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Expression of PNA-Binding Sites on Specific Glycoproteins by Human Melanoma Cells Is Associated With a High Metastatic Potential

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Abstract Lectin-binding patterns of seven human melanoma clones and variants selected from the same parental cell line and differing in their spontaneous metastatic potential in an animal model were compared by flow cytometry and Scatchard analysis. Human melanoma clones and variants with high and low metastatic potential could be distinguished by their peanut agglutinin (PNA)–binding patterns, but not by their wheat germ agglutinin (WGA)–, *Ulex europaeus* agglutinin I (UEA I)–, and soybean agglutinin (SBA)–binding patterns. Low metastatatic clones and variants proved to be made up of a single poorly peanut agglutinin–binding cell population (2.20–3.52 $\times 10^6$ sites/cell, Ka = 2.48–2.75 $\times 10^6$ M^{-1}). By contrast, highly metastatic variants were found to be constituted by two cellular subpopulations, exhibiting respectively a moderate (2.62–3.72 $\times 10^6$ sites/cell) and a high peanut agglutinin staining (17.68–18.76 $\times 10^6$ sites/cell). One highly metastatic clone was found to be homogeneously constituted by a single population of cells strongly binding this lectin (18.86 $\times 10^6$ sites/cell) with an association constant of 4.06 $\times 10^6$ M^{-1}. Using an EPICS V cytometer, these two subpopulations were sorted from a highly metastatic variant and tested for their metastatic abilities: cells with high PNA binding generated a higher frequency of metastases than did moderately PNA-binding cells. Following treatment with *Vibrio cholerae* neuraminidase, all cells from all variants and clones were brightly labeled by PNA, collecting in a single peak with similar fluorescence intensities. Electrophoresis of total cellular proteins and subsequent detection with labeled PNA on Western blots show two major PNA-reactive glycoproteins with apparent molecular weights of 140 and 110 kDa (MAGP1 and MAGP2), expressed only in highly metastatic cells, but which can be strongly labeled by PNA in slightly metastatic cells following a treatment with neuraminidase. These results provide evidence that the expression of terminal galactose (β1–3)N-acetyl galactosamine structures, positioned on MAGP1 and MAGP2 glycoproteins, is associated with the metastatic potential of human melanoma cells.

Key words: human melanoma, metastasis, peanut agglutinin, glycoproteins, flow cytometry

The dissemination of malignant cells from a primary tumor to near and/or distant secondary sites represents the most important phase in the pathogenesis of cancer and is responsible for the majority of cancer deaths. The metastatic process involves numerous sequential steps in which cancer cells escape from the primary tumor, invade the surrounding host tissues, enter lymphatic and blood vessels, and reach distant tissues where they extravasate and proliferate to form new tumor foci [Nicolson, 1984]. At each step, the tumor cells must avoid recognition and destruction by host defenses.

There is substantial evidence that cell surface glycoconjugates are important for tumor cells to complete each of these steps [Poste and Nicolson, 1980; Dennis and Laferté, 1987; Hakomori, 1989]. Neoplastic transformation is generally accompanied by structural alterations in cell surface oligosaccharides [Hakomori, 1989] that can broadly be divided into two categories: expression of truncated or incomplete structures and expression of sequences not found in non-transformed tissues surrounding the tumor [Dennis, 1991]. However, alterations in glycosylation appear at different times during tumor progression and correlations between particular cell surface changes and specific events in the

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metastatic process are far from being fully characterized. The identification of cell surface carbohydrates associated with tumor cell metastasis should result from the comparison of closely related high and low metastatic tumor cells.

As a first approach, glycosylation mutants have been selected from highly metastatic experimental tumor cell lines using toxic concentrations of plant lectins. Following the pioneering work by Tao and Burger [1977], who selected undersialylated mutants from the murine B16 melanoma through WGA-resistance, other WGA-resistant mutants have been selected from the murine PG19 melanoma [Bramwell and Harris, 1978], the murine lymphosarcomas RAW110 [Reading et al., 1980] and MDAY-D2 [Kerbel, 1979], and from the murine Lewis lung carcinoma [Dus et al., 1990], which were found to be less metastatic than their respective parental tumor lines. Other lectin-resistant mutants [Tao and Burger, 1982; Reading et al., 1980], obtained by treatment with Concanavalin A [mannose-binding lectin] or *Ricinus communis* agglutinin [galactose-binding lectin], did not present a decrease in their metastatic potential.

Another approach was based on the comparative study of experimental tumors selected in vitro and/or in vivo in appropriate animal models. Thus, Schirrmacher et al. [1982] described a positive relationship between the binding of the PNA lectin by murine Eb lymphoma cells and their metastatic ability. This result was later confirmed, and even extended to murine sarcoma cells [Altevogt et al., 1983; Schwartz et al., 1985]. In murine mammary carcinomas, the loss of PNA-recognized glycoproteins is associated with malignant transformation [Rak et al., 1992] and with metastasis [Steck and Nicolson, 1983; Barnett and Eccles, 1984; Bresalier et al., 1990]. Other studies have demonstrated that highly metastatic cells express unsialylated structures accessible to soybean agglutinin [Lang et al., 1987; Buckley and Carlsen, 1988] or to peanut agglutinin [Badenoch-Jones et al., 1987]. In addition, subtle changes in the positioning of sialic acid [Yogeeswaran and Salk, 1981; Passaniti and Hart, 1988] and variability in glycoconjugate profiles [Raz et al., 1980; Narita, 1989; Welch et al., 1990] have been related to the metastatic phenotype. More recently, Yamamura et al. [1991] described an increase in sialylated tetra-antennary sugar chains lacking N-acetyl lactosamine on B16 melanoma cells characterized by a high lung colonizing ability.

In human melanoma, metastatic variants have been selected using different experimental animal models [Kerbel et al., 1984; Bailly and Doré, 1991], but little is known of the nature of the carbohydrate moieties associated with metastasis. The only data currently available are derived from the study of two glycosylation mutants with different degrees of resistance to WGA isolated from the MeWo cell line: while one of these showed extensive lung colony formation and wide dissemination to extrapulmonary sites when xenografted to nude mice, the other was virtually devoid of metastatic ability [Ishikawa et al., 1988; Ishikawa and Kerbel, 1989].

A new metastasis model has recently been developed in our laboratory [Bailly and Doré, 1987; Bailly et al., 1989]. This model, using orthotopic inoculation in immunosuppressed newborn rats, proved to be a suitable tool to study the spontaneous metastatic ability of human tumor cells [Bailly et al., 1991]. A large panel of clones and variants were selected by in vitro and in vivo procedures from the parental M4Be melanoma cell line; their metastasis incidence (10% to 100%) and their median lung nodule formation (0 to more than 250 nodules/rat) proved to be stable and characteristic for each clone/variant [Bailly and Doré, 1991]. To investigate the cell surface carbohydrate moieties associated with the metastatic phenotype of human melanoma cells, we studied the parental M4Be human melanoma cell line and seven of its clones and variants, chosen for their high (HM group) or low (LM group) spontaneous metastatic propensity in newborn rats. The expression of the common terminal and penultimate saccharides were compared by flow cytometric and Scatchard analyses, using lectins. To gain further information on the glycoproteins responsible for lectin binding, extracts of human melanoma cells were analyzed by Western blot. This report presents evidence that human melanoma clones/variants with high and low metastatic potential could be distinguished by their PNA-binding characteristics, but not by their WGA-, UEA I-, and SBA-binding characteristics. The presence of terminal unsialylated Galβ1–3GalNAc structures, specifically positioned on two glycoproteins with respective molecular weights of 140 kDa and 110 kDa (MAGP1 and MAGP2, respectively), could be associated with the lung metastatic potential of human melanoma cells.

MATERIALS AND METHODS

Human Melanoma Cells

Clone IC8 and variants 7GP122, 0PP20, 6PP177, 9PP257–262, and T1P26 used in these experiments were selected from the human melanoma cell line M4Be, established from a patient's lymph node metastasis [Jacubovich and Doré, 1979]. The selection and cloning procedures have been fully described elsewhere [Bailly and Doré, 1991]. Clone T1C3 was obtained from the T1P26 variant by limiting dilution. These eight human melanoma cell populations are characterized by their ability to produce spontaneous metastases and by either a low or a high number of pulmonary metastatic nodules in immunosuppressed newborn rats (Table I). The low metastatic (LM) cells displayed metastatic behavior not statistically different from that of the parental M4Be cells, while the highly metastatic (HM) cells exhibited a metastatic ability significantly superior to that of the parental cells (nonparametric Wilcoxon test). No significant differences were seen in the in vitro growth rates of cells in these two groups, and the doubling times were similar for all the melanoma cells used in these studies: 30 ± 0.5 hr.

These cells were cultured as monolayers in plastic Corning flasks (GIBCO, Paisley, Scotland), and maintained at 37°C in a 5% CO_2 humidified atmosphere in McCoy 5A medium (GIBCO) supplemented with 10% fetal bovine serum (INTEGRO B.V., Zaandam, The Netherlands). Cultures were routinely checked and found free of mycoplasma, using the Hoechst 33258 fluorescence staining procedure.

Lung Metastasis Assay

The lung metastatic potential was assessed using a standardized routine protocol [Bailly et al., 1991; Bailly and Doré, 1991]. Briefly, Wistar rats less than 24 hr old received, on day 0, a subcutaneous injection of 10^6 melanoma cells in 0.1 ml phosphate-buffered saline (PBS) in the abdomen, and a subcutaneous injection of an optimal dose of antithymocyte serum (ATS) in the dorsum. The ATS injection was repeated on days 2, 7, and 14. Animals were killed on day 21 and the metastatic potential was determined by direct counting of the pulmonary nodules.

Lectins

Fluorescein-labeled lectins, peanut agglutinin (PNA), soybean agglutinin (SBA), *Ulex europaeus* agglutinin I (UEA I), and wheat germ agglutinin (WGA), respectively specific for β-D-galactose(1–3)N-acetyl-D-galactosamine, N-acetyl-D-galactosamine, α-L-fucose, and N-acetylneuraminic acid, were purchased from E.Y. Laboratories (San Mateo, CA).

Neuraminidase Pretreatment

Subconfluent monolayer cells (48 hr after trypsinization) were treated in situ with 20 U/ml *Vibrio cholerae* neuraminidase (Boehringer Mannheim, Mannheim, Germany) at 37°C for 30 min (Berthier-Vergnes et al., 1985a,b) and subsequently subjected to flow cytometric analysis and electrophoresis.

TABLE I. Metastases of M4Be Human Melanoma Cells and Derived Clones and Variants in Immunosuppressed Newborn Rats*

Cells	Derivation[a]	Metastasis incidence[b]	Lung nodules per rat		
			Range	Median	
M4Be	Parental line	5/14	0–30	0	LM[c]
9PP257-262	Variant	11/22	0–150	0	
0PP20	Variant	13/23	0–>250	1	
IC8	M4Be clone	4/34	0–100	0	
7GP122	Variant	24/27	0–≫300	100	HM[c]
6PP177	Variant	11/12	0–≫300	112	
T1P26	Variant	21/21	2–≫300	>250	
T1C3	T1P26 clone	12/12	28–≫300	200	

*Data from Bailly and Doré [1991 and 1992].

[a]M4Be variants were selected following 1–10 in vivo passages in immunosuppressed newborn rats [Bailly and Doré, 1991]. Clones were isolated in vitro by the limiting dilution technique.

[b]Number of rats with lung metastasis/number injected rats.

[c]LM: low metastatic cells; HM: high metastatic cells (see Materials and Methods).

Flow Cytofluorimetric Analysis of Lectin-Binding Sites

Melanoma cells cultured for 48 hr were rinsed in their flasks three times with PBS, pH 7.4, and harvested using 0.5 mM ethylenediaminetetraacetic acid (EDTA) in PBS. After centrifugation, cell pellets were washed twice with PBS and aliquots of 10^6 viable cells were incubated with fluorescein-labeled lectins (20 μg/ml) in a 200 μl final volume for 20 min. Cells were then washed twice with PBS at room temperature and fixed with 3% formaldehyde in PBS for 30 min. Control experiments were conducted either in the absence of lectin or in the presence of lectin preincubated with its appropriate inhibitory sugar (0.3 M). The stained cells were then subjected to flow cytometric analysis using a FACStar cytometer (Becton Dickinson, Mountain View, CA). In each experiment 10^4 cells were analyzed using the green fluorescence emission channel (530 nm). The same scattered fluorescence gates were used for each clone/variant regardless of the size, morphology, or melanin content of the cells. The mean specific fluorescence intensity was calculated by subtraction of the mean fluorescence intensity of cells incubated with sugar-inactivated lectin from that of cells stained with the lectin alone.

Flow Cytometric Cell Sorting

The PNA–fluorescein isothiocyanate (PNA-FITC) cell surface labeling was performed as described above without fixation. The analysis and sorting were carried out in a sterile environment at room temperature using an EPICS V flow cytometer (EPICS V, Coulter, France), equipped with a 100 μm nozzle, at a rate of 1,000 cells per second. The optical configuration was 488 nm, 300 mW (Spectrophysics Argon LASER), green emission 515–535 nm. Objects were sorted by forward-angle-light-scatter (FALS) and integral fluorescence (IF).

Quantitation of Cell Surface Sialic Acid

Total cell surface sialic acid was measured by mild periodate oxidation. Melanoma cell suspensions were treated with 0.5 mM of sodium metaperiodate (Merck AG, Darmstadt, Germany) for 15 min at 4°C in the dark [Berthier-Vergnes et al., 1985b]. After centrifugation, the amount of formaldehyde released in the supernatant was assayed by a colorimetric method as described by Massamiri et al. [1978]. Standard curves were obtained using periodate-treated commercial N-acetylneuraminic acid (Sigma Chimie, St Quentin Fallavier, France).

To measure cell surface sialic acid accessible to neuraminidase, monolayers of human melanoma cells were treated in situ with *Vibrio cholerae* neuraminidase under optimal conditions [Berthier-Vergnes et al., 1985b], and the amount of sialic acid released under such conditions was determined by a sensitive fluorimetric method [Hammond and Papermaster, 1976].

Scatchard Analysis of PNA-Binding Sites

Quantitative binding of the peanut agglutinin to duplicate samples of tumor cell suspensions was performed using a fluorimetric method. Subconfluent monolayers of melanoma cells were harvested with 0.5 mM EDTA in PBS. Then, 2×10^5 cells were incubated for 30 min at 4°C in a 200 μl final volume of PBS, pH 7.4, supplemented with 0.5% bovine serum albumin (BSA), 0.05% NaN_3, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ (Sigma). Serially diluted PNA-FITC was added to the cell suspension to obtain final concentrations ranging from 0 to 40 μg/ml. For control experiments, 0.3 M of the inhibitory saccharide D-galactose was added. After the incubation period, the cells were washed twice with PBS and the cell pellets were then lysed with 50 μl 0.1 N NaOH and adjusted to a final volume of 2 ml with distilled water. The emission at 516 nm was monitored by excitation at 494 nm of the resulting lysates using a Jobin Yvon JY3D spectrofluorometer. For each cell line, the nonspecific binding was always less than 10% of the total binding. The binding data were plotted according to the Scatchard method in order to determine the number of PNA-binding sites and the corresponding apparent association constants.

Electrophoresis and Glycoprotein Staining

Freshly harvested melanoma cells were collected by centrifugation in cold PBS, pH 7.4, and the cell pellet was lysed on ice using a 10 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 0.1 mM EDTA, 2 mM phenylmethylsulfonylfluoride (PMSF), 1 mM diisopropyl fluorophosphate (DIPF), and 200 Kallikrein international units (KIU)/ml aprotinin. After centrifugation at 100,000 *g* for 5 min (Airfuge, Beckman, Palo Alto, CA), aliquots of the supernatants were taken for pro-

tein assay [Bradford, 1976] and then subjected to electrophoresis. One hundred micrograms of protein were dissolved in 50 mM Tris-HCl buffer, pH 6.8, containing 2.3% SDS, 2 mM EDTA, 5% β-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue, and heated at 100°C for 5 min. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed on a 5–15% gradient of polyacrylamide under reducing conditions. The gels were then incubated for 30 min in a 25 mM Tris buffer, pH 8.3, containing 0.192 M glycine and 20% methanol (v/v), and electrotransferred to nitrocellulose (Hybond-C, Amersham, UK) using a Trans Blot apparatus (Biorad). The nitrocellulose sheet was incubated with a blocking solution (Boehringer Mannheim) for 30 min at room temperature. The sheet was washed three times in 50 mM Tris-HCl, 150 mM NaCl buffer, pH 7.5 (TBS), for 10 min, then incubated with digoxigenin-labeled PNA (Boehringer Mannheim) in TBS containing 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ for 1 hr. Following three washes, the nitrocellulose paper was incubated with 0.75 U/ml alkaline phosphatase–coupled anti-digoxigenin antibody (Boehringer Mannheim) in TBS for 1 hr and PNA-binding glycoproteins were visualized using 0.1 M Tris-HCl buffer, pH 9.5, containing 50 mM $MgCl_2$, 0.1 M NaCl, 0.47 mM 4–nitro blue tetrazolium chloride (NBT), and 0.43 mM 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). The gray to almost black color developed in a few minutes, and the sheets were rinsed several times with distilled water to stop the staining reaction.

RESULTS

Flow Cytofluorimetric Analysis of Lectin-Binding Sites at the Surface of Human Melanoma Cells

Histograms in Figure 1 show that human melanoma cells displayed WGA-, UEA I-, and PNA-binding sites but no SBA-binding sites. Staining with WGA or UEA I did not reveal any significant difference between the LM group and the HM group. In contrast, striking differences were observed in PNA binding between these two groups: the clones/variants having the lowest ability to metastasize exhibited the lowest fluorescence intensities (mean fluorescence intensity range: 6–18 arbitrary units, a.u.), with a single cell population lightly stained with PNA-FITC. In the HM group, all three 7GP122, 6PP177, and T1P26 variants proved to be made up of two subpopulations, one exhibiting moderate PNA staining (peak 1), and the other being highly stained by PNA (peak 2). In these variants, peak 1, corresponding to a fluorescence intensity of 30–50 a.u., encompassed 84, 30, and 77% of the above cell population of 7GP122, 6PP177, and T1P26 variants, while peak 2, corresponding to a fluorescence intensity of 400–900 a.u., included 16, 70, and 23% of the whole cell population, respectively. All cells from the highly metastatic clone T1C3, derived from the T1P26 variant, were highly stained by PNA, and collected in a single peak (550 a.u.) corresponding to the peak 2 of the T1P26 variant.

This high PNA binding by cells from the HM group, as opposed to weak PNA binding by cells from the LM group, was found to be a constant and reproducible feature, independent of in vitro cell proliferation (data not shown). In addition, the lectin recognition of cell surface carbohydrates was not influenced by culture conditions, since substitution of fetal calf serum by a synthetic serum (Ultroser G, IBF, France) gave FACS (fluorescence activated cell sorter) profiles similar to those in Figure 1.

Following treatment with *Vibrio cholerae* neuraminidase, all the cells from all variants and clones were brightly stained with PNA, collecting in a single peak of fluorescence intensity ranging from 700 to 1,000 a.u. (Fig. 2). In addition, all the melanoma cells studied displayed similar amounts of cell surface sialic acid ranging from 125 ± 10 to 149 ± 13 nmoles/mg protein as measured by mild periodate oxidization. By contrast, the level of sialic acid released by neuraminidase was higher in cells from the LM group (18.87 ± 1.35 to 27.26 ± 0.4 pmoles/mg protein) than in those from the HM group (4.68 ± 0.07 to 15.11 ± 1.28 pmoles/mg protein).

Quantitative Analysis of PNA Binding by Human Melanoma Cells

Consistent with the above mentioned FACS findings, quantitative analysis of PNA binding by M4Be, IC8, and T1C3 cells indicated a single binding affinity (Fig. 3A), while 7GP122 and T1P26 cells exhibited more complex binding patterns, which were analyzed according to a dual affinity model (Fig. 3B). Assuming a molecular weight of 110 kDa for the tetrameric PNA molecule, the Scatchard plots yielded the binding parameters indicated in Figure 3. HM T1C3 cells displayed six to eight times more PNA-binding sites (18.86×10^6 sites/cell) than M4Be or IC8 LM cells ($2.20–3.52 \times 10^6$ sites/cell), with

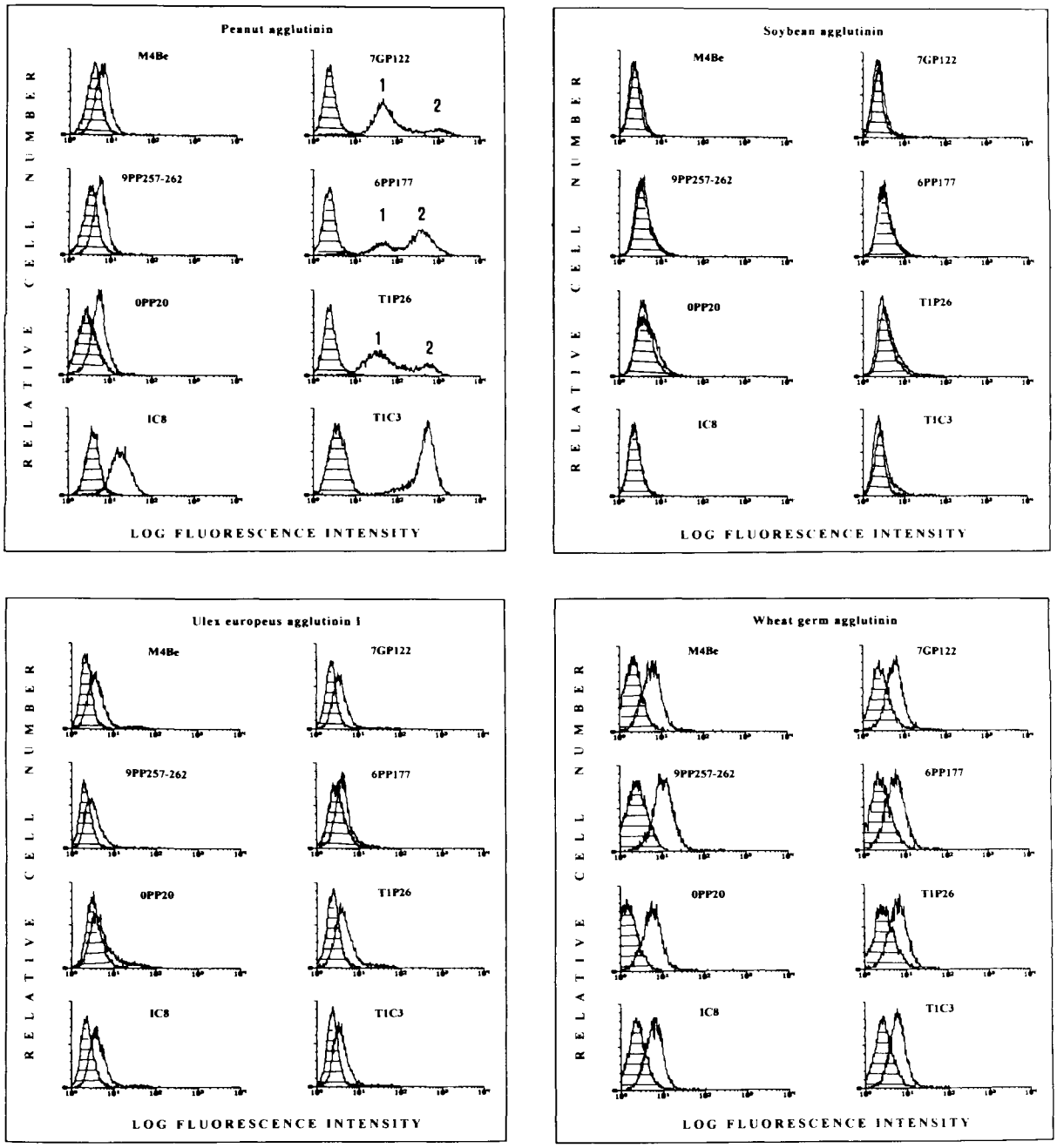

Fig. 1. Flow cytofluorimetric analysis of expression of cell surface lectin-binding sites by human melanoma cells. Staining with FITC-labeled lectin (20 μg/ml) in the absence (open areas) or in the presence of 0.3 M specific inhibitory sugar (hatched areas) as described in Materials and Methods. M4Be, 9PP257-262, 0PP20, and IC8 are low metastatic cells; 7GP122, 6PP177, T1P26, and T1C3 are high metastatic cells. Graphs represent the relative number of cells on the Y axis (linear scale) and the associated fluorescence intensities on the X axis (logarithmic scale of arbitrary units).

$4.06 \times 10^6\ M^{-1}$ and $2.48–2.75 \times 10^6\ M^{-1}$ affinity constants, respectively. While the PNA-binding data of M4Be and IC8 cells were representative of PNA binding by low metastatic cells, the PNA-binding parameters of the T1C3 clone were not identical to those of the other HM cells. Indeed, consistent with their FACS profiles, these are constituted by two subpopulations displaying low and high numbers of PNA-binding sites ($2.62–3.72 \times 10^6$ sites/cell and $17.68–18.76 \times 10^6$ sites/cell) with apparent association constants of $8.24–11.94 \times 10^6\ M^{-1}$ and

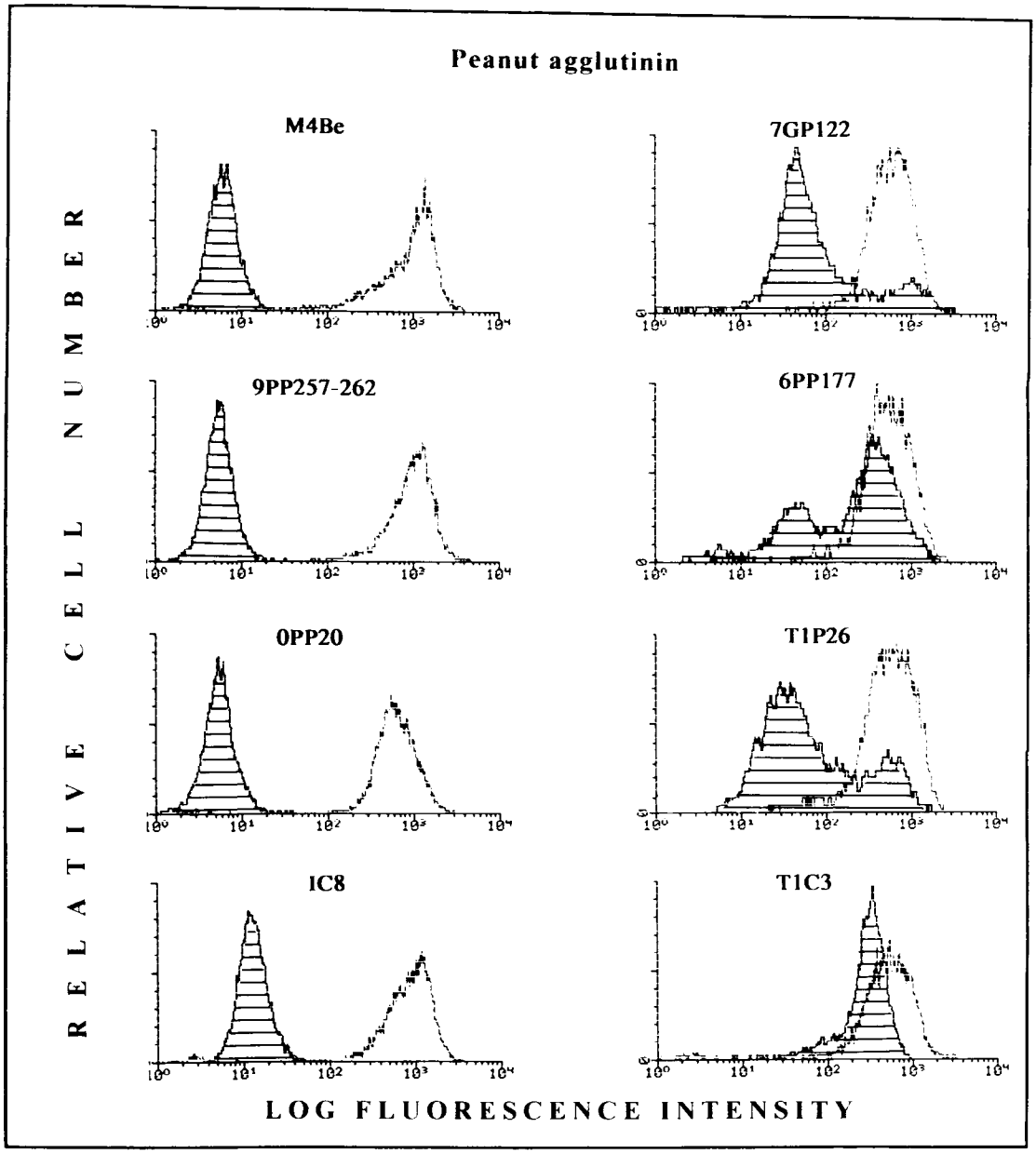

Fig. 2. Flow cytometric analysis of PNA binding by low and high metastatic melanoma cells. Staining with FITC-labeled PNA (20 μg/ml) of cells pretreated (open areas) or not (hatched areas) with 20 U/ml neuraminidase as described in Materials and Methods. Control staining in the presence of 0.3 M galactose is omitted from the figure (see Fig. 1). Cells and units as in Figure 1.

0.78–1.03 × 10^6 M^{-1}, respectively; however, these affinity constants can only be regarded as approximations, due to the non-linearity of the Scatchard plots (biphasic curves).

Western Blot Analysis of Human Melanoma PNA-Binding Glycoproteins

When digoxigenin-labeled PNA was made to react on nitrocellulose membranes to which melanoma cell proteins had been transferred, two high molecular bands of approximately 140 kDa and 110 kDa, designated MAGP1 and MAGP2, were faintly revealed, both in the parental cell line M4Be and in the three low metastatic clones and variants (Fig. 4A). By contrast, a particularly heavy staining of these two bands was observed in extracts of cells from the high metastatic T1C3 clone and 6PP177 variant. Among the HM group, 7GP122 and T1P26 variants displayed lower staining intensities of

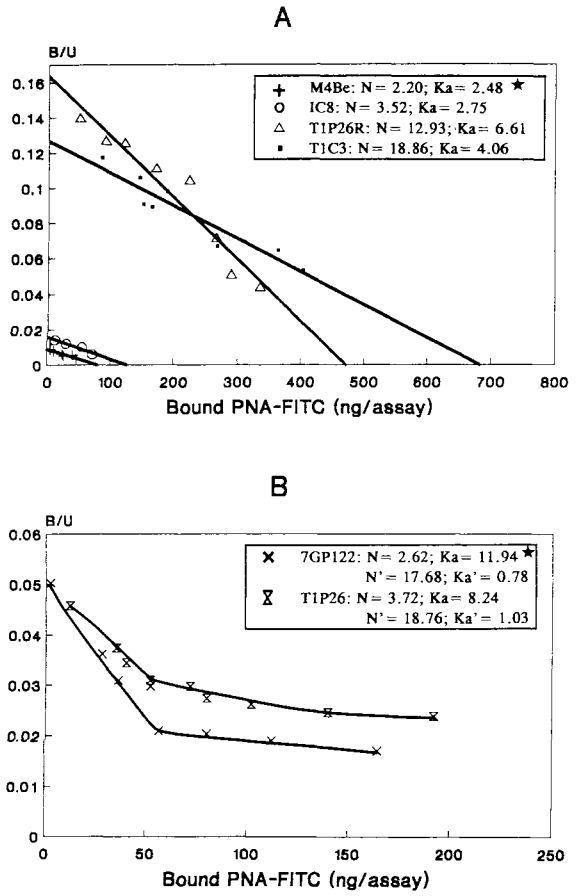

Fig. 3. Quantitative analysis of PNA binding by human melanoma cells. Scatchard plots obtained from a fluorimetric binding assay (see Materials and Methods). **A:** Homogeneously stained cells; **B:** heterogeneously stained variants. B/U: Bound PNA-FITC/ng unbound lectin by assay. Individual points are averages of at least two determinations. ★ N and Ka are, respectively, the number of PNA-accessible sites per cell ($\times 10^{-6}$) and the related apparent association constant ($\times 10^{-6}$ M^{-1}) calculated from the Scatchard plots.

MAGP1 and MAGP2 than did T1C3 and 6PP177, probably due to the lower proportion of cells exhibiting high PNA-binding sites (peak 2, Fig. 1). Such a diversity probably reflects the presence in the high metastatic variants of the two cellular subpopulations characterized either by a moderate or a high number of PNA-binding sites, as detailed above. However, it should be stressed that there was no difference in the total cellular protein profiles of these eight melanoma cells, as assessed by Coomassie brilliant blue staining or silver staining (data not shown). Moreover, following treatment of the monolayer cells by *V. cholerae* neuraminidase, the parental melanoma cell line, as well as its clones and variants, exhibited similar PNA-reactive glycoprotein patterns, which suggests that specific sialylation of MAGP1 and MAGP2 contributes to the differences observed (Fig. 4B).

Flow Cytometric Cell Sorting of Highly Metastatic Cells From the T1P26 Variant

To further define the contribution to the metastatic phenotype of the subpopulation of melanoma cells expressing the greatest number of high affinity PNA-recognized sites (MAGP1 and MAGP2 PNA-binding glycoproteins), the two cellular subpopulations evidenced by flow cytometry analysis in the HM T1P26 variant (Fig. 1) were sorted using an EPICS V cytometer into T1P26L (left, peak 1) and T1P26R (right, peak 2) cells, which were thereafter separately recultured in vitro. Flow cytometric analysis of the two sorted sublines and quantitative studies of PNA binding revealed that the T1P26L subline was constituted by a single poorly PNA-binding cell population corresponding to peak 1 (data not shown). By contrast, the T1P26R subline, although still containing a proportion of peak 1 cells, was considerably enriched in peak 2 cells, binding PNA in a comparable manner to the highly metastatic T1P26-derived T1C3 clone (12.93×10^6 sites/cell; Ka: 6.61×10^6 M^{-1}, Fig. 3). As demonstrated in Figure 5A, the PNA-binding glycoprotein profile of T1P26R sorted cells was close to that of the highly metastatic T1C3 clone, whereas the PNA-recognized glycoprotein pattern of T1P26L cells resembled that of the heterogeneous T1P26 variant. *V. cholerae* neuraminidase cleavage was able to abrogate these differences (Fig. 5B), as mentioned above. These data suggest an efficient selection of the two subpopulations constituting the T1P26 variant. The sorted subpopulations were further grown in vitro and were shown to proliferate in culture with comparable growth rates (data not shown). When xenografted into immunosuppressed newborn rats, T1P26R cells generated significantly higher numbers of lung nodules per rat than T1P26L cells ($P = 0.02$, Wilcoxon test), the respective medians of pulmonary metastatic nodules per rat being more than 250 (comparable to the HM group) and 50 (comparable to the LM group) (Fig. 6).

DISCUSSION

To identify cell surface oligosaccharides associated with metastasis in human tumors and more especially in melanoma, we have compared the lectin-binding properties of closely related

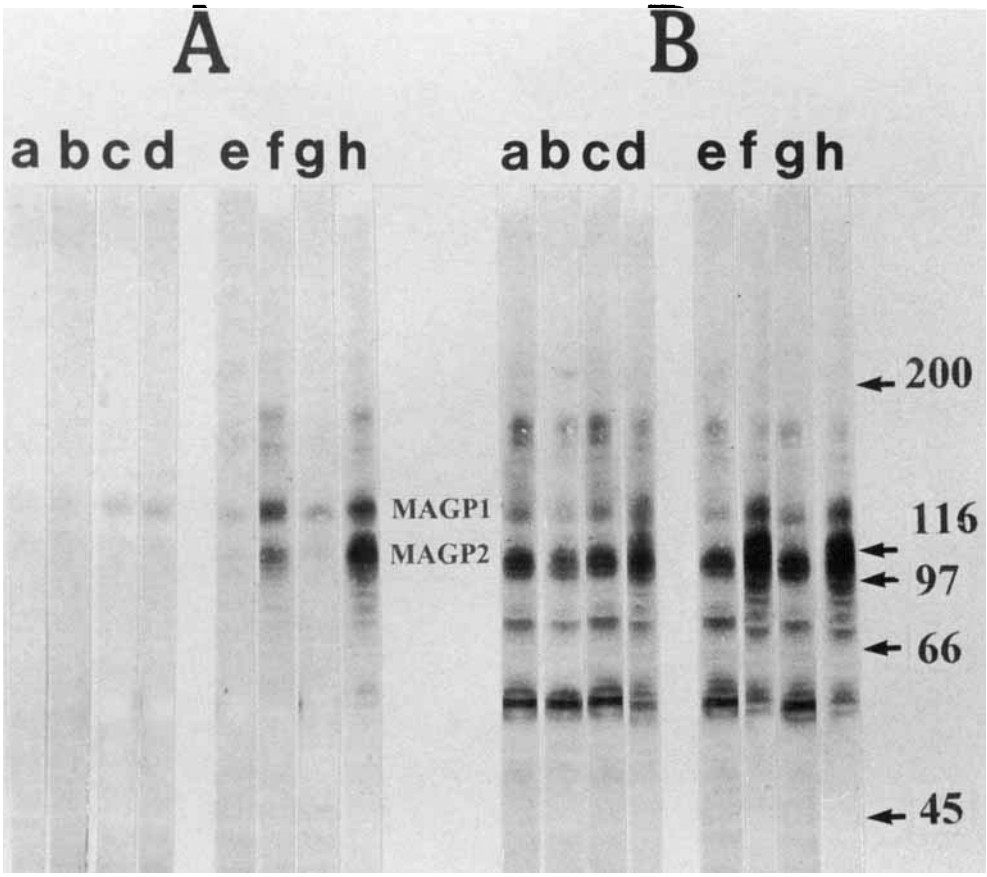

Fig. 4. PNA staining of human melanoma cell glycoproteins. Glycoproteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, stained with digoxigenin-labeled PNA, and revealed with alkaline phosphatase–labeled anti-digoxigenin antibody. **A:** No pretreatment with neuraminidase; **B:** cells pretreated as monolayers with 20 U/ml *V. cholerae* neuraminidase. **Lanes a–d:** low metastatic cells (M4Be, 9PP257-262, 0PP20, and IC8 cells, respectively); **lanes e–h:** high metastatic cells (7GP122, 6PP177, T1P26, and T1C3 cells, respectively). Arrows: standard molecular weight markers.

human melanoma clones and variants, selected for their high (HM group) or low (LM group) metastatic ability when subcutaneously grafted in immunosuppressed newborn rats. Using cells selected in this spontaneous metastasis model, we showed that the expression of a high number of cell surface PNA-binding sites by human melanoma cells correlates with a high metastatic ability. No such correlation could be found using SBA, UEA-I, and WGA.

The study, by flow cytometry and quantitative analysis, of PNA-binding parameters of human melanoma cells, revealed clear-cut differences between the low metastatic and the high metastatic cells. All the low metastatic cells, be they cell lines, variants, or cloned cells, appeared to be constituted, at least as far as PNA binding is concerned, by a single homogeneous cell population expressing 2.20–3.52 $\times$ 10^6 PNA-binding sites/cell, with apparent association constants strikingly comparable from one cell to another (2.48–2.75 $\times$ 10^6 M^{-1}). By contrast, the HM variants 7GP122, 6PP177, and T1P26 appeared to be constituted by varying proportions of two cell subpopulations: one subset (peak 1, Fig. 1) including cells five to eight times more intensely labeled than cells from the LM group, the second one (peak 2) including cells 70–150 times more intensely labeled than cells from the parental line. Accordingly, quantitative analysis of PNA binding by HM variants showed nonlinear Scatchard plots, denoting the presence within these variants of two subpopulations: one expressing a number of PNA-binding sites comparable to that of the low metastatic cells, the other expressing six times more PNA-binding sites. Binding affinities could only be approximated, but corresponded to either a fourfold

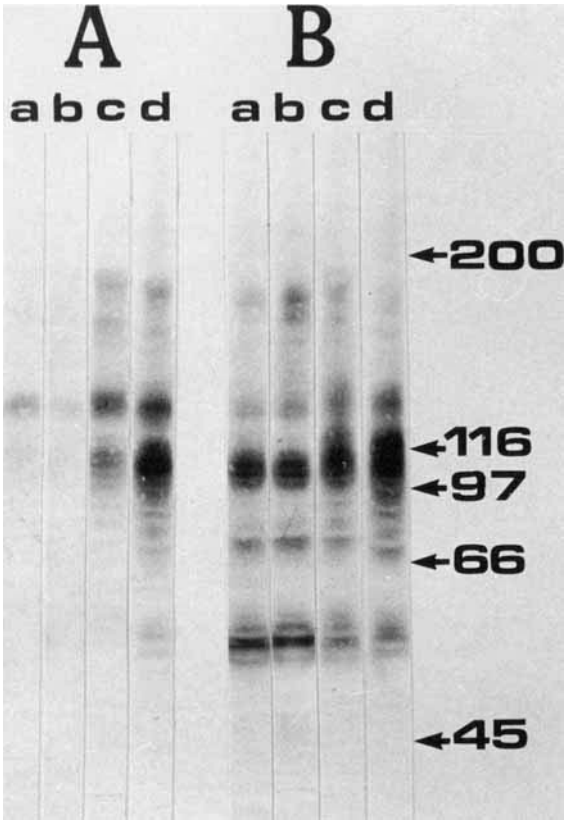

Fig. 5. PNA staining of melanoma glycoproteins from highly metastatic T1P26 cells and related sublines and clones. Same methods as in Figure 4. **A:** No pretreatment with neuraminidase; **B:** cells pretreated as monolayers with 20 U/ml *V. cholerae* neuraminidase. **Lanes a–d:** T1P26, T1P26L, T1P26R, and T1C3 cells, respectively. Arrows: standard molecular weight markers.

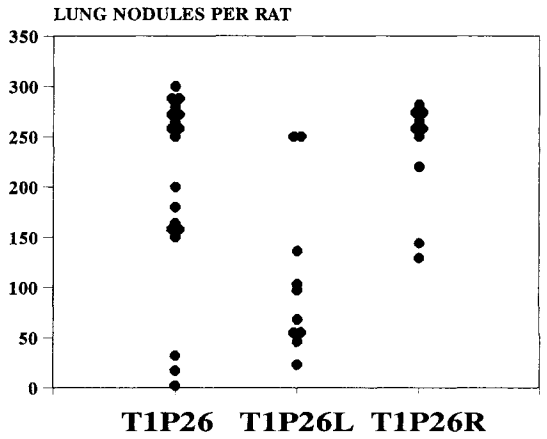

Fig. 6. Spontaneous metastases of T1P26 cells and of sorted T1P26 sublines in immunosuppressed newborn rats.

greater, or to a slightly lower affinity as compared to the PNA-binding affinity of the low metastatic cells. Consistent with these experimental data, cells from the T1P26-derived T1C3 highly metastatic clone were 100 times more intensely PNA labeled than the low metastatic parental M4Be cell line, expressing nine times more PNA-binding sites of higher affinity. These PNA-binding characteristics of LM and HM cells were remarkably constant: repeated PNA staining experiments showed that the quantitative differences in PNA binding between the two groups of melanoma cells were constant over time in culture and were not cell cycle dependent.

Since human melanoma cell lines may differ in the composition and architectural organization of their glycoconjugates within the plasma membrane [Berthier-Vergnes et al., 1985b], the low density of the PNA-binding sites observed at the surface of the low metastatic cells could reflect a nonaccessibility of these carbohydrate epitopes to this lectin. Thus, we examined binding of PNA to electrophoretically separated whole cell lysates from melanoma cells belonging to the LM and HM groups. Differences in the PNA-binding glycoproteins were observed as assessed by staining intensities of glycoprotein bands recognized by this lectin. In cells from the HM group, two major PNA-labeled glycoproteins (MAGP1 and MAGP2) could be identified, while these glycoproteins were only poorly labeled by PNA in LM cells.

When monolayer cells were treated in situ by *Vibrio cholerae* neuraminidase, the PNA-labeled glycoprotein profiles of cells from the LM group appeared qualitatively and quantitatively similar to those of HM cells. This suggests that the same PNA-binding glycoproteins are expressed in all melanoma cells, but that the terminal PNA-binding sugar moieties of these glycoproteins are specifically sialylated in the low metastatic cells. These findings were corroborated by flow cytofluorimetric analyses which show that a treatment of the LM cells with *Vibrio cholerae* neuraminidase induced a 500-fold increase in the cell surface binding of PNA, while in the HM cells neuraminidase treatment resulted in only a slight increase, if any, in cell surface PNA binding. In all cell lines tested, the total amount of cell surface sialic acid was found to be similar, whether by biochemical measurements (125–149 nmoles/mg cellular proteins) or flow cytometric analyses of the ability of cells to bind WGA, a lectin known to be specific for sialic acid residues. However, *Vibrio cholerae* neuraminidase was able to release three times more sialic

acid from the cell surface of LM cells than from that of HM cells.

Taken together, our findings strongly suggest that the high density of PNA-binding sites at the surface of human melanoma cells may be attributable to the expression of specific glycoproteins recognized by PNA. These same glycoproteins are also expressed by cells with a low density of PNA-binding sites, but in fact under a highly sialylated form so that their PNA-recognized moieties are not accessible to this lectin. Nevertheless, the metastatic potential of a given tumor could be dependent on the number of tumor cells with high levels of PNA-binding sites or on clonal interactions between different cellular subpopulations. Since the highly metastatic variants appeared to be constituted from two different tumor cell populations with, respectively, a high and a low density of PNA-binding sites, we sorted two such subpopulations from the T1P26 variant. The two sorted subpopulations, T1P26L and T1P26R, exhibited in vitro growth characteristics similar to those of their parental T1P26 variant. However, when xenografted to immunosuppressed newborn rats, T1P26R cells were shown to be significantly more metastatic than T1P26L cells. It is noteworthy that the T1P26R cells exhibited PNA-binding characteristics and a PNA-recognized glycoprotein pattern closely similar to those of the high metastatic T1P26-derived T1C3 clone, while the T1P26L cells could be characterized by a PNA-recognized glycoprotein pattern comparable to that of their parental T1P26 variant. Thus, we may hypothesize that the presence within tumor cell populations of specific subsets of cells exhibiting high numbers of PNA-binding sites shared by MAGP1 and MAGP2 is sufficient to result in an enhanced metastatic ability.

We have previously shown that normal human melanocytes grown in vitro in the presence of dialysed hypothalamic extract do not bind PNA, and that a high level of terminal sialic acid residues contributes to the absence of PNA-binding sites [Berthier-Vergnes et al., 1990]. Histochemical studies using the lectin PNA also demonstrated that human melanocytes in the skin of healthy donors do not express any PNA-recognized component at their cell surface [Réano et al., 1982; Nau and Schaumburg-Lever, 1990]. By contrast, we have also shown that human melanoma cells express PNA-binding glycoproteins, and that such an expression correlates with their tumorigenicity in nude mice [Berthier-Vergnes et al., 1986]. Thus, the PNA-recognized carbohydrates expressed on MAGP1 and MAGP2 glycoproteins by human melanoma cells appear to be specific for metastatic melanoma cells, representing a promising biochemical progression marker for this type of cancer. Indeed, preliminary results of PNA staining of tissue sections from benign naevi and melanomas showed that PNA-reactive cells were almost exclusively found in malignant lesions and increased in proportion together with the depth and thickness of the dermal invasion [Berthier-Vergnes et al., 1993].

Peanut (*Arachis hypogaea*) agglutinin specifically recognizes saccharides with terminal D-galactose residues with a strong preference for Galβ1–3GalNAc [Lotan et al., 1975]. Thus, our results suggest a key role of the Galβ1–3GalNAc disaccharide on specific MAGP1 and MAGP2 glycoproteins in the acquisition of metastatic potential. Terminal D-galactose may be a determining factor in cellular interactions during the metastatic process, serving as a complementary molecule for endogenous lectins [Raz and Lotan, 1987]. Such galactose-binding proteins have been found both on human malignant melanoma cells [Lotan and Raz, 1990] and in rat and human lungs [Kasai and Hirabayashi, 1990], suggesting that rather than a single carbohydrate–lectin interaction pathway, there may exist a complex network involving interactions between Galβ1–3GalNAc moieties on metastatic melanoma cells and specific endogenous lectins from host cells and other tumor cells.

Cell surface glycoproteins are produced during the post-translational glycosylation of proteins, involving the sequential action of various glycosyltransferases [Hakomori, 1989]. The glycosylation pathway may constitute a regulatory mechanism by masking or unmasking penultimate saccharide moieties. The type and pattern of oligosaccharide structures found on mammalian cells appear to be tissue specific and developmentally controlled. Recent studies have confirmed that during embryogenesis, expression of PNA-binding sites is involved in the delineation of migration pathways for neural crest–derived cells [Oakley and Tosney, 1991; Schroeter et al., 1990]. We would therefore predict that these developmentally regulated changes in the sialylation of Galβ1–3GalNAc may play a key role in the modulation of adhesion and motility of human malignant melanoma cells, and may be fundamental processes in tumor progression.

Further studies are needed to identify the nature of the carbohydrates involved in PNA binding by metastatic human melanoma cells, as well as the structure and function of proteins expressing these PNA-reactive sugars.

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