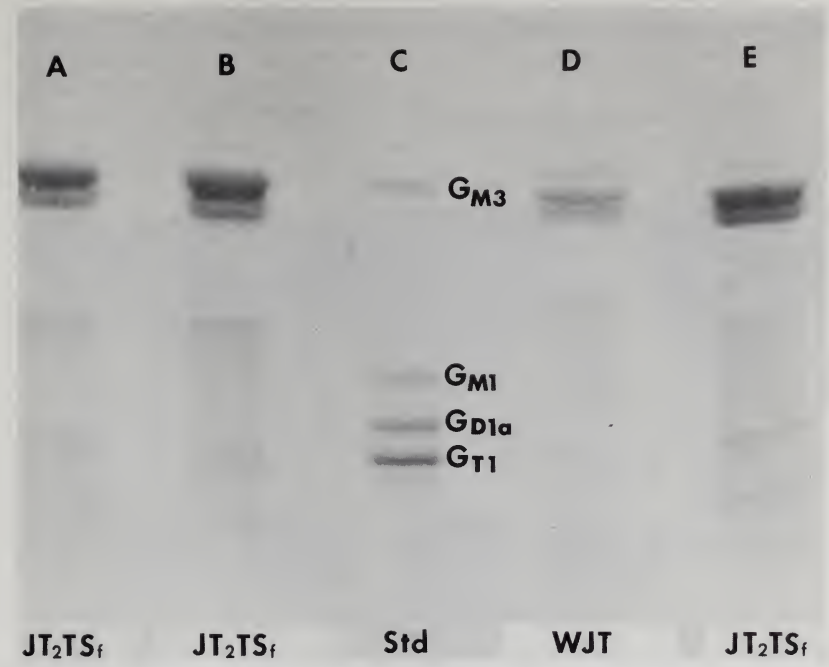

FIGURE 7. *Thin layer chromatogram of gangliosides from rat jaw tumors. (A) JT_2TF_{fs}, (B) JT_2TS_f, (C) Standard mixed brain gangliosides, (D) WJT, (E) JT_2TS_f which had a high degree of necrosis.*

ganglioside band not present in the non-metastatic tumors (Figure 9). No available ganglioside standard was found to migrate in the region of this ganglioside band.

Similarly increased in the metastatic line were higher neutral glycosphingolipids (Figure 10). Especially striking was the absence of neutral glycosphingolipids migrating in the vicinity of ceramide tetrahexosides (C4H).

Discussion

Two tumor lines, one metastatic and the other non-metastatic, of similar origins provide an excellent opportunity to compare various cell surface constituents potentially related to malignancy. Also, the WJT is an unusual metastatic primary tumor. Generally, metastatic primary tumors are invasive, poorly circumscribed and fast growing. The WJT does not conform to these criteria in that it mimics the non-invasive phenotype, is well circumscribed and slow growing. The many similarities of the metastatic and non-metastatic lines may eventually prove beneficial in understanding the metastatic process. Our observations indicate that the WJT line is able to release cells to form secondary metastases but still retain the gross characteristics of a benign neoplasm.

The similarity of the two lines is apparent at both the light and electron microscope levels. The more abundant lysosome population of the metastatic isolate may be significant as a source of hydrolytic enzymes to disrupt the intracellular matrix and thus aid the disassociation and migration of invading cells.

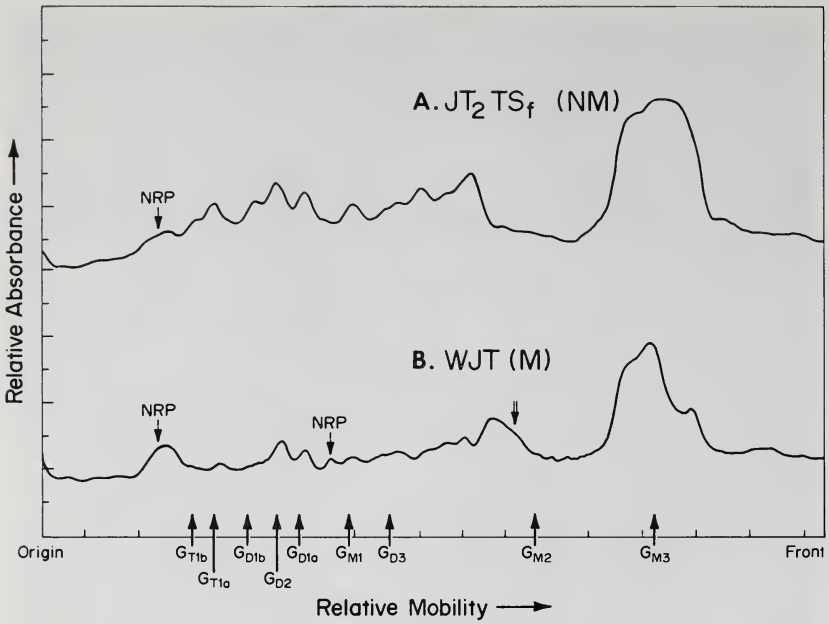

FIGURE 8. *Densitometry scans of rat jaw tumor gangliosides. (A) Scan of the non-metastatic JT_2TS_f from lane B of Fig. 7 (B) Scan of the metastatic WJT tumor (lane D of Fig. 7). Arrows indicate the relative mobilities of the standard gangliosides indicated. Note the reductions in the regions of the scans corresponding to the gangliosides GD_{1b} and GT_{1b}. ABBREVIATIONS USED: Cer = ceramide; G_{D1a} = NAN-Gal-Ga1NAc-(NAN)-Gal-Glc-Cer; G_{D1b} = Gal-Ga1NAc-$(NAN)_2$-Gal-Glc-Cer; G_{D2} = Ga1NAc-$(NAN)_2$- Gal-Glc-Cer; G_{D3} = $(NAN)_2$-Gal-Glc-Cer; G_{M1} = Gal-GalNAc-(NAN)-Gal-Glc-Cer; G_{M2} = Ga1NAc-(NAN)-Gal-Glc-Cer; G_{M3} = NAN-Gal-Glc-Cer; G_T = $(NAN)_2$-Gal-GalNAc-(NAN)-Gal-Glc-Cer (G_{T1a}), NAN-Gal-GalNAc-$(NAN)_2$-Gal-Glc-Cer (G_{T1b}), or $(NAN)_3$-Gal-Glc-Cer (G_{T3}); G_X = G_Q and other unidentified higher ganglioside homologs; Gal = galactose; Ga1NAc = N-acetylgalactosamine; Glc = glucose; NAN = N-acetylneuraminic acid (sialic acid).*

The ganglioside composition of tumors appears to be highly dependent upon their stage of development (12). Generally, most tumor or transformed cells appear to have an increased level of ganglioside-associated sialic acid (8). Yet, except for a few rat hepatoma lines (7, 8), no studies have compared the ganglioside composition of metastatic and non-metastatic tumor lines of similar origins.

At least 15 distinct ganglioside bands were resolved in the extracts prepared from the non-metastatic tumor line (Figure 8). At least one band was present in the metastatic line that could not be detected in the non-metastatic line. Since it did not co-migrate with any of the standard gangliosides available, it may represent a novel structure containing fucose (11), glucosamine (2) or some other unusual constituent (16). Structural studies will be necessary to confirm this supposition.

More important, however, in the context of the present study was the absence of certain ganglioside bands in the extracts of the metastatic line (Figure 8).

Striking in this regard were the almost complete absence of gangliosides G_{D1b} and G_{T1b}. Both these gangliosides have been implicated recently as potential receptors for fibronectins, proteins that link cells to underlying substrata and may be important to cell adhesion (3, 5).

Recent evidence shows that di- and trisialogangliosides inhibit the fibronectins mediated binding of cells to collagen-coated plates (5, 6). Data from our laboratory (14) indicate that the gangliosides G_{D1a}, G_{D1b} and G_{T1} all bind fibronectins.

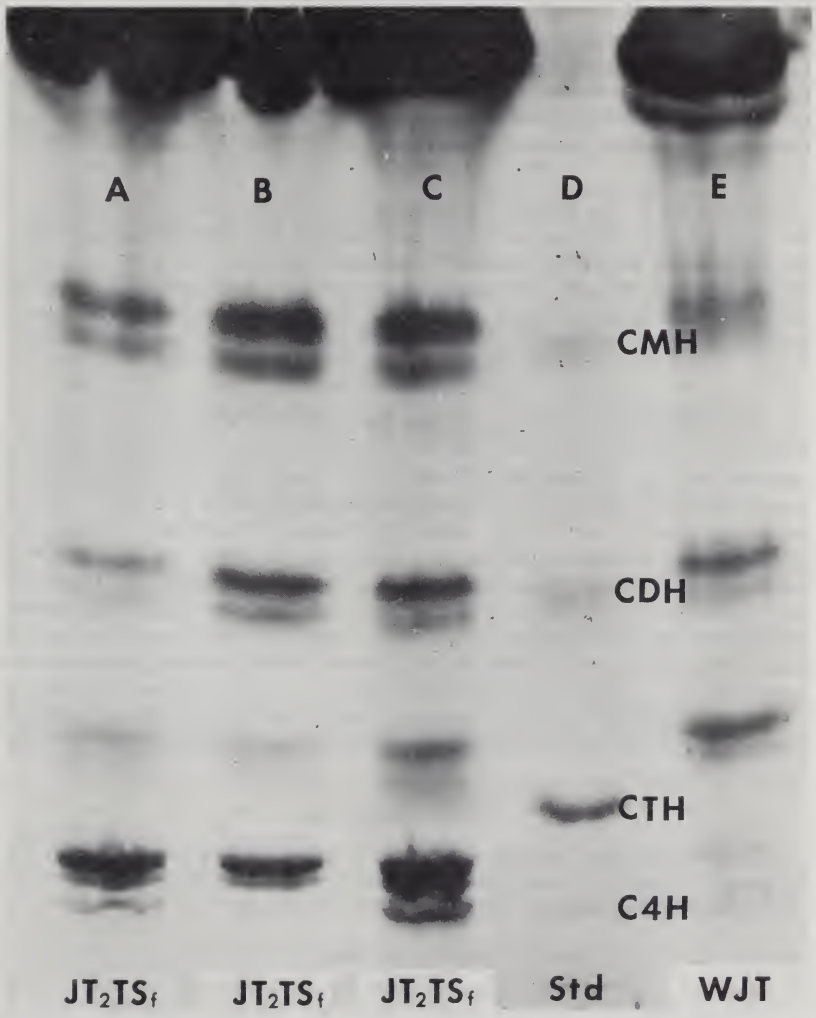

FIGURE 9. *Thin layer chromatogram of neutral glycosphingolipids from rat jaw tumors. (A) JT_2TS_f (B) JT_2TS_f (C) JT_2TS_f which had a high degree of necrosis (D) Standards (E) WJT Note the reductions in the relative amount of material migrating in the vicinity of C4H. Abbreviations Used: CHM = ceramide monohexoside; CDH = ceramide dihexoside; CTH = ceramide trihexoside; C-4H = ceramide tetrahexoside.*

Additionally, there is a strong correlation between the loss of the ability of cells to bind fibronectins and the ability of cells to invade and form tumor metastases (1). This loss of ability to bind fibronectins has little or no effect on the ability of cells to synthesize fibronectins (1, 4).

Evidence presented here indicates that the metastatic tumor line, WJT, loses the gangliosides that migrate in the region of G_{T1} and G_{D1b}. Thus it would appear that loss of the complex fibronectin-receptor gangliosides may contribute to the metastatic potential of the WJT tumor line.

The mechanism whereby the gangliosides are lost is unknown. One possibility is that lysosomal enzymes may be involved in their degradation. Another is that their biosynthesis may be blocked. In 2-acetylaminofluorene-treated rats, tumors exhibit a block in the branchpoint enzyme, CMP-sialic acid: G_{M3} sialyltransferase (13) which results in a decrease in the disialo- and trisialogangliosides in those tissues.

The proof of the fibronectin receptor hypothesis of ganglioside action in metastasis will be aided substantially by structural characterization of the missing gangliosides and of the fibronectin-binding ability of the two tumor lines, both of which are in progress. We anticipate that the continued investigation of the metastatic and non-metastatic squamous cell carcinomas reported here will aid in the eventual understanding of the metastatic process during tumor progression.

Summary

Two rat tumors, a spontaneous jaw tumor (WJT) which, in 47 transfers, exhibited a metastatic incidence of 100% and a jaw tumor (JT_2TS_f) induced by the chemical carcinogen 2-acetylaminofluorene and which has not metastasized in 54 generations were compared. Criteria for comparison utilized both light and electron microscopy as well as gross morphological characteristics and glycosphingolipid composition. The two lines exhibited similar morphologies with the metastatic line being more homogeneous and less stratified. Ultrastructurally, more lysosomes were observed in the metastatic line. Certain di- and trisialogangliosides were reduced or absent from extracts of the metastatic line that were present in the non-metastatic line. The reduced or missing gangliosides are those thought to function in fibronectin binding and cell attachment. It is suggested that both the increased lysosome content and the reduction in putative fibronectin receptor gangliosides may be important to the ability of the WJT line to form metastases.

Acknowledgements

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