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Phenotyping of 76 Human Bladder Tumors with a Panel of Monoclonal Antibodies: Correlation Between Pathology, Surface Immunofluorescence and DNA Content

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Abstract—*Phenotyping of* 76 *bladder tumors* (11 *grade I,* 33 *grade II and* 32 *grade III) has been carried out by flow cytometry on cell suspensions with simultaneous determination of DNA content and surface immunofluorescence using G4 and* 5 *new monoclonal antibodies* (10*D*1, 7*C*12, 6*D*1, 3*C*6 *and* 12*F*6) *directed against bladder tumor cells. Ten normal bladder samples were used as control. Antibodies* 6*D*1 *and* 12*F*6 *were specific for tumor cells whereas the others also labelled umbrella cells. Cells from grade I tumors were labelled with* 10*D*1, 6*D*1, 7*C*12 *and* 12*F*6 *antibodies, and cells of grade II tumors with* 7*C*12 *and to a lesser degree with* 12*F*6 *but not with* 10*D*1 *and* 6*D*1. *Grade III tumor cells were specifically labelled with antibodies* 3*C*6 *and G*4. *Reactivity of antibodies with tissue sections was well correlated with cytometry results, except for the antibody* 3*C*6. *Finally, most of the cells stained by* 3*C*6 *and G*4 *were shown to have a DNA index greater than* 1.0. *In conclusion cells of low grade tumors can be identified with* 10*D*1 *and* 6*D*1 *antibodies, and antigens recognized by* 3*C*6 *and G*4 *antibodies are mostly expressed by aneuploid cells.*

INTRODUCTION

MALIGNANT TRANSFORMATION can be accompanied by modifications of cell phenotype as shown by the loss of antigens usually present at the cell surface or by the appearance of new antigens. For instance, the expression of blood group antigens was reported to be defective on tumor cells from patients with invasive bladder tumors [1]. Recently several monoclonal antibodies (Mabs) were produced by immunization with bladder tumor cells or cell lines. Some of these Mabs may represent useful reagents for identification of bladder tumor associated antigens (BTAA). According to immunohistochemical studies, some of these Mabs recognize both BTAA and antigenic structures present on normal urothelial cells. Other Mabs cross-react with elements of non-urothelial origin as for instance endothelium, lymphocytes, granulocytes or fibroblasts. Mabs G4 and E7 were reported by Chopin *et al.* to react specifically with high grade tumors and also with *in situ* carcinoma [2–4]. Altogether about 20 Mabs have been described and their patterns of reactivity analyzed by immunocytochemical methods on urinary cells or by imunofluorescence on tissue sections [5–10]. Our previous studies on flow cytometry analysis of DNA content of bladder tumor cells [11, 12] showed that DNA profiles varied according to the grade of the tumor. In the present study we have performed a combined analysis of DNA content and expression of antigens defined by the antibody G4 [2, 4] and by five new Mabs. We report here that the expression of these antigens by tumor cells varies according to the DNA content of the cells. Moreover it is shown that two of these Mabs selectively stain grade I tumor cells, whereas two others preferentially react with aneuploid cells.

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MATERIALS AND METHODS

(a) *Bladder biopsies*

Biopsies were obtained from 86 patients, of whom 76 had a primary or recurrent bladder tumor treated by endo-urethral resection only. Eleven of these 76

tumors were of grade I, 33 of grade II and 32 of grade III according to the World Health Organization classification [13]. The 10 remaining samples belonged to patients who underwent an endoscopic examination for an urological pathology of non-tumoral origin. From each biopsy one sample was frozen in liquid nitrogen for cytometry analysis and another processed for pathological examination. All 11 grade I tumors were of the non-infiltrating stage Pa. Among the 33 grade II tumors, 24 were of stage Pa and 9 of stage P1, infiltrating the chorion. In the 28 grade III tumors, eight belonged to stage Pa, three to stage P1 and 17 to P2 or a higher stage, infiltrating the muscular layer. The last four tumors were *in situ* carcinomas.

(b) *Preparation of cell suspensions*

Frozen tumor fragments were thawed and mechanically dissociated on a mesh in phosphate buffered saline (PBS)-ethylene diamine tetracetate (EDTA) buffer (140 mM NaCl, 8 mM $Na_2HPO_4 \cdot 2H_2O$, 1.67 mM $NaH_2PO_4 \cdot H_2O$, 1 mM EDTA, pH 7.2). The cell suspension was filtered then centrifuged at 250 ***g*** during 15 min. The pellet was washed once in PBS-EDTA. Cells were resuspended in 0.5 ml buffer and adjusted to 2×10^6 cells/ml.

(c) *Monoclonal antibodies*

Mabs 10D1, 7C12, 6D1, 3C6 and 12F6 were kindly provided by J.C. Laurent (Sanofi Research Center, Montpellier, France). Their preparation and properties will be described in other reports (G. Escourrou *et al.*, manuscript in preparation, A. Longin *et al.*, submitted). The G4 Mab was kindly given by D. Chopin [2, 3] (Hôpital Henri Mondor, Créteil, France).

Mab 10D1 (IgM) was obtained by immunization of a mouse with human bladder cancer T24 cell line mixed with grades I and II stage P1 human bladder tumor cells. Mabs 7C12 (IgM) 6D1 (IgG1) 3C6 (IgM) and 12F6 (IgG1) were obtained in mice by immunization with stage P1, grades II and III bladder tumor cells.

Primary screening of these antibodies was carried out by a binding assay on RT4, T24 JM and Raji cell lines, followed up by immunohistochemical screening on frozen sections of normal and tumoral bladders. The patterns of reactivity of these Mabs are summarized in Table 1.

Antibodies were used at a final optimal concentration of 20 μg/ml, in flow cytometry. The second antibody was a fluorescein isothiocyanate (FITC)-$F(ab')_2$ fragment of a goat anti-mouse immunoglobulin (Eurobio Paris, France) used at a 1:50 dilution.

(d) *Double labelling procedure*

Labelling of urothelial cells concerns membrane antigens as well as DNA. Our method is a combination of those of Clevenger *et al.* [14] and Oud *et al.* [15].

For surface antigen labelling, urothelial cells were incubated with the Mabs during 60 min at +4°C, then washed twice at 250 ***g*** in a PBS–bovine serum albumin (BSA) buffer (140 mM NaCl, 8 mM $Na_2HPO_4 \cdot 2H_2O$, 1.67 mM $NaH_2PO_4 \cdot H_2O$, 2% BSA). The pellet was then resuspended in the FITC-$F(ab')_2$ solution, out of light, for 30 min at +4°C. Following two washings, cells were then fixed in a 4 ml total volume of a freshly prepared 1% paraformaldehyde solution during 30 min at room temperature. Cells were further washed and resuspended in 100 μl PBS-EDTA buffer.

For DNA staining, cells were incubated at 37°C for 30 min in 0.1 ml PBS-EDTA buffer containing 0.1% ribonuclease A. A 0.5 ml volume of a 25 μg/ml propidium iodide solution (75 mM $Na_2HPO_4 \cdot 2H_2O$, 75 mM $NaHPO_4 \cdot H_2O$, 50 mg propidium iodide, pH 7.0) was finally added. Labelled and stained cells were examined by means of an ORTHO 50 H cytofluorograph within 3 h of staining. A first control suspension was made of cells labelled for their DNA content and incubated with FITC-$F(ab')_2$ without Mab. They provided the non-specific fluorescence level and allowed the determination of a threshold above which cells were considered positive as to their labelling by Mabs. Another control was made of a suspension of chicken red blood cells which is a reference for DNA index calculation [11, 12].

(e) *Analysis of labelled cells*

Cytometry analysis required a preliminary step during which debris, doublets and aggregates were climinated by defining the F1 window on cytogram 1 (red fluorescence peak and area) as shown in Fig. 1. Cytogram 2 shows within the F1 window two cellular populations that are distinct as to their DNA content. A second window F2 was defined by excluding cells with the lowest DNA content among those included in F1. This cytogram allowed the calculation of the DNA index. DNA index was defined as the ratio of the mode (or mean) of the relative DNA content of the $G_{0/1}$ cells of the sample divided by the mode (or mean) of the relative DNA measurement of the diploid $G_{0/1}$ reference cells [16]. Cells with a normal diploid karyotype have, by definition, a DNA index of 1.0. Histograms of cell frequency according to DNA content (parts A1 B1 C1 D1 of Fig. 1) were constructed. Using the same F1 and F2 windows of cytogram 2 surface immunofluorescence was analyzed according to DNA content (columns A2, B2, C2 and D2). In the negative control (A2) cells were only treated with the fluorescent conjugate and propodium iodide. In histogram A2, the IF_e peak represents the non-specific green fluorescence. It allows the determination of a threshold channel, at the intersection of the

Table 1. *Reactivity of the five Mabs with cell lines and normal tissues*

Cell lines	10D1	7C12	6D1	3C6	12F6
Bladder					
RT4	+	+	+	+	+
T24	+	+	+	+	+
T lymphocytes					
CEM	+	+	−	+	−
Jurkat	−	−	−	−	−
HPB-ALL	−	−	−	−	−
HSB2	−	−	−	−	−
B lymphocytes					
64-10	(+)*	(+)	−	(+)	−
Ramos	−	−	−	−	−
Daudi	−	−	−	−	−
Nalm 6	−	−	−	−	−
Raji	−	−	−	−	−
Mono/myelocytes					
K 562	−	−	−	−	−
HL 60	−	−	−	−	−
U 937	−	−	−	−	−
Tumor lines					
Breast MC7	+	+	ND†	+	ND
Lung					
NCIH 69	+	+	ND	+	−
GLCA2	+	+	−	+	−
GLC1	−	−	−	−	−
GLCP1	−	−	−	−	−
Colon Lovo	−	−	+	+	(+)
Melanoma M1477	−	−	−	−	−
Neuroblastoma Lan 1	−	−	−	−	−
Leukocytes					
Lymphocytes	−	−	−	−	−
Monocytes	−	−	−	−	−
Granulocytes	+	+	−	+	−
Normal tissues					
Tonsils, spleen	−	−	−	−	−
Thymus	−	−	−	−	−
Bladder	−‡	−‡	+	−‡	+§
Prostate	−	−	−	−	−
Kidney (proximal tubules)	+	−	−	−	−
Liver	+‖	−	+¶	−	−
Lung (alveolar epithelium)	+	+	−	+	−

*(+) indicates a weak or inconstant reactivity.
†ND: not determined.
‡Except 5–20% labelled cells in superficial urothelium (umbrella cells).
§Positive on the urothelium of about 50% of the individual specimens examined.
‖Labelling restricted to Küppfer cells.
¶Labelling restricted to biliary ducts.

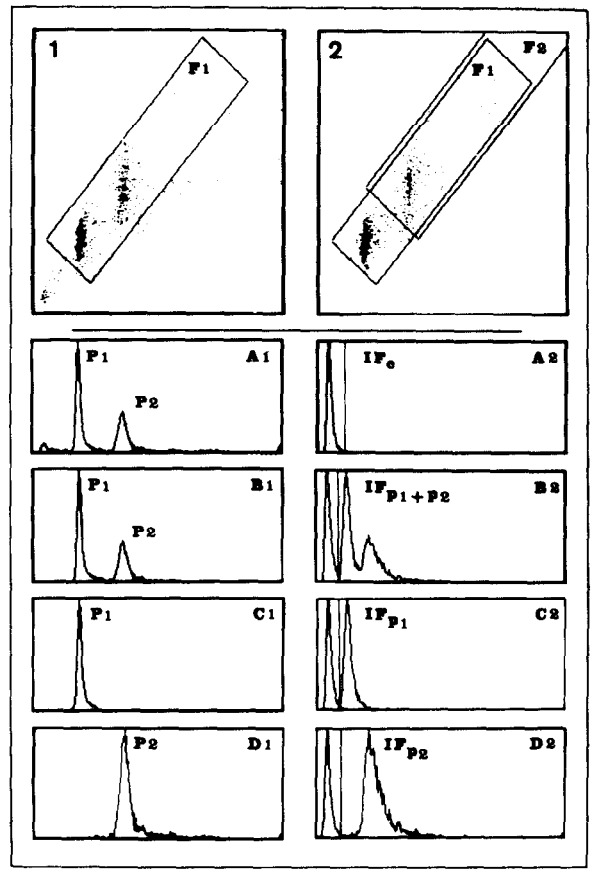

Fig. 1. *Determination of the percentages of double fluorescent cells. Top = Cytogram* 1. *Bladder tumor cells treated with propidium iodide and FITC-F(ab')$_2$ fragment of goat anti-mouse immunoglobulin without monoclonal antibody. Cytogram* 2. *Same cell suspension stained with the above mentioned reagents and* 3C6 *antibody. Abcissa: 'red surface' fluorescence intensity at* 610 nm, *arithmetical scale. Ordinate: 'red peak' of the same fluorescence intensity. Bottom = Histograms describing number of cells per channel (ordinate) according to fluorescence intensity in abcissa (arbitrary units, arithmetical scale). On the left (columns A1, B1, C1 and D1), histograms of DNA content (red). On the right (columns A2, B2, C2 and D2), histograms of FITC-F(ab')$_2$ labelling (green). A1 and A2 = cell suspension from cytogram* 1. *B1 C1 D1 B2 C2 D2 = cell suspension from cytogram* 2.

extended slope of peak IF_c with the X axis. Histogram B2 represents fluorescence intensities of cells included in the F1 window. Three peaks of green fluorescence may be seen. The first relates to non-labelled cells and bare nuclei, the two last correspond to labelled cells in the whole urothelial suspension. Histogram C2 represents the distribution of fluorescence intensities of cells included in F1 but excluded from F2, that is cells with a DNA index close to 1.0 (histogram C1). A single peak of fluorescence IF_{p1} can be attributed to this cell population. The second subpopulation side window F2 was similarly studied, and we obtained the peak IF_{p2} of fluorescence intensity on histogram D2, which corresponds exclusively to cells with high DNA content, as shown in histogram D1.

RESULTS

(a) *Distribution patterns of DNA content*

According to their DNA content, the 76 examined bladder tumors could be separated in two groups. Thirty-eight tumors (50%) had a unimodal distribution (unimodal tumors) with a single peak, and a DNA index close to 1.0. All grade I tumors, 23 grade II and four grade III tumors displayed this

pattern, as did the 10 normal bladders. The second group (bimodal tumors) had a bimodal distribution with two peaks of fluorescence, the first one with a DNA index of 1.0, the second one with a DNA index greater than 1.0. Ten grade II and 28 grade III tumors belonged to this group.

(b) Binding of monoclonal antibodies to normal and neoplastic urothelial cells

Four Mabs among six (10D1, 7C12, 3C6 and G4) showed a positive reaction with 4–6% of normal bladder cells. Immumohistochemical data showed that the positive reaction with normal urothelium concerned umbrella cells only. Mabs 6D1 and 12F6 were devoid of this reactivity. All Mabs reacted with all tumor samples, but the percentage of labelled cells and the intensity of labelling varied. Therefore we investigated these parameters according to the grade of the tumors and then the expression of the epitopes recognized by the six Mabs according to the DNA content at single cell level.

(c) *Reactivity of monoclonal antibodies in relation tumor grade*

1. *Percentage of fluorescent cells.* The 11 grade I tumors were essentially labelled with 10D1, 7C12, 6D1 and 12F6 Mabs. About 60% of cells were positive with the three first antibodies, and 69% with the fourth (Fig. 2). The 33 grade II tumors were almost the only ones to be labelled by 7C12 which recognized in average 55% of urothelial cells. These tumors were less intensely labelled with 12F6 which recognized 44% of cells. The 32 grade III tumors (including four *in situ* carcinoma) were labelled with Mabs 3C6 and G4, with an average of 54 and 58% of positive cells. Thus Mabs 10D1 and 6D1 labelled especially tumors of low grade whereas Mabs 7C12 and 12F6 labelled tumors of low and medium grade. Percentage of cells stained by these antibodies diminished as tumor grade elevated. Conversely, G4 and 3C6 antibodies labelled only a few cells in low grade tumors but a much higher proportion of cells among grade III tumors. Except for the Mab 12F6 which labelled 69% of tumor cells, the maximal percentage of labelled cells with the five other antibodies remained around 60%.

2. *Mean fluorescence intensity (MFI).* This parameter reflects the number of antigenic sites in cell membrane. Values (Fig. 2) varied slightly according to Mabs or tumor grades. The MFI for a given Mab was not significantly different between grades. However, for each Mab there was a tendency toward a slight increase of MFI when the percentage of fluorescent cells diminished and *vice-versa* but changes in MFI did not reach statistical significance.

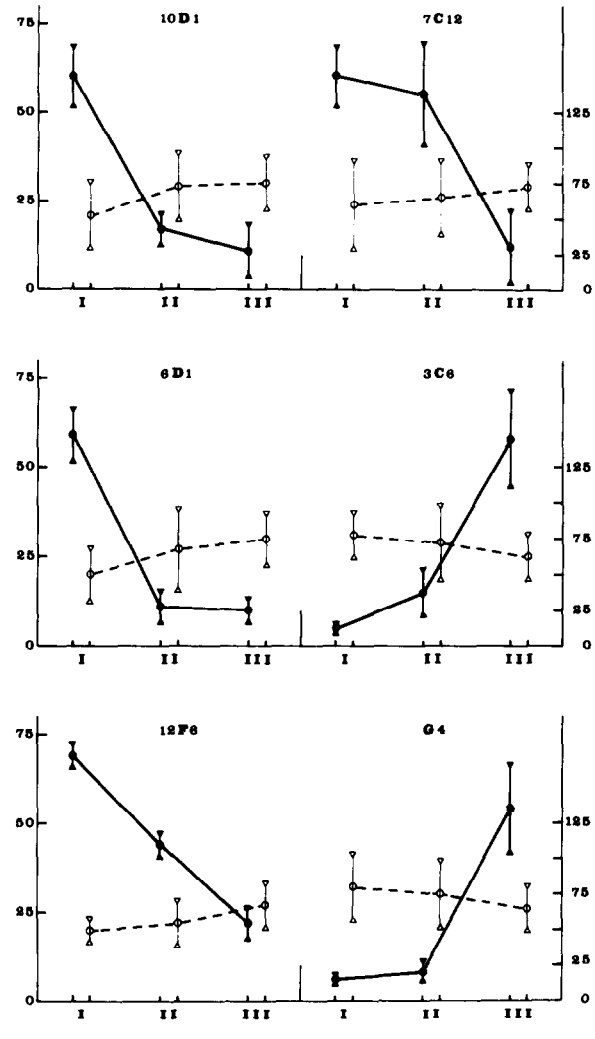

Fig. 2. Reactivity of monoclonal antibodies according to tumor grade (mean ± standard deviation). Abscissa: tumor grade. Left ordinate: percentage of fluorescent cells (thick line). Right ordinate: mean fluorescence intensity (discontinuous line).

(d) Reactivity of monoclonal antibodies in relation to DNA content

For each tumor, the percentage of fluorescent cells and MFI was calculated for cells with a DNA index close to 1.0 (IF_{p1} outside window F2, Fig. 1) and for those with a DNA index greater than 1.0 (IF_{p2} inside window F2), as well as for the total population (IF_{p1+p2}). Results are presented in Table 2. For grade I tumors the distribution of DNA was only unimodal and the pattern of reactivity is that reported above. Among grade II tumors, 23 had a unimodal profile of DNA content and 10 a bimodal one. The unimodal group was remarkable by the low percentage of cells labelled by Mabs 10D1 and 6D1 as compared to the grade I group. Conversely comparable high percentages of cells were labelled with Mabs 7C12 and 12F6 in both

groups. For the 10 bimodal tumors of grade II, total fluorescence with each of the six Mabs was similar to that of the whole group of grade II tumors and also to that of the 23 unimodal tumors of the same grade. However, differences appeared when IF_{p1} and IF_{p2} data were analyzed separately (Table 2). The percentage of cells labelled with Mabs 7C12 and 12F6 was much higher among cells with high DNA content than among other cells. Unimodal grade II tumors could be distinguished from grade I tumors by much lower percentages of cells labelled with 10D1 and 6D1 (Table 2). The 32 grade III tumors comprised four tumors of unimodal type and 28 of bimodal type.

The four unimodal tumors differed from the unimodal ones of grade I or II by a low percentage of cells labelled with 7C12 and high percentages of cells stained by 3C6 and G4. In bimodal tumors, analysis of each peak showed that the expression of antigens associated with high grade tumors and recognized by Mabs 3C6, G4 and 12F6 was much more frequent in cells with high DNA content than among other cells of the tumor.

DISCUSSION

We report here the patterns of reactivity on normal and tumoral urothelial cells of five new Mabs obtained after immunization of mice with human bladder tumor cells and cell lines. Two Mabs (6D1 and 12F6) can be regarded as specific for BTAA since they do not react with normal urothelial cells as shown by cell surface immunofluorescence. Three other Mabs, 10D1, 7C12 and 3C6, react with tumor cells but also with some umbrella cells on bladder sections. This reactivity accounts for the labelling of 4–6% of normal bladder cells by these Mabs in flow cytometry analysis. Mab G4 [2, 3] was also found to react with umbrella cells. Other authors have obtained Mabs specific for BTAA. For instance Mabs T23, T43 and T138 described by Fradet *et al.* [5, 6] react with bladder tumors but not with normal urothelium. Others have reported Mabs which react with high grade bladder tumors but their specificity and possible cross-reactivity with normal tissues has not been investigated [7–9]. Several other Mabs recognize epitopes shared by bladder tumors and normal tissues (e.g. Mabs T16, T87 and J143 of Fradet *et al.* [6]). One of the Mabs (Om5) prepared by Fradet *et al.* reacted with normal adult urothelium of 12 out of 22 tested specimens.

Thus, obtaining BTAA-specific Mabs has met limited success so far and antibodies 6D1 and 12F6 may be added to the list of the reported Mabs sharing this property. Since the four other Mabs used in the present study have only limited and well defined cross-reactivity with normal urothelium, the set of six Mabs may be useful for characterization of bladder tumors.

In the present study we have determined the

*Table 2. Percentage of cells stained with monoclonal antibodies directed against bladder tumor associated antigens according to DNA profile**

Tumor	DNA profile	*N*	Percentage of cells stained with 10D1	7C12	6D1	3D6	12F6	G4
Grade I								
	Unimodal	11	60 ± 9	60 ± 9	59 ± 8	5 ± 2	69 ± 4	6 ± 2
	Bimodal	0						
Grade II		33	17 ± 5	55 ± 15	11 ± 5	15 ± 7	44 ± 4	8 ± 3
	Unimodal	23						
	IFt		17 ± 5	55 ± 15	11 ± 3	13 ± 5	43 ± 4	7 ± 2
	Bimodal	10						
	IFt		17 ± 5	56 ± 16	12 ± 8	18 ± 10	45 ± 4	9 ± 4
	IFp1		10 ± 8	26 ± 12	6 ± 3	9 ± 4	23 ± 5	3 ± 1
	IFp2		10 ± 3	67 ± 6	8 ± 2	13 ± 6	58 ± 8	8 ± 2
Grade III		32	11 ± 8	12 ± 11	10 ± 4	58 ± 14	22 ± 4	54 ± 13
	Unimodal	4						
	IFt		8 ± 4	8 ± 5	17 ± 5	57 ± 7	14 ± 5	37 ± 16
	Bimodal	28						
	IFt		9 ± 4	9 ± 4	10 ± 4	61 ± 10	22 ± 4	57 ± 10
	IFp1		6 ± 2	5 ± 2	5 ± 2	23 ± 5	14 ± 5	21 ± 10
	IFp2		8 ± 2	8 ± 3	8 ± 3	77 ± 12	63 ± 11	68 ± 15

N: number of cases. IFt: Total fluorescence in whole urothelial suspension.
IFp1: Fluorescent cells in the first peak with a DNA index 1.0.
IFp2: Fluorescent cells in the second peak with a DNA index greater than 1.0.
*Average ± standard deviation.

reactivity of the six Mabs on bladder tumors by indirect surface immunofluorescence and quantification by flow cytometry. This method essentially detects the binding of Mabs to cell surface antigens as opposed to immunohistochemical methods on tissue sections which also reveal the binding of Mabs to intracellular antigens and which are less sensitive for the detection of cell surface antigens. Flow cytometry permits the measurement of fluorescence intensity at a single cell level, the calculation of MFI and the objective determination of the percentages of positive cells. Our results indicate that the reactivity of the six Mabs differs according to the grade of the tumor. For instance, tumors in which more than 30% of cells react with 3C6 and/or G4 belong to grade III. The most important and new finding is that two Mabs, 10D1 and 6D1, strongly react with grade I tumors and much less so with grade II or III tumors (Table 2). These two Mabs permit a precise identification of grade I tumors since there was no overlapping in the distribution of the percentage of labelled cells between grade I and grade II tumors. Furthermore tumors of grade II and III could be clearly distinguished on the basis of the percentages of cells labelled with the 7C12 Mab. The same set of six Mabs except G4 was used for immunohistochemical staining of tissue sections of bladder tumors. Detailed results will be presented in another report. Briefly an excellent concordance was found between the two methods with the exception of the 3C6 Mab which stained most of the grade I tumors on tissue sections whereas it reacted with an average of 58% cells of grade III tumors versus 5% of cells of grade I tumors in cytometry. This discrepancy may suggest that the antigen recognized by 3C6 may be localized only in the cytoplasm of grade I cells but expressed on the membrane in grade III cells. So far, attempts to correlate Mabs reactivity with pathological characteristics of bladder tumors had not been successful [10].

In previous studies we have described the alterations of DNA content of bladder tumor cells according to the grade of the tumor [11]. Therefore it was of great interest to investigate the expression of antigens defined by our six Mabs according to the DNA content at a single cell level. This was made possible by combining cell surface immunofluorescence with DNA staining by propidium iodide. All the methodological problems related to the determination of DNA content in whole fixed cells from bladder tumor have been extensively dealt with in previous reports, especially the gating of the cytograms in order to exclude debris, doublets and background signal [12]. Our results reveal two important findings. Firstly the clear-cut difference of reactivity of Mabs 10D1 and 6D1 between grade I and grade II tumors is still demonstrable when grade II tumors with a unimodal distribution of DNA content (DNA index close to 1.0) are considered. Therefore the difference is not linked with the alteration in DNA content which occur in some grade II but not in grade I tumors (Table 2). The second observation is the association within each bimodal grade II and grade III tumors between expression of antigens recognized by Mabs 3C6, 12F6 and G4 and a DNA index ranging from 1.5 to 1.8 (peaks IF_{p2} in Table 2). Conversely, in the same tumors, cells with a DNA content close to 1.0 (peak IF_{p1}) showed a decreased expression of these antigens as compared with cells of unimodal tumors of the same grade. One may speculate that cells of the first peak accumulate tumor antigens in their cytoplasm and that expression on the surface membrane occurs only after a transitional step in malignant evolution which is defined by a change in cell DNA content. Alternatively, cells of the first peak with a DNA index close to 1.0 may comprise only few tumor cells and a majority of normal urothelial cells and infiltrating leukocytes. Evidence in favor of the latter explanation comes from the observation that, whatever the Mab and the grade of the tumor, no more than 69% of the cells from each specimen were labelled. Furthermore distinct subset of infiltrating leukocytes could be demonstrated in the same specimens (Hijazi *et al.*, manuscript in preparation). A comparable relationship between aneuploidy and tumor antigen expression was reported by Czerniac *et al.* [17, 18] who showed that the Mab Ca1 which recognizes an epitope of the carcinoembryonic antigen 'Ca', labelled only 11% of cells in unimodal tumors versus 57% in bimodal ones, suggesting that 'Ca' was preferentially expressed in high grade invasive tumors with alterations of DNA content.

In conclusion, we describe five new Mabs which recognize antigens expressed on bladder tumor cells, including grade I tumors. Two of these Mabs specifically react with BTAA. As regards the three other Mabs, their cross-reactivity with normal tissues is restricted to some umbrella cells. Therefore these antibodies along with the G4 Mab can be used for identification of urothelial neoplastic cells. Their patterns of reactivity defined by the percentage of labelled cells in each tumor provides a clear-cut discrimination between tumors of grade I, II and III. Furthermore the expression of the antigens defined by some of these Mabs is associated with alterations of the cell DNA content.

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