Differences in Lectin Binding in Tissue Sections of Human and Murine Malignant Tumors and Their Metastases

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Lectin binding to tumor cells in tissue sections of 16 nonmetastatic and 24 metastatic human adenocarcinomas and 5 nonmetastatic and 5 metastatic murine Lewis lung carcinomas (LLCs) was assessed with an avidin–biotin peroxidase technique. In human tumors, *Ulex europaeus* agglutinin I (UEA I) showed no binding; whereas concanavalin A (Con A), *Ricinus communis* agglutinin I (RCA I), wheat germ agglutinin (WGA), soybean agglutinin (SBA), and *Dolichos biflorus* agglutinin (DBA) bound equally to primaries and metastases. However, peanut agglutinin (PNA) bound to <5% of cells in 37 of 40 primaries but to >50% of cells in 18 of 24 metastases. In LLC tumors, UEA I and DBA showed no binding; whereas Con A, RCA I, and WGA bound equally to primaries and metastases. SBA bound to >50% of cells in 5 metastases but not to the 5 primaries. There was <5% binding of PNA to 10 primary murine tumors after neuraminidase pretreatment of tissue sections but >50% binding in 3 of 5 metastases. These studies indicate, in both human adenocarcinomas and an experimental tumor system, that most tumor cells which metastasize show preferential binding of PNA and SBA. (Am J Pathol 1985, 119:420–429)

LECTINS are proteins which bind to complex saccharide sequences on cell surfaces, and binding can be blocked by high concentrations of certain monosaccharides.[1-3] Peanut agglutinin (PNA), derived from Arachis hypogaea, binds to terminal β-D-galactose and β-D-galactose (1→3)-N-acetyl-D-galactosamine residues.[4] If these sugars are blocked by sialic acid, neuraminidase must be used to remove this residue and enable cells to react with PNA.[5]

Neoplastic cell transformation is associated with an altered carbohydrate composition of plasma membranes.[6,7] Changes in lectin binding have been employed in examining tissue sections from various neoplastic and nonneoplastic pathologic conditions to characterize these alterations.[8-13] In addition, demonstration of altered lectin binding by tumor cells has been used to predict the likelihood of recurrence[14] and the invasive potential[15] of a neoplasm and to distinguish benign from malignant tumors.[16-21] In the present study, we assessed lectin binding by tumor cells in human adenocarcinomas of the breast, colon, and stomach and the murine Lewis lung carcinoma (LLC) to determine whether the carbohydrate composition of malignant cells differs in primary tumors and metastases.

Materials and Methods

We obtained tissue sections from 40 cases of adenocarcinoma of the breast (17 cases), stomach (11 cases),

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and colon (12 cases) from the surgical pathology files of Women's College Hospital. The tumors were categorized as well or poorly differentiated on the basis of light microscopy with the use of H&E stained tissue sections. The presence or absence of metastases in sections of draining lymph nodes was also determined. Formalin-fixed, paraffin-embedded blocks of primary tumors and lymph node metastases were then sectioned and stained with biotinylated lectins (Vector Laboratories, Burlingame, Calif) with the use of an avidin-biotin peroxidase technique.

The lectins and their nominal sugar specificities are listed in Table 1. Staining was performed at 37 C for 45 minutes, and the lectins were used at the following concentrations: concanavalin A (Con A), 20 μg/ml; *Ricinus communis* agglutinin I (RCA), peanut agglutinin (PNA), wheat germ agglutinin (WGA), 50 μg/ml; *Ulex europaeus* agglutinin I (UEA I), soybean agglutinin (SBA) and *Dolichos biflorus* agglutinin (DBA), 200 μg/ml. The specificity of the staining obtained with each lectin was tested by preincubating them in a 0.2 M solution of the appropriate inhibiting sugar before the staining was performed. The inhibiting sugars were obtained from Sigma Chemical Co. (St. Louis, Mo). Negative controls consisted of avidin alone and biotinylated lectins without avidin peroxidase.

The degree of lectin binding to tumor cells was assessed semiquantitatively as negative, ie, no staining (−), and positive when <5% (+), 5–50% (++), and >50% (+++) of tumor cells stained. To assess binding of PNA to tumor cells, we also treated some of the tissue sections with *Vibrio cholerae* neuraminidase, 1 unit/ml) (Calbiochem, La Jolla, Calif) at a 1:10 concentration at 4 C overnight before staining with the PNA to remove sialic acid and allow the PNA to react with newly exposed sugar residues.

Table 1—Lectins Studied for Their Ability to Bind to Primary Tumors and Metastases

Lectin	Nominal sugar specificity	Inhibiting sugar
Ulex europaeus agglutinin I	α-L-fucose Blood group O	L-fucose
Concanavalin A	α-D-glucose α-D-mannose	α-methyl-mannoside
Ricinus communis agglutinin I	β-D-galactose	D-galactose
Wheat germ agglutinin	β-(1-4)-N-acetyl-glucosamine Sialic acid	N-acetylglucosamine
Soybean agglutinin	β-D-galactose N-acetyl-D-galactosamine	N-acetylgalactosamine
Dolichos biflorus agglutinin	N-acetyl-D-galactosamine Blood Group A	N-acetylgalactosamine
Peanut agglutinin	β-D-galactose β-D-galactose (1-3)-N-acetyl-D-galactosamine	D-galactose

Table 2—Lectin Binding to Tumor Cells in 2 Cases of Breast Adenocarcinoma With and Without Axillary Lymph Node Metastases

Lectin	Adenocarcinomas of the breast		
	Primary with no metastases	Primary with metastases	
		Primary	Metastases
Ulex europaeus agglutinin I	−	−	−
Concanavalin A	+++	+++	+++
Ricinus communis agglutinin I	++	++	++
Wheat germ agglutinin	++	++	++
Soybean agglutinin	++	++	++
Dolichos biflorus agglutinin	++	++	++
Peanut agglutinin	+	+	+++

−, negative; +, <5% of tumor cells stained positively; ++, 5–50% of tumor cells stained positively; +++, >50% of tumor cells stained positively.

The LLC tumor was adapted to tissue culture and grown as a monolayer in Alpha medium (Flow Laboratories, Mississauga, Ontario) supplemented with 15% fetal calf serum.[22] When 1×10^6 cells were injected into the footpads of C57 mice, tumors appeared within 45 days. To induce pulmonary metastases, LLC tumor-bearing hind limbs were amputated, which resulted in a 100% incidence of metastases, in contrast to their absence when no hind limb amputation was performed.[23] Five primary tumors from mice which did not undergo leg amputation and 5 primary tumors and lung metastases from mice subjected to leg amputation were examined with the biotinylated lectins, as above.

Results

Human Tumors

Binding of Different Lectins to Human Primary Adenocarcinomas and Lymph Node Metastases

Binding of the seven lectins (Table 2) to 2 cases of adenocarcinoma of the breast, with or without lymph node metastases, was assessed. No binding of UEA I was seen. No difference in binding of Con A, RCA I, WGA, SBA, or DBA was noted in the primary tumor, whether metastases were present or not, or in the case of the metastatic adenocarcinoma, in the primary tumor or metastases (Table 2). With PNA, there was <5% binding by tumor cells at the primary site, whether metastases were present or not. However, in the metastatic adenocarinoma, PNA bound to >50% of tumor cells in the metastases. Similar results were observed in 2 cases each of metastatic and nonmetastatic adenocarcinomas of the colon and stomach. Binding

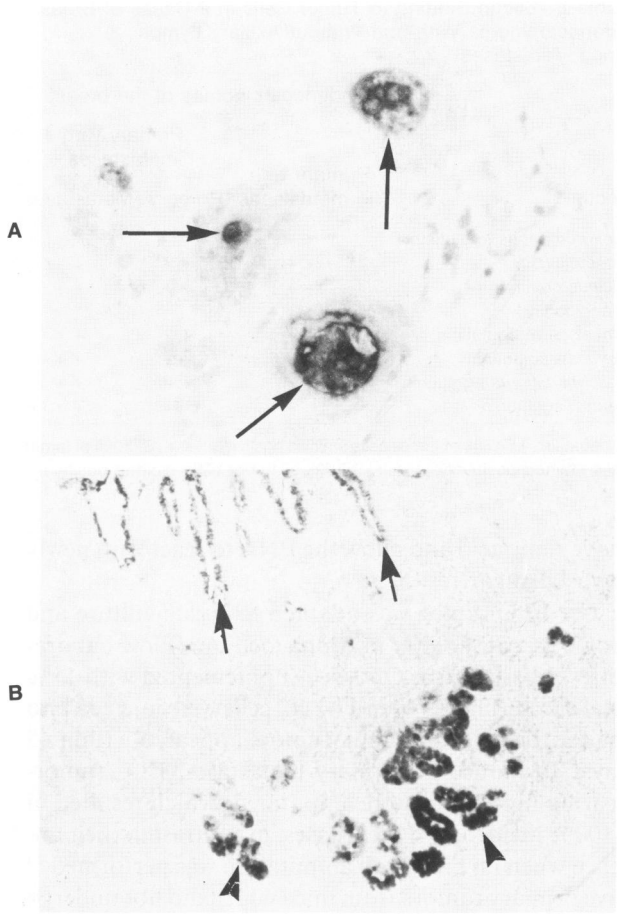

Figure 1—Normal human tissue. **A**—Breast, showing binding of PNA to secretions within ducts (*arrows*). (Avidin–biotin peroxidase, ×350) **B**—Stomach, showing binding of PNA to superficial mucosal glands (*arrows*) and to the cytoplasm of cells lining deep mucosal glands (*arrowheads*). (Avidin–biotin peroxidase, ×140)

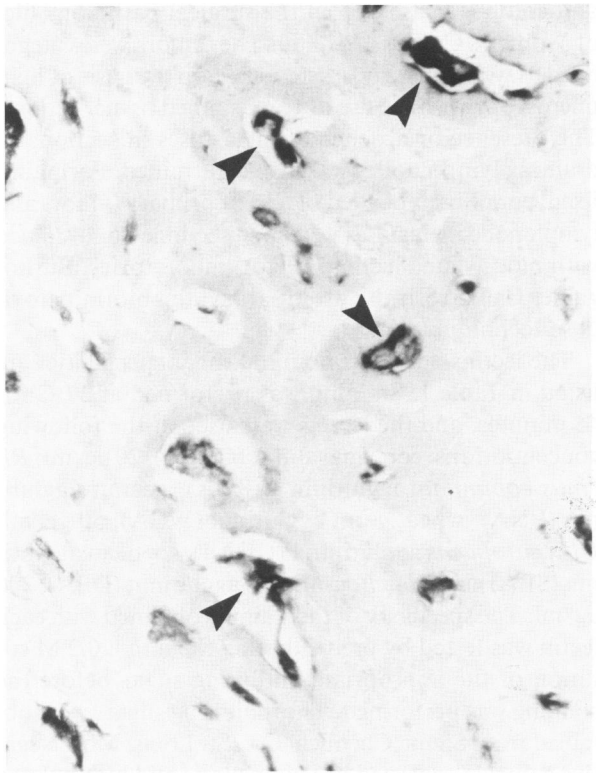

Figure 2—Human adenocarcinoma. Well-differentiated adenocarcinoma of the colon, showing binding of peanut agglutinin to secretions within gland lumens (*arrowheads*). (Avidin–biotin peroxidase, ×350).

of PNA was examined more closely for determination of the frequency of the above results.

PNA Binding to Normal Breast, Colon, and Stomach; Primary Human Adenocarcinomas of Breast, Colon, and Stomach; and Lymph Node Metastases

PNA bound to secretions within duct lumens and the apical surfaces of duct epithelium in normal breast (Figure 1A). In contrast, no PNA binding was observed with normal colon. Normal stomach showed PNA binding by secretions, with surface accentuation on superficial mucosal glands and cytoplasmic staining of deep mucosal glands (Figure 1B). Three patterns of staining were observed in the adenocarcinomas of the breast, colon, and stomach: staining of secretions (Figure 2); plasma membrane staining of tumor cells (Figure 3A and B), and staining of the cytoplasm of tumor cells, especially in poorly differentiated adenocarcinomas (Figure 4A and B). PNA binding was studied in 16 cases of adenocarcinoma (breast, 7 cases; colon, 6 cases; and stomach, 3 cases) without regional lymph node metastases and 24 (breast, 10 cases; colon, 6 cases; and stomach, 8 cases) with metastases. Staining of the cytoplasm of tumor cells varied but was either negative or <5% in 37 of 40 primary adenocarcinomas (ie, 15 of 16 without metastases and 22 of 24 with metastases) (Figures 3A and 4A and Table 3). The tumor cells which bound PNA were often aggregated into small clusters. In contrast, there was >50% binding of PNA by metastatic tumor cells within lymph nodes in 18 of 24 adenocarcinomas of the breast (7 of 10 cases), colon (5 of 6 cases), and stomach (6 of 8 cases) (Figure 4B). In 9 of these cases, PNA binding approached 100%. Of the 6 cases which did not show an increase in PNA binding in metastases, 5 demonstrated similar binding in both the primary tumor and the metastases, while in one there was no PNA binding by tumor cells in metastases. Many of the adenocarcinomas which demonstrated only positive staining of secretions with PNA in the primary tumor showed plasma membrane and cytoplasmic positivity of tumor cells in metastases. When tissue sections were treated with neuraminidase before assessment of PNA binding, all tumor cells in 38 of 40 adenocarcinomas, both primary tumors and

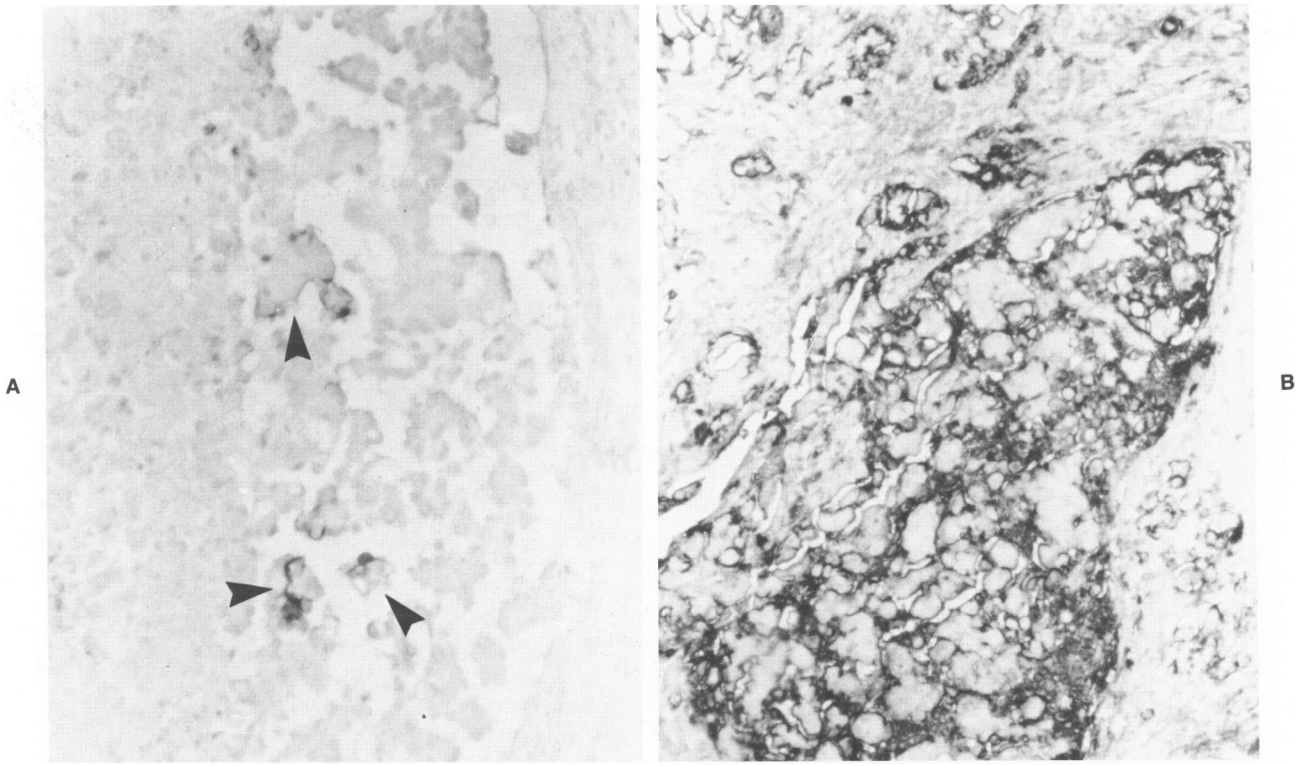

Figure 3—Human adenocarcinoma. Poorly differentiated infiltrating duct adenocarcinoma of the breast, showing (**A**) PNA binding to the plasma membrane of occasional tumor cells (*arrowheads*) (avidin–biotin peroxidase, ×350) and (**B**) PNA binding to the plasma membrane of all tumor cells after treatment of the tissue section with neuraminidase (avidin–biotin peroxidase, ×350).

metastases, showed plasma membrane and/or cytoplasmic binding (Figure 3B).

Murine Tumor

Binding of Different Lectins to the Murine Primary LLC Tumor and Pulmonary Metastases

LLC cells injected into the footpads of C57 mice formed tumors within 45 days. LM examination showed that they were composed of ovoid cells with eosinophilic cytoplasm and enlarged, pleomorphic nuclei. There were extensive areas of necrosis. Pulmonary metastases could be induced by amputation of the tumor-bearing hind limb. There was no binding of UEA I or DBA to primary LLC tumors from 2 mice, one with and the other without pulmonary metastases, or to the pulmonary metastases (Table 4). Similar staining of tumor cells in the nonmetastatic and metastatic primary tumors and metastases was seen using Con A, RCA I, and WGA. Neither SBA nor PNA bound to primary nonmetastatic or metastatic LLC tumors. In the case of PNA, pretreatment of the tissue sections with neuraminidase led to <5% binding of tumor cells. In metastases, SBA bound to >50% of tumor cells, as did PNA following neuramindiase pretreatment (Table 4). A larger number of LLC tumors with and without metastases was assessed more closely for PNA and SBA binding.

PNA and SBA Binding to the Murine Primary LLC Tumor and Pulmonary Metastases

Five primary LLC tumors without and 5 with pulmonary metastases were examined for SBA and for PNA binding without and with neuraminidase pretreatment of tissue sections (Table 5). No binding of PNA by tumor cells was seen in the 10 primary tumors, with or without pulmonary metastases, in the absence of neuraminidase pretreatment. Following neuraminidase pretreatment to remove sialic acid, tumor cells remained negative or showed <5% binding of PNA to the plasma membrane and/or cytoplasm of tumor cells (Figure 5A). Under these conditions, PNA binding to >50% of tumor cells in pulmonary metastases was seen in 3 of 5 cases (Figure 5B). In addition, SBA did not bind to the 5 primary tumors with pulmonary metastases but did to >50% of tumor cells in the metastases.

Discussion

The aim of our study was to determine whether lectin binding patterns differed in primary tumors with

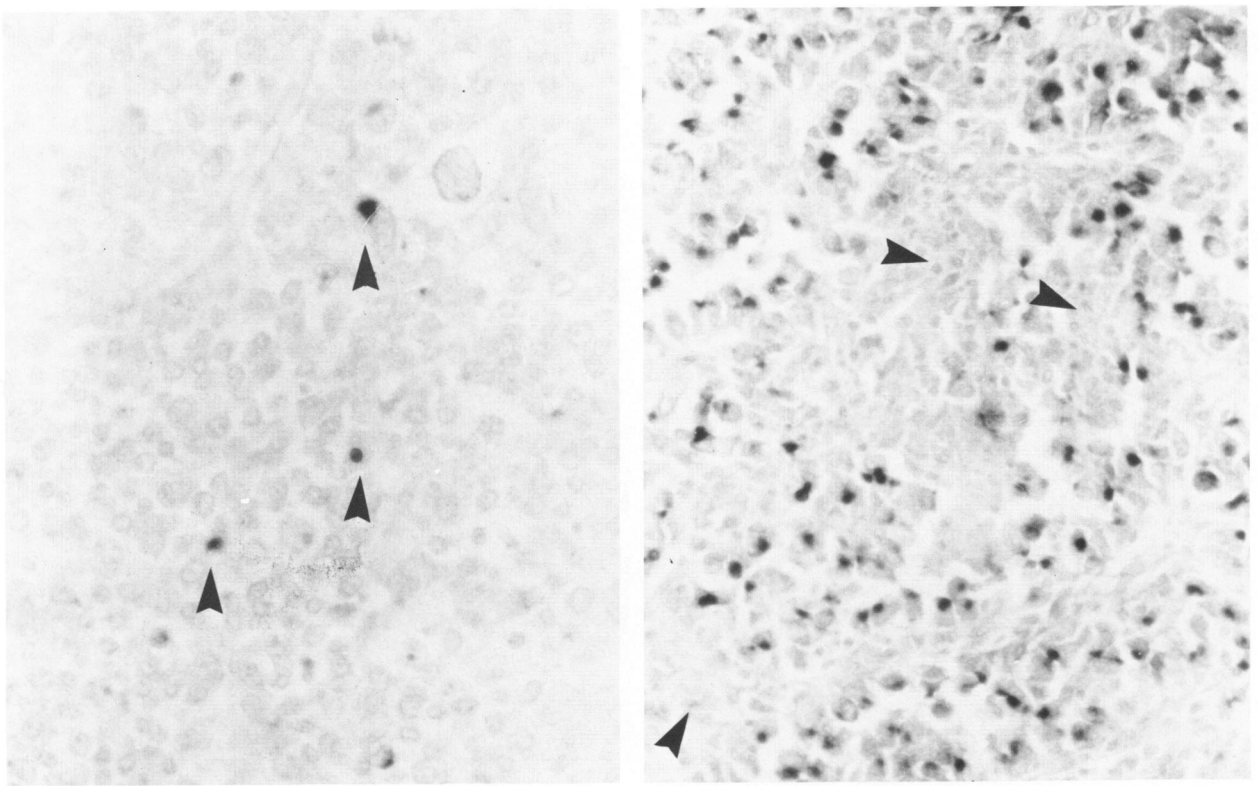

Figure 4—Human adenocarcinoma. Poorly differentiated adenocarcinoma of the stomach, showing (**A**) PNA binding to the cytoplasm of occasional tumor cells in the primary tumor (*arrowheads*) (avidin–biotin peroxidase, ×350) and (**B**) PNA binding to the cytoplasm of most of the tumor cells in a lymph node metastasis (the surrounding lymphocytes do not stain positively [*arrowheads*] (Avidin–biotin peroxidase ×350)

metastases and those without. If a difference could be identified, it would enable us to predict whether a tumor had already or would eventually metastasize. In the human adenocarcinomas and the murine tumor we examined, lectin binding by tumor cells at the primary site was the same whether or not metastases were present. However, when the primary tumor was compared with its metastases, there was a marked difference in the percentage of tumor cells which bound certain lectins, for example, PNA in human adenocarcinomas of the breast, colon, and stomach and PNA and SBA in the murine LLC tumor. In 75% of the human adenocarcinomas (18 of 24 cases), the primary tumors contained many fewer PNA-binding tumor cells than lymph node metastases, as shown in tissue sections which were not subjected to neuraminidase treatment. A similar finding was obtained in 100% (5 of 5 cases) of murine LLC tumors with the use of SBA. In addition, in LLC tumors, <5% of tumor cells at the primary site bound to PNA, whether pulmonary metastases were present or not, while >50% of tumor cells in 60% (3 of 5 cases) of pulmonary metastases did, but only after neuraminidase was used to remove sialic acid from the surface glycoproteins on the tumor cells. These observations

Table 3—PNA Binding by Cytoplasm and/or Plasma Membranes of Tumor Cells in Human Adenocarcinomas of Breast, Colon, and Stomach With or Without Lymph Node Metastases

	PNA binding of human adenocarcinomas			
	No neuraminidase pretreatment			Neuraminidase pretreatment
Percentage of cells binding to PNA	Primary without metastases (n = 16)	Primary with metastases (n = 24)		Primary ± metastases (n = 40)
		Primary	Metastases	
0	9	10	1	1
<5	6	12	3	1
5–50	1	2	2	0
>50	0	0	18	38

Table 4—Lectin Binding to Tumor Cells in 2 Cases of Murine Lewis Lung Carcinoma Tumor With and Without Pulmonary Metastases

Lectin	Lewis lung carinoma		
	Primary without metastases	Primary with metastases	
		Primary	Metastases
Ulex europaeus agglutinin I	–	–	–
Concanavalin A	+ + +	+ + +	+ + +
Ricinus communis agglutinin I	+ + +	+ + +	+ + +
Wheat germ agglutinin	+ + +	+ + +	+ + +
Soybean agglutinin	–	–	+ + +
Dolichos biflorus agglutinin	–	–	–
Peanut agglutinin			
– Neuraminidase	–	–	–
+ Neuraminidase	+	+	+ + +

–, negative; +, <5% of tumor cells stained positively; + +, 5–50% of tumor cells stained positively; + + +, >50% of tumor cells stained positively.

suggest that in primary tumors, both clinical and experimental, a small number of tumor cells with the ability to bind PNA and SBA have metastatic potential. An advantage of having made this observation in an experimental murine tumor system, as well as in human tumors, is the fact that the murine system can be manipulated for study of the mechanism of metastasis of tumor cells which bind these lectins.

Since PNA and SBA bind to galactose derivates, similar results might be expected with the use of these lectins, as was the case in the murine LLC tumor. However, RCA I and DBA also bind to galactose and its derivatives; yet these lectins did not show differential binding between primary and metastatic tumors. A larger number of different human and experimental tumor systems must be examined for determination of whether our results have general applicability. In addition, the changes in lectin binding we have described may have occurred as a result of the metastatic potential of tumor cells, rather than as a cause. However, the latter explanation is favored because alterations in lectin reactivity have been shown by many workers to be correlated with metastatic behavior.[24–27]

Tumor cells binding PNA were present in roughly equal numbers in both primary human adenocarcinomas and murine LLC tumors, with or without metastases. Two explanations may account for the finding of PNA-binding cells in tumors with no known metastases: they may possess cells which are capable of metastasizing but have not yet done so; or they may have metastasized already, and if a more complete search were made, as, for example, by serial sectioning of all lymph nodes draining a tumor, metastases might be detected.

Lectin binding by tumor cells in tissue sections has been used for study of various normal, dysplastic, and neoplastic tissues. In normal tissues, PNA binding has been described in normal lymphocytes and histiocytes,[28–31] in normal breast secretions, and on the luminal surface of glands in the breast.[18] Some workers have noted binding of PNA to normal colonic mucosa after treatment with neuraminidase, but others have not.[16,32] However, in normal mucosa adjacent to adenocarcinomas of the colon, PNA binding following neuraminidase has been reported in secretions and lumens.[33,34] In the stomach, PNA binding has been found in the cytoplasm of cells of deep mucosal glands and more superficially in secretions.[35] Variations in lectin binding, especially PNA, have been noted with age in prostatic epithelium[10] and with abnormal epidermal maturation, such as in psoriasis.[11,36,37]

With neoplasms, PNA binding has been noted in adenomas, carcinomas in situ,[17] and invasive carcinomas of the colon[16]; malignant lymphomas[12,13]; transitional cell carcinomas of the bladder[8]; and breast neoplasms.[18,20] In adenocarcinomas of the breast, colon, and stomach, PNA binding by tumor cells was characterized by alteration from secretion or luminal positivity to plasma membrane or cytoplasmic staining.[9,19] Less differentiated breast tumors, which tended to be more aggressive and had a worse prognosis, showed less PNA

Table 5—PNA Binding by Cytoplasm and/or Plasma Membranes of Tumor Cells in Murine Lewis Lung Carcinomas With or Without Pulmonary Metastases

Percentage of cells showing PNA binding	PNA binding of murine Lewis lung carcinomas				
	No neuraminidase pretreatment		Neuraminidase pretreatment		
	Primary without metastases (n = 5)	Primary with metastases (n = 5)	Primary without metastases (n = 5)	Primary with metastases (n = 5)	
				Primary	Metastases
0	5	5	3	3	0
<5	0	0	2	2	1
5–50	0	0	0	0	1
>50	0	0	0	0	3

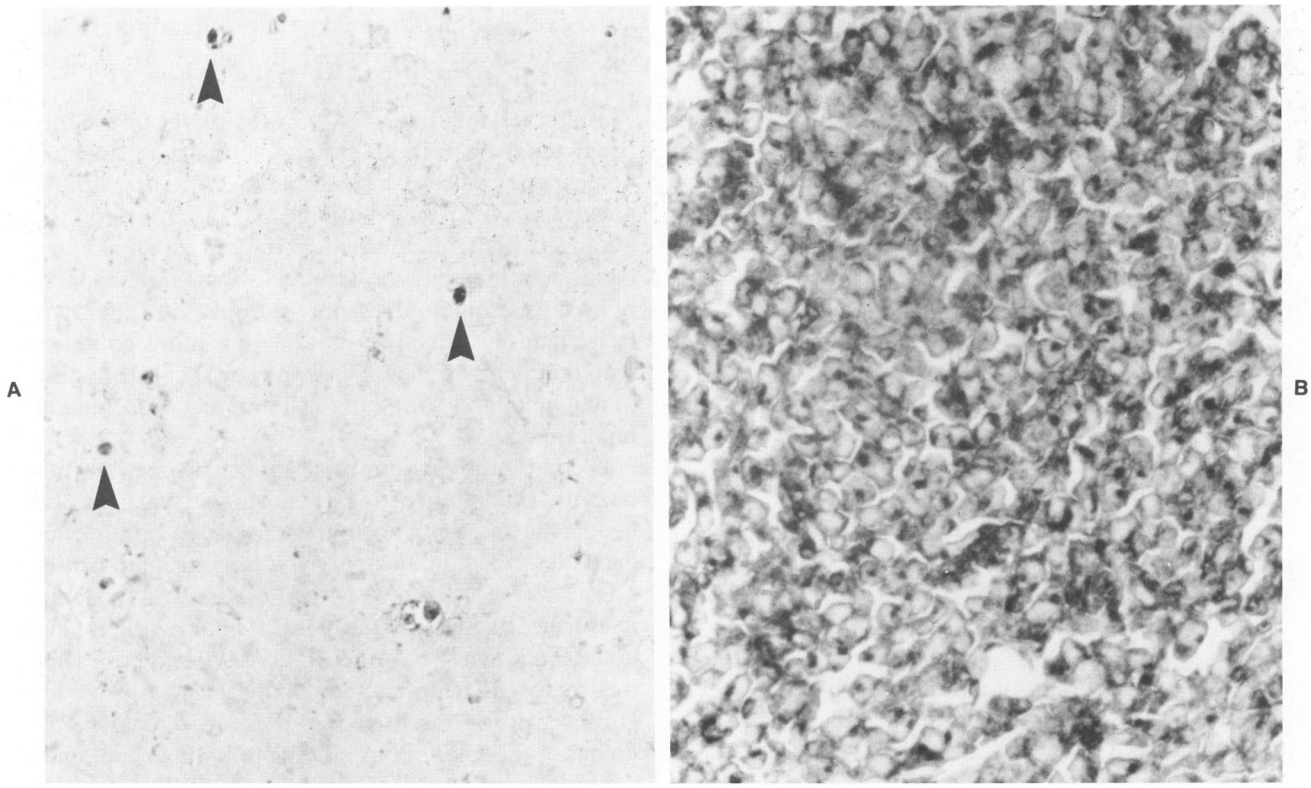

Figure 5—Murine tumor. Lewis lung carcinoma, showing (**A**) binding of PNA to a few tumor cells in the primary hind limb tumor (such a result was seen only when the tissue section was first treated with neuraminidase) (avidin–biotin peroxidase, ×350) and (**B**) binding of PNA to the cytoplasm of >50% of tumor cells in pulmonary metastases after treatment of the tissue section with neuraminidase (avidin–biotin peroxidase, ×350).

binding than well-differentiated tumors.[20] In our study using PNA, well-differentiated human adenocarcinomas showed staining of secretions with luminal accentuation. The pattern was similar to that noted in normal breast and in benign breast and colonic tumors.[16–18] These tumors also showed staining of plasma membranes and the cytoplasm of tumor cells. In contrast, poorly differentiated human adenocarcinomas showed predominantly cytoplasmic staining with PNA. We did not detect cytoplasmic and plasma membrane staining in normal tissues, except in the deep glands of the stomach. Therefore, such staining appears to be an index of malignant transformation, as others have noted.[38]

In addition to the foregoing morphologic studies, alterations in lectin binding by tumor cells have been used to characterize various aspects of tumor biology. Benign and malignant skin, breast, and colon tumors have been distinguished with the use of Con A, PNA, UEA I, and SBA[18,20,21,39]; whereas differentiation by germ-cell tumors has been studied with the use of PNA, UEA I, and DBA.[40] The possibility of predicting recurrence of breast carcinoma with Con A[14] and muscle invasion by bladder carcinoma with PNA[15] has been described. In an experimental rat mammary carcinoma and in human mammary carcinomas an increase in PNA binding was shown to coincide with the hormonal dependence of the tumor.[41,42] In a recent study of human breast adenocarcinoma with lymph node metastases, there was no difference in PNA binding by tumor cells in tissue sections of the primary tumor or its lymph node metastases in 5 of 7 cases. In a sixth case, tumor cells in metastases bound PNA, and those in the primary tumor did not. The opposite occurred in the seventh case.[43] However, one table in this study actually shows that PNA binding by tumor cells was greater in metastases than in primary tumors in 4 of 7 cases. Steck and Nicolson used tumor cell suspensions and ^{125}I-labeled lectins to study differences in the surface glycoproteins of primary tumors and their metastases. They demonstrated that there were twice as many PNA binding sites in cells from a highly metastatic clone of a rat mammary adenocarcinoma as in cells from low-metastatic clones.[24]

In most of the foregoing morphologic and biologic tumor studies, either fluorescein- or peroxidase-conjugated lectins were used. In the present study, binding of biotinylated lections was assessed with routinely

processed (formalin-fixed, paraffin-embedded) tissue sections and in avidin–biotin peroxidase technique because of our desire to measure the metastatic phenotype of tumors with routine material available in any surgical pathology laboratory. Conceivably, different results might be obtained with the use of a fluorescent technique on frozen tissue sections or quantitative flow cytometric analysis of isolated tumor-cell suspensions. Moreover, different methods of fixation and processing of tissue sections might also result in alterations in the lectin binding patterns of tumor cells.[44] For example, although UEA I did not bind to the human adenocarcinomas or the murine LLC tumor with routinely processed tissue sections, binding did occur when the sections were first digested briefly with trypsin[45] (unpublished observations).

Intraneoplastic heterogeneity is a well-known phenomenon in which subpopulations of tumor cells within a single malignant neoplasm have different characteristics, such as cellular morphology, karyotypes, receptors, and growth rates.[46] We interpret our finding of PNA-positive tumor cells within primary tumors as an indication of tumor-cell heterogeneity. In support of this, PNA-binding tumor cells in the present study were often present in focal clusters that resembled the localized or zonal sublines described by others.[47]

The metastatic process involves a complex series of steps in which malignant cells detach from a tumor, traverse vascular or lymphatic basement membranes, and circulate to distant sites, where they are trapped, cross basement membranes again, and enter tissues.[48] Metastasis by malignant tumors is a nonrandom process associated with preexisting variant tumor cells that have high metastatic potential.[49] Highly metastatic tumor cells differ from low-metastatic tumor cells in various properties, including chromosome number,[50] surface antigens (such as the amount and/or distribution of glycoproteins),[25,26,51] and the ability to home to various organs.[47] Ultrastructural studies have also shown a decrease in the amount of actin and fibronectin in a murine tumor cell line of high metastatic potential, compared with one of low metastatic potential.[52]

Sialic acid on the surface of tumor cells may play an important role in determining their ability to metastasize.[53] Some previous studies have shown that the quantity of sialic acid is increased on highly metastatic tumor cells, whereas others have found it decreased.[53,54] Because the number of PNA-binding tumor cells in our study increased after tissue sections from primary human adenocarcinomas, with or without metastases, were treated with neuraminidase, there were probably large amounts of sialic acid on these cells. However, because 75% of the human adenocarcinoma metastases had more PNA-binding tumor cells than the primary human tumors, in the absence of neuraminidase treatment of tissue sections, tumor cells without sialic acid appear to have preferentially metastasized. With the murine LLC tumor, PNA binding of tumor cells could not be demonstrated at the primary site before tissue sections were treated with neuraminidase. After neuraminidase, PNA binding remained negative or occurred in <5% of tumor cells. However, in pulmonary metastases, large numbers of PNA-binding tumor cells were demonstrated in 60% of LLC tumors after treatment with neuraminidase; this suggests that there were large amounts of sialic acid on metastatic LLC cells.

In summary, we were not able to detect any difference in the lectin-binding profiles of tumor cells in primary human adenocarcinomas of the breast, colon, or stomach, or in the primary murine LLC tumor, whether metastases were present or not. However, we found that the numbers of PNA-binding tumor cells were greater in the lymph node metastases of 75% of human adenocarcinomas and in the pulmonary metastases of 60% of murine LLC tumors than in the primary tumors. In addition, metastases of LLC tumors contained large numbers of SBA-binding cells. In the human tumors, PNA-binding cells were identified in metastases even when tissue sections were not treated with neuraminidase. However, neuraminidase treatment was necessary to show altered PNA binding by metastases of the murine LLC tumor. This difference between the adenocarcinomas and LLC tumor may have been due to alterations in the terminal sialic acid content on the surface of the human and murine tumor cells.

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