Flow Cytometric Analysis of Heterogeneity in Blood Group-related Antigen Expression in a Human Urinary Bladder Carcinoma Cell Line, 647V

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ABSTRACT

Previous immunohistological studies showed a relationship between expression of blood group-related antigens (BG-Ag) and invasive potential in human urinary bladder carcinoma, but the marked variability of antigen staining within many individual tumors has obscured the biological basis of this finding. We studied the expression of the A, H, and T (Thomsen-Friedenreich) BG-Ag by flow cytometry in a human bladder carcinoma cell line (647V) using fluoresceinated BG-Ag-specific lectins (Dolichos bifloris, Ulex europaeus, and Arachis hypogaea). Cell cycle compartments were quantified by flow cytometry using propidium iodide staining. Expression of all three antigens was highly variable, but staining for each antigen produced a distinct profile. T antigen expression appeared independent of A or H antigen expression. Cell populations sorted by T antigen expression showed heritable antigenic differences persistent over many weeks in culture. However, much of the T antigen variability was nonheritable, since the stable staining profiles of the sorted cells were intermediate between the parental and the profiles obtained immediately after sorting. The nonheritable antigenic variation did not appear entirely explainable by cell size or cell cycle fluctuation. These results were confirmed by isolating 64 clones that were intermediate between the parental and the profiles obtained immediately after sorting. The variability of BG-Ag expression in human bladder carcinoma cells in vitro may explain the staining patterns observed in the study of antigen expression in resected human bladder carcinomas.

INTRODUCTION

Numerous retrospective immunohistological studies have shown a correlation between deletion of normal epithelial ABH BG-Ag from noninvasive urinary bladder carcinomas and development of recurrent carcinomas with deep muscle invasion (1, 2). We have recently shown that detection of the normally cryptic T antigen in tumors provides additional prognostic information, especially in ABH-negative bladder carcinomas (4, 5). Thus, patients whose noninvasive carcinomas are both ABH negative and T positive are more likely to suffer recurrence and invasion than patients with either antigen abnormality alone. However, nothing is known of the biological basis for the correlations between BG-Ag on tumor cell surfaces and malignant phenotype. A major problem which has obscured the biological basis of these correlations and limited their clinical usefulness is the marked variability of staining patterns among different tumors and among different areas of the same tumor (6, 7). Some tumors stain predominantly along the basal cell layer, some along the luminal membrane, whereas others express the antigens in all layers, similar to normal urothelium. In some tumors, BG-Ag are detectable in only sharply circumscribed geographical areas of the tumor or in isolated clusters of tumor cells. It is unknown whether staining variability is dependent upon clonal diversity within the tumors, cell cycle-dependent antigen fluctuation, antigen fluctuation related to variable growth conditions in different areas of the tumor, or other undefined factors. Because immunohistological study of fixed tumors did not appear to allow dissection of the factors responsible for variability of BG-Ag expression, we studied the expression of these antigens in a human urinary bladder carcinoma cell line by multiparameter FCM and sorting. We found that several factors, some heritable, some not, have a role in producing the complex antigen profile in these cells.

MATERIALS AND METHODS

Tumor Cells and Culture Conditions. The human bladder carcinoma cell line, 647V, was obtained from Dr. Jorgen Fogh, Sloan-Kettering Institute for Cancer Research, Rye, NY, at passage 87. The tumor arose as a Grade II transitional cell carcinoma of the urinary bladder in a 59-year-old male of unknown ABO blood type (8). The patient was untreated prior to explantation. The tumor cell line is hyperdiploid and tumorigenic in nude mice, in which it grows with the histology of a transitional cell carcinoma (8). The line was grown as adherent cells on 80-sq cm tissue culture flasks (Nunc, Vanguard, Int., Neptune, NJ). The cells were grown in MEM with 2 mM L-glutamine, nonessential amino acids, and vitamins (Grand Island Biological Co., Grand Island, NY) and supplemented with 10% FBS (KC Biologicals, Lenexa, KS), penicillin (100 units/ml), and streptomycin (100 g/ml) (Sigma Chemical Co., St. Louis, MO). The medium pH was 7.2 to 7.4. The cells were grown in a fully humidified atmosphere containing 5% CO2 and were split at 1:5 twice weekly for routine maintenance. Cells of passages less than 100 were routinely used for all experiments; after 10 to 12 passages in vitro, new aliquots of cells were thawed and used except where noted in the text. 647V carcinoma cells were checked for the presence of Mycoplasma by the fluorescent assay of Chen (9). The results described are from cultures that were free of Mycoplasma.

Immunofluorescence Staining for BG-Ag. The cells for immunofluorescence staining were harvested in a 2-stage procedure. Twenty-four h prior to staining, the exponentially growing tumor cells were harvested from the tissue culture flasks using 0.15% trypsin (DIFCO, Detroit, MI) (at 37°C) and then reseeded at 1:2 to 1:3 to give an approximate 30 to 40% confluent monolayer of cells. These monolayers (seeded 18 to 24 h previously) were then harvested for staining by incubating the flasks with 5 ml of 10 mM EDTA for 3 min and then striking the flasks sharply from the side with the palm of the hand to dislodge the cells from the surface of the flasks. The flasks were then further incubated at 37°C for 1 min and then harvested using a 10-ml pipet. The flasks were further washed down with 10 ml of ice-cold MEM supplemented with 10% FBS.
to dislodge any additional cells. These procedures resulted in a single cell suspension with a viability of 80 to 90% as determined by trypan blue exclusion (10). The pretreatment with trypsin and resuspending did not alter the cell staining distribution of the cells compared to those dislodged with EDTA alone, but it resulted in fewer aggregates and higher viability. In contrast, cells analyzed immediately after harvesting with trypsin showed a significant shift in the BC-Ag profile compared to cells harvested with EDTA. These observations caused us to adopt the 2-stage harvesting procedure described above. The cell suspension was then washed once with Ringer's salts solution supplemented with 1% bovine serum albumin and 1 mM MgCl₂ (washing buffer), counted, and resuspended at the appropriate concentration. The cells were then reacted with one of 3 FITC-conjugated lectins known to have relative BG-Ag specificity, at 2.5 μg/ml/10⁶ viable cells in 1 ml. Optimal lectin concentration and absolute amount of lectin per 10⁶ cells were determined empirically by titration. The lectins used were obtained as lyophilized powder in their native state or as an FITC conjugate from Vector Laboratories, Burlingame, CA. The following lectins were used: PNL to detect the T antigen; UEL for the H antigen; and DBL for the A antigen. The cells were then incubated on ice for 45 min, washed once with washing buffer, and resuspended in 2 ml of washing buffer containing propidium iodide (0.15 mg/ml; Sigma) for FCM analysis. The sugars α-galactose, L-fucose, and N-acetyl-α-galactosamine were obtained from Sigma and were used as inhibitors of PNL, UEL, and DBL, respectively. For inhibition studies of the specificity of the lectin staining, 0.2 M sugar was added simultaneously with the FITC:lectin and then incubated and washed as above.

Cytchemistry for Correlation of BG-Ag Expression with Cell Cycle Compartment. For correlated cell cycle analysis and membrane immunofluorescence, the cells were first stained for cell surface BG-Ag as above. However, in some cases, the staining intensity was augmented by the use of heterologous anti-lectin antibodies (1:100; Vector Laboratories) followed by an FITC-conjugated anti-lgG antibody (Litton Bionetics, Kensington, MD). After staining of the cells, they were resuspended in 1 ml of 0.25% sucrose:0.005 M MgCl₂:0.02 M Tris-HCl, pH 8.5 (11), containing DNase (0.5 mg/ml) and RNase (1 mg/ml) (Sigma) for 15 min at 37°C to digest the nucleic acids from any dead cells before the propidium iodide staining. The cells were then washed 3 times with 2 ml of washing buffer, an essential step to remove all DNase activity. The cells were then resuspended in 1 ml of washing buffer, and then 1 ml of 100% ethanol was added with vortexing. The cells were then incubated in the fixative for 30 min at 4°C and then washed with washing buffer. The fixed cells were then digested with RNase (1 mg/ml) for 15 min at 37°C and then washed once. Finally, the cells were resuspended in 2 ml of washing buffer containing propidium iodide (0.15 mg/ml) for correlated FCM analysis.

FCM. Flow cytometric analysis of stained tumor cells was performed using a Coulter Epics V flow cytometer with confocal optics and a 3-decade logarithmic amplifier in the green fluorescence channel (Coulter Electronics, Hialeah, FL). Excitation was provided with an argon ion laser (Coherent, Worcester, MA) operating at 200 milliwatts at 488 nm. Forward-angle light scatter was measured from 0.75⁰ to 18⁰. The fluorescent light was separated from the exciting laser light using a 495-nm absorbance and a 515-nm interference filter. A 560-nm dichrom mirror was used to separate the red and green signals, with a 530-nm short pass filter in front of the green detector and a 610-nm long pass filter in front of the red detector. After setting light scatter and red fluorescence gates to exclude debris and dead cells (dead cells were excluded by uptake of propidium iodide), 1- or 2-parameter correlated histograms were collected for 5,000 to 10,000 viable cells. The instrument was calibrated daily to allow comparison of staining profiles obtained on different days using 10-μm ½ bright beads (Coulter Electronics).

For correlated cell cycle and membrane immunofluorescence studies, the red and green amplifier high voltages were adjusted by using the pulse subtractor compensation module, so that cells stained only with propidium iodide and cells stained only with FITC gave orthogonal signals on a red-green correlated plot. Cells which were nonviable at the time of immunofluorescence surface staining were excluded by their failure to take up propidium iodide (cells were treated with DNase before fixation). The data for approximately 80,000 to 90,000 cells were then collected using list mode to allow for multiple gating and analysis of the data after collection. The list mode data were then analyzed for quantification of cells in different compartments of the cell cycle using a Coulter Terak data analyzer and the Coulter Para-I program.

Cell Sorting. For sorting of 647V cells expressing varying amounts of BG-Ag, the cells were plated and harvested using the standard 2-stage harvesting procedure (above), washed, and then counted. The cells were then stained with lectin (sterile FITC:PNL, 2.5 μg/ml/10⁶ viable cells) and incubated on ice for 45 min. After incubation, the cells were washed twice, resuspended at 2 x 10⁶/ml in MEM containing propidium iodide (0.15 mg/ml), and then passed through a 25-gauge needle to disrupt doublets. The cells were then analyzed by FCM to collect a fluorescence histogram, gating out debris and dead cells as described above. Sort windows were then set to collect the desired populations. After 1 to 3 h of sorting into 2 ml of FBS in 15-ml conical glass culture tubes, we typically collected 5 x 10⁷ to 1 x 10⁸ cells. These were then centrifuged and resuspended in 2 ml of MEM supplemented with 10% FBS plus antibiotics. An aliquot was then reanalyzed by FCM to assess purity of the sort. The sorted cells were then grown in 24-well plates (Costar, Cambridge, MA) and, when confluent, expanded into Nunc tissue culture flasks. Aliquots were frozen using 10% dimethyl sulfoxide with 10% FBS in MEM.

Cloning. To isolate clones of tumor cells with different intensities of T antigen staining, we sorted cells using the Coulter Autoclone, an automatic single cell deposition system. After setting sort windows as described above, we set the Autoclone to deposit a single cell fitting the criteria of the sort window into each well of a 96-well microtiter plate (Costar) containing 200 μl of growth medium. These plates were then examined at 48 and 96 h for cell growth, and the resultant clones (30 to 40/plate) expanded and were analyzed as above.

RESULTS

BG-Ag Profiles in Human Urinary Bladder Carcinoma Cells, 647V. The staining profiles were distinctive for each of the BG-Ag, A, H, and T (Chart 1). Since all profiles showed a large range of staining intensity, we elected to use a 3-decade logarithmic scale. The profile for H antigen consisted of a single peak of brightly stained cells, with a "tail" of dimmer cells comprising less than 10% of the population. In addition to allowing us to get all cells on scale, the log amplifier emphasized the low end of the fluorescence curve (the tail) containing the dimmest cells. This logarithmic staining profile was consistent with the fluorescence microscopic appearance of the cells; most of the cells stained brightly, with a minority being much dimmer. Staining for the T antigen resulted in a somewhat different profile, in that dimmer cells were more numerous (approximately 15%) and appeared to form a second broad peak which partially overlapped the brighter peak. Antigen A expression produced another pattern, in which most of the cells stained weakly, with a minority (approximately 20%) of the cells forming a second, broad, more strongly staining peak. Inhibition studies with hapten sugars for A, H, and T antigens revealed that all cells in the major peaks and some cells in the dimmer tails were specifically stained (Chart 1). Unstained cells had no measurable green fluorescence on the scale used for stained cells. The lectin concentrations used did not cause appreciable agglutination as judged by change in forward angle light scatter compared to unstained cells on the flow cytometer, yet appeared to nearly saturate the specific

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binding sites, since increasing the lectin concentration 10 times caused only a minor change in position and no change in the shape of the profiles. The staining profiles for all antigens were highly reproducible and remained quite stable over 4 months in culture. However, because some profiles did change somewhat after 20 or more passages, antigen profiles were routinely determined with cells grown at less than 10 passages after thawing.

Relationship of T Antigen Expression to Other Cellular Parameters. The marked, unexplained variability of 647V cells for expression of the BG-Ag (Chart 1) led us to study the biological basis of this variability. Since the profile for each of the BG-Ag was significantly different, and because expression of the T antigen appeared to be independent of the A and H antigens (see below), we will focus only on analysis of variability for expression of the T antigen in this paper.

It seemed unlikely that the variation in staining intensity merely reflected the presence of cells of different size, since the staining intensity varied so widely, and because dim and bright cells of all sizes could be identified by fluorescence microscopy. Nevertheless, we explored this relationship quantitatively by measuring forward angle light scatter versus log integral green fluorescence in 647V cells stained for the T antigen (Chart 2a). Although scatter measurements are imperfectly related to cell size, being affected by cellular granularity and other factors (12), they adequately reflect cell surface area among members of the same cell line (12). Ethanol fixation had little effect on forward light scatter on these cells and did not alter the relationship between T antigen staining and scatter (data not shown). The scatter of the brightest cells was larger than that of the dimmest, as would be expected, since the flow cytometer measured total fluorescence per cell. However, the light scatter distribution of the brighter cells overlapped that of the dimmer to such a great extent that size cannot be the major determinant of staining intensity (correlation coefficient, 0.31).

The relationship between T antigen expression and the cell cycle in 647V cells is shown in Chart 2c. This analysis was performed on logarithmically growing cells doubly labeled with FITC:PNL for the T antigen and propidium iodide for DNA quantitation. A great degree of overlap of the log green fluorescence (T antigen) values for the G1-G0, S, and G2-M cell cycle compartments was apparent (correlation coefficient, 0.30). To clarify the relationship between T antigen expression and the cell cycle, we approximated the antigen staining and light scatter profiles for cells in each of 3 cell cycle compartments by computer gating (Chart 2d). Comparison of cells in Gi-G0 versus those in G2-M is shown in Chart 3. Scatter and log green fluorescence profiles for S-phase cells were intermediate (not shown). Comparison of the peak channels of the log green fluorescence histograms for cells in the G1-G0 (Channel 124), S (Channel 133), and G2-M (Channel 144) cell-cycle compartments showed relatively minor differences compared to the total range of staining intensity of 647V cells (Channels 33 to 197). Furthermore, cells representing all components of this range could be found in each cell cycle compartment. The somewhat greater peak channel of log green fluorescence for G2-M cells than for G1-G0 cells appeared attributable to the larger size of G2-M cells, as was demonstrated by gated analysis of light scatter of cells in the G1-G0 (Peak Channel 102) and G2-M peaks (Peak Channel 134) (Chart 3b). Thus, fluctuation of the T antigen with the cell cycle appeared to be a minor factor in producing variable T antigen expression, and the cell cycle-dependent variability which did exist appeared dependent upon attendant size fluctuation. However, the considerable overlap between the light scatter histograms of G1-G0 and G2-M cells implied that size variability was also not totally determined by the cell cycle.

To clarify whether the cells with dimmest and brightest T antigen expression commonly occurred in the same cell cycle compartment and among cells of similar size, we obtained the DNA and scatter histograms from the brightest and dimmest 15% of cells (Chart 4). The green fluorescence gates thus corresponded closely to the "sort gates" used for physical isolation of the dimmest and brightest staining cells (see below). Both the dimmest and brightest cells contained many cells from
FCM OF ANTIGENS IN BLADDER CARCINOMA

Chart 2. Cell surface T antigen expression correlated with cell size and the cell cycle (simultaneous analysis of 3 parameters). a, 2-parameter analysis of forward angle light scatter and log integral green fluorescence of FITC:PNL-stained, ethanol-fixed 647V cells. Data are represented as a contour plot with 6 levels showing at least 1, 2, 5, 7, 9, and 17 cells per channel in a 64 x 64-channel matrix. Plots are of 5000 cells as quantified by FCM. b, forward scatter profile corresponding to 2-dimensional plot above (a). c, 2-parameter analysis of T antigen staining versus the cell cycle. FITC:PNL-stained 647V cells were fixed and subsequently stained with propidium iodide. Data are represented as a contour plot with 5 levels showing at least 1, 2, 6, 9, and 17 cells per channel in a 64 x 64-channel matrix. Simultaneous T antigen and DNA determinations were made for 5000 cells. d, DNA histogram corresponding to 2-dimensional plot above (c). 

Investigation of Possible Heritable Basis of T Antigen Variability. To determine whether the observed antigenic variation had a heritable component, we attempted to physically isolate populations of 647V cells with markedly different expression of the T antigen, using the sorting capability of the flow cytometer. We labeled 647V cells with FITC:PNL and collected cells corresponding to the dimmest and brightest 15% of the T antigen histogram using sort windows as shown in Chart 5. The cells were collected for 3 h, and then aliquots were immediately reanalyzed by FCM. Both the bright and dim cells were initially more than 90% pure, i.e., composed of cells whose green fluorescence fell within the respective sort windows. The sorted subpopulations were then expanded in tissue culture for 21 days, and then progeny of the dim and bright sorted cells were stained with FITC:PNL to assess stability of the T antigen profile in culture. The reanalysis revealed that the dim and bright populations differed dramatically from both the parental population and the corresponding profiles observed immediately after sorting but before expansion in culture. The "bright" cells displayed a major peak similar to the parental population, but the overlapping population of dimmer cells was considerably reduced (approximately 5% of cells). In contrast, the T antigen histogram of the "dim" cells revealed a distinctly bimodal distribution. The curve in Chart 5e shows a distinctly increased number of dim cells (approximately 30%) with a second population of bright cells corresponding to the major peak of the parental line. Light scatter and DNA histograms of the bright and dim sorted cells were superimposable, eliminating size or simple cell cycle differences among the total population. The peak light scatter channel of the brightest cells was 127 compared to 88 for the dimmest. However, the light scatter profiles overlapped extensively, indicating that large dim and small bright cells were not unusual. Our conclusion from this analysis is that, although antigen expression did show some correlation with size and therefore fluctuated with the cell cycle, neither factor accounted for the wide staining variability among cells.

The dimmest cells had a reduction of G2-M and an increase of G1-G0 cells compared to the total population. Conversely, the fraction of the brightest cells in the S- and G2-M phases was increased compared with Table 1. The dimmest cells had a reduction of G2-M and an increase of G1-G0 cells compared to the total population. Conversely, the fraction of the brightest cells in the S- and G2-M phases was increased compared with
as described above has been repeated 3 times with very similar cells in culture with weekly reanalysis for over 30 weeks with between 647V cells which represent the brightest dim cells did indeed display a histogram with a greater proportion with less than 10% of the recovered cells outside the sort analysis of the sorted cells again showed excellent separation enriched for dim cells as shown in Chart 5, e and f. Immediate to derive a 647V variant population which would be further results.

very little drift in antigen expression in either population. Sorting of the parental population with analysis of the sorted populations as described above has been repeated 3 times with very similar results.

We sorted the population enriched for dim cells a second time to derive a 647V variant population which would be further enriched for dim cells as shown in Chart 5, e and f. Immediate analysis of the sorted cells again showed excellent separation with less than 10% of the recovered cells outside the sort window. After expansion in culture over 21 days, the resorted dim cells did indeed display a histogram with a greater proportion of dim cells than in the initial sorted "dims." The dim cells (approximately 45%) appeared to be present in approximately equal proportion to those corresponding to the main peak of the parental population. The doubly sorted cells thus displayed a profile which differed markedly from that of the parental population and which was stable over 10 weeks in culture. However, compared with the profile immediately after sorting, the stable profile again shifted markedly toward that of the parental population.

To further characterize the cells comprising the dim "tail" of 647V cells stained for the T antigen, we cloned sorted dim cells into individual wells of microtiter plates using the Coulter Autoclone device. The sort window for dim cells was identical to that used in the initial sort (Chart 5a). All wells were checked and contained single cells initially. A total of 64 clones was successfully expanded in culture for reanalysis by FCM. Most of the clones, e.g., clone 3, gave staining profiles with major peaks similar to the parental population except for a much smaller tail of dim cells, similar to the mass-sorted bright cells (Chart 6). However, 19 of the clones (e.g., clone 1) had a very different profile, with a peak almost totally overlaying the dim population of the parental population. The clones illustrated had essentially identical forward scatter and DNA histograms, eliminating size or cell cycle differences as explanations for different T antigen expression. This experiment seemed consistent with the partial "snap back" in culture of the mass-sorted 647V cells. However, unlike the mass-sorted cells, we were unable to verify that essentially all of the autocloned cells were dim immediately after sorting. Despite this reservation, we interpret the sorting experiments to show that the dim tail of the parental 647V cells stained for the T antigen consisted of at least 2 cellular components present in different proportions. Most of the cells (approximately 2 of 3) were only transiently dim and rapidly reproduced the major peak of the parental 647V population, while a second subpopulation was persistently dim with a distribution corresponding to clone 1.

Expression of A and H Antigens by T Antigen Variants of 647V. Because the profiles for each of the BG-Ag A, H, and T were different, it seemed likely that expression of the antigens might be independent. To probe this question further, we analyzed 2 of the T antigen clones with markedly different T antigen staining (clones 1 and 3) for expression of A and H by FCM (Chart 6). Neither of the A or H antigen histograms was significantly different for the T antigen clones or from that of the parental population, indicating that T antigen expression was not linked to expression of A and H antigens in these cells.

**DISCUSSION**

Accurately determining intratumor heterogeneity and its significance in the crucial functional properties of invasion, metastasis, and resistance to immunotherapy and chemotherapy is one of the most important topics in oncology (13). Pathologists have known for decades that individual cells from the same tumor differ in appearance (14), but the biological basis and

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**Table 1**

<table>
<thead>
<tr>
<th>Cell cycle</th>
<th>Total cells (%)</th>
<th>Dimmest 15%</th>
<th>Brightest 15%</th>
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<td>G0-G1</td>
<td>41.4</td>
<td>52.7</td>
<td>20.5</td>
</tr>
<tr>
<td>S</td>
<td>28.2</td>
<td>36.1</td>
<td>50.7</td>
</tr>
<tr>
<td>G2-M</td>
<td>30.4</td>
<td>11.1</td>
<td>28.8</td>
</tr>
</tbody>
</table>

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**Chart 4.** Cell cycle and cell size differences between 647V cells which represent the brightest 15% and the dimmest 15% of the T antigen profile. Single-parameter histograms of DNA analysis for 5000 cells are shown for dim (a) and bright (b) 647V cells. Single-parameter histograms of forward angle light scatter of 5000 cells are shown for dim (c) and bright (d) 647V cells. This chart represents gated analysis of the experiment shown in Chart 2.
significance of this variability have not been defined. Experimental evidence suggests that variability among tumor cells may be related to cell cycle fluctuation (15), differences in cell differentiation (16), altered growth conditions in different areas of the tumor (17), as well as the presence of subpopulations with heritable differences (18). Nonheritable as well as heritable variability may be significant in tumor behavior, as exemplified by the concept of the transient metastatic compartment advocated by Weiss (19).

Characterizing the variability of many important tumor cell properties is difficult, because of the cumbersome nature of the assays for these properties. Thus, it is impossible to measure invasive or metastatic ability for individual cells; one can only measure the capacity of a population as a whole. Analysis of clones is not a satisfactory solution to this problem, because one does not know whether the phenotype of cloned variants is expressed in the parental population (13). Also, it is difficult to test the hypothesis that cells may periodically enter a transient state or "compartment" from which they are more likely to invade or metastasize. It appears worthwhile, therefore, to characterize tumor heterogeneity with respect to other properties, especially cell surface antigens which may serve as markers for functional tumor properties. Surface antigens can be instantaneously and quantitatively measured for each cell in a population and interrelated to other relevant cell properties, e.g., cell cycle compartment, using multiparameter FCM. Variable expression of cell surface antigens clearly seems relevant to susceptibility of tumor cells to attack by immune effector cells (20). Considerable evidence also suggests that specific cell surface carbohydrate antigens may be important in the processes of invasion and metastasis. For example, lectin-resistant tumor variants with altered metastatic behavior also have specific alterations of cell surface oligosaccharide antigens (21). We chose to study the variability of expression of the human carbohydrate BG-Ag, ABH and T, by human urinary bladder carcinoma cells because of the extensive literature linking their expression with recurrence and muscle invasion by urinary bladder carcinomas in patients (1, 2, 4, 5). The term BG-Ag is a misnomer, since they are not restricted to erythrocytes but are expressed in normal endothelium and many epithelia throughout the body (2). Although their expression evolves with differentiation (22) and oncogenesis (23), their function is unknown.

In contrast to the many retrospective immunohistological studies of BG-Ag expression by bladder carcinoma cells, our study represents the first quantitative characterization of these antigens in bladder carcinoma cells in vitro. By studying the expression of the A, H, and T antigens by a human bladder carcinoma cell line, we have shown that these antigens may be expressed by bladder carcinoma cells grown in vitro. Since we do not know the ABO blood group of the patient from whom the tumor was derived, we do not know whether the A antigen expression was homologous or inappropriate, both of which have been reported in carcinomas (23). Normal bladder epithelium and tumors may express the H antigen regardless of ABO blood group (24). T antigen expression has been observed in some bladder carcinomas but not in normal urothelium (4). Although the structure of BG-Ag on these or other epithelial cells has not been fully defined, the lectins we used for staining have a high degree of BG-Ag specificity and are used routinely as blood-banking reagents (25). We believe that their high avidity, chemical purity, and ready availability make them highly useful reagents for the study of BG-Ag. However, we recognize that some authorities might prefer to speak of, for example, the "PNL receptor" rather than the T antigen to distinguish our data from those using other anti-T reagents. Our finding that T antigen expression varied independently of A and H antigens in 647V antigen variants is consistent with the lack of correlation between expression of T and ABH antigens in resected human bladder carcinomas (4). The great variability of antigen staining in 647V cells, as quantitated by FCM, is also consistent with the marked antigen variability seen in most resected urinary bladder carcinomas (6). In contrast, cells in normal urothelium are uniformly ABH positive and T antigen negative (4, 6).

Heritable heterogeneity made a significant contribution to the
variability of the T antigen staining of 647V cells, unlike the antigen recently studied by FCM by Bahler et al. (26). This was most convincingly demonstrated by the initial sort, which produced bright and dim subpopulations, both of which differed from the parental population through many generations in culture. Unlike experiments in which antigen variants were produced by cloning (16, 26), we have demonstrated the direct descent of a variant phenotype from the parental phenotype. Furthermore, we have shown that this variant phenotype is expressed in the parental population. However, the stable bright and dim subpopulations also differed markedly from the antigen profile of their progenitor cells immediately after isolation from the cell sorter. Liebson et al. (27) reported a similar phenomenon in murine myeloma cells sorted for high and low expression of surface immunoglobulin. This rapid partial reversion or “snap-back” toward the parental phenotype demonstrated the role of nonheritable variation in producing the broad parental staining profile. The fact that the profiles were subsequently stable through many passages in culture argues against a selective growth advantage related to T antigen expression as an explanation for the initial snap-back. The snap-back also occurred after the stable dim population was again sorted for further purification of dim cells. Furthermore, only 19 of 64 clones isolated from the dim tail of the parental population showed a stable dim phenotype. Thus, we demonstrated a predominance of nonheritable variation in producing the parental phenotype, consistent with earlier, non-quantitative reports of BG-Ag expression in vitro (28). This was most striking for cells comprising the dim tail (below the main peak) of the parental population which was apparently composed of overlapping populations of persistently dim cells and cells which reproduced the main peak when expanded in culture. Presumably, the altered profile of the sorted bright cells was due to deletion of persistently dim cells. The basis for the large, nonheritable variability of T antigen expression of 647V cells needs further elucidation. The variability among cells was not an artifact of FCM and had a duration of at least 3 h, since populations with much more restricted variability corresponding to the sort windows were detected immediately after sorting. Although the cells were very variable in size, the variation in antigen-specific fluorescence was much greater (requiring a logarithmic scale), and the fluorescence distributions of large and small cells overlapped extensively. Although a relationship between antigen expression and the cell cycle was also observed, apparently secondary to size fluctuation with the cell cycle, the extensive overlap among the antigen histograms of the cells in different cell cycle compartments precluded this as a satisfactory explanation for the variability, consistent with the findings of Bahler et al. (26) and Taupier et al. (29).

Our findings may interrelate with some of the observations from the immunohistological study of BG-Ag in bladder carcinoma. The frequent variation of antigen expression among clusters of cells in adjacent areas of the same tumor (2, 4) and the common staining stratification from basal to superficial cells (6, 7) are presumably attributable to transient cell variation but are difficult to reconcile totally with a cell cycle effect. Our present data confirm that considerable nonheritable antigen variation may not be attributable to the cell cycle. Immunohistological studies show that human bladder carcinomas may vary from none to virtually 100% of the cells expressing a given BG-Ag. Empirically, it has been found that tumors with less than approximately 30%...
of the cells expressing the ABH antigens have the same relatively high probability to invade the bladder wall as those with no antigen staining (2, 3). This is intriguing, since our data suggest that all of the cells in the “30% positive” tumor may transiently express BG-Ag. The difference between BG-Ag “positive” and “negative” tumors may thus relate to the kinetics of BG-Ag expression or the responsiveness of the cells to environmental influences which induce BG-Ag expression. Whether transient changes in cell surface BG-Ag are associated with alteration in the functional properties of tumor cells is unknown but potentially testable by using cells freshly sorted on the basis of antigen expression. The observation that the BG-Ag expressions of invasive and noninvasive parts of the same tumor are indistinguishable (2) lends some support to the idea that reversible (not heritable) phenotypic alterations may be important in tumor invasion. Finally, the heritable or clonal component to antigen variability which we have directly demonstrated may parallel the rather frequent immunohistological finding of totally dissimilar antigen expression in different blocks from the same bladder carcinoma (2). Whether functional properties differ in 647V subpopulations with stable differences in BG-Ag expression is currently under investigation.

REFERENCES

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