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# SCANNING ELECTRON MICROSCOPY OF IMMUNO-GOLD LABELED ANTIGENS ASSOCIATED WITH BLADDER CANCER

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## Abstract

Scanning electron microscopy (SEM), in the backscattered electron imaging (BEI) mode, has been used to study the topographical distribution of colloidal gold labeled antigens expressed on the luminal surface of the bladder urothelium in biopsies from three categories of patients: 1) normal controls; 2) patients with a history of bladder cancer but no pathological diagnosis at time of cystoscopy; and 3) patients with overt transitional cell carcinoma (TCC) of various histopathological stages and grades. Cold cup biopsies were processed for immuno-SEM according to a previously described method. Antigens under investigation were: 1) ABH blood group antigens; and 2) those identified by the following murine monoclonal antibodies (mAbs): LEU-M1, T16, 19A211, G4 and E7. In most cases labeling patterns were correlated with the surface features of the superficial urothelial cells as revealed in the secondary electron imaging (SEI) mode of the SEM. Results, to date, indicate that the immuno-gold labeling method is more sensitive than immuno-peroxidase, and that phenotypic heterogeneity of antigenic expression (or deletion) is a frequent observation of potential diagnostic or prognostic value.

Key Words: Urinary bladder, Carcinoma, Antigens, Monoclonal antibodies, Immuno-labeing, Colloidal gold, Scanning electron microscopy, Backscattered electrons.

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## Introduction

A significant number of patients with superficial bladder cancer progress to muscle invasive disease. Currently, there is no accurate method to identify these patients (14). The aim of our study is to characterize the antigenic phenotype of superficial cells of the bladder urothelium, normal and transformed at the resolution offered by the scanning electron microscope. Immuno-labeling of the ABH antigens on the surface of the bladder urothelium has been reported previously, using bacteriophage T4 as marker recognizable with the scanning electron microscope (12). Immuno-labeling of the ABH antigens with colloidal gold has been reported by Nakajima et al. in a transmission electron microscope study of human salivary glands (17). Other antigens have been recently identified in the transformed bladder urothelium (2, 8, 9, 11); they have not, sofar, been studied at the resolution offered by electron microscope immuno-cytochemistry.

In our studies, we took advantage of the striking elemental contrast generated by gold particles when observed in the backscattered electron imaging (BEI) mode of the scanning electron microscope (SEM)(4). The advantages of using backscattered electron imaging to study antigenic expression on cell surfaces became obvious to us during initial SEM studies on human peripheral blood leukocytes and have been recently reviewed (5,7). The method was then used to study antigenic expression on the luminal surface of the bladder urothelium. Our initial targets were ABH blood group antigens, known for being partially deleted after malignant transformation, especially in cases of invasive bladder neoplasia (3,14,18,19,21). Phenotypic heterogeneity in the deletion of the ABH antigens was demonstrated in many cases of non- invasive TCCs and was described as a "Mosaic" labeling pattern (6). The

Abbreviations Used

| | |
|---|---|
| SEM: | scanning electron microscopy |
| SE: | secondary electrons |
| BE: | backscattered electrons |
| TCC: | transitional cell carcinoma |
| mAb: | monoclonal antibody |
| NPD/TH: | no pathological diagnosis but tumour history |
| PBS: | phosphate buffer saline |
| ABH: | blood group antigens |

word "Mosaic" was chosen to emphasize different levels of expression of the antigen on the luminal surface of adjacent cells, sharply delineated along well recognized, polygonal cell borders.

We now report results dealing not only with antigens deleted during some steps of the transformation process, but also with antigens characteristically expressed by the transformed bladder urothelium, such as those described over the past few years by Chopin et al. (2), Fradet et al. (8,9) and Hoshi et al. (11). The significance of our investigations has been enhanced by access to two distinct groups of patients: 1) a "normal control" group undergoing surgery for the correction of urinary stress incontinence or other non-neoplastic and non-inflammatory bladder conditions; and 2) a group of patients which is hereby referred to as "NPD/TH", i.e. no pathological diagnosis at time of biopsy of the bladder mucosa but with well documented bladder tumor history.

The rationale of our studies is linked to the following hypotheses:
1) high resolution immuno-gold labeling of bladder biopsies with the SEM reveals phenotypic changes in antigenic expression which are of prognostic significance and would not have been recognized with immuno-peroxidase or with flow cytometry methods; and 2) the antigenic phenotype of the superficial cells of the urothelium reflects, as a slightly delayed testimony, primary transformation events occuring in the basal cell layer of the urothelium.

## Material and Methods

Cold cup or excision biopsies were collected at cystoscopy using "Plasma-lyte" (Travenol Canada Inc., Mississauga, Ontario) as an irrigation fluid and processed for immuno-SEM according to a previously described method (6) which included the following main steps: a) immediate transfer of the biopsied tissue fragment to a fixative solution made of buffered 0.1% glutaraldehyde and 3% freshly prepared paraformaldehyde. "Prefixation" time was routinely 10 min, at room temperature; b) after appropriate rinses in PBS supplemented with 1% ovalbumin and dissection of the mucosal layer of the biopsy fragment, the mucosa was incubated for 45 min with a 1/10 dilution of the relevant monoclonal antibody (mAb); c) additional rinses with PBS/ovalbumin were followed by a second incubation, this time with a 1/10 dilution of a class specific goat anti-murine Ig adsorbed on the surface of 40 or 30 nm colloidal gold particles (GAM-G40, or G30, Janssen Pharmaceutica, Beerse, Belgium); d) "postfixation" for 2 hours with 2.5% buffered glutaraldehyde; e) graded ethanol dehydration and drying at the critical point of $CO_2$, preceded conductive coating with evaporated carbon; f) storage of the samples in desiccators until examination with the SEM; g) for scanning electron microscopy a JEOL JSM #840 instrument was used throughout. The microscope was equipped with a lanthanum hexaboride ($LaB_6$) cathode (Denka, Mitsui & Cy, Tokyo, Japan), and with a "GW #115" Summing Unit (GW Electronics, Inc., Norcross, GA) to permit the mixing of secondary (SE) and backscattered electron (BE) signals in adequate proportions.

In all control experiments step b) was omitted or substituted by an irrelevant mAb.

Biopsies of bladder mucosa were collected from: 1) "normal" control patients, after written consent, and during surgery for the correction of urinary stress incontinence or of other non-neoplastic or non-inflammatory urinary conditions; 2) a group of patients with a well documented history of bladder cancer but with no pathological diagnosis (NPD/TH) at the time of cystoscopy and cold cup biopsy of bladder mucosa; 3) cases of bladder transitional cell carcinoma (Ta to T3, grades 1 to 3); in these cases, the cold cup biopsies were taken from the tumor itself, not from adjacent or surrounding mucosa.

Biopsies were frequently divided to test several antibodies on the same sample. The total number of labeling experiments is reported in Tables 1 and 2.

Surface expressed antigens investigated in this study were: 1) ABH blood group antigens recognized by appropriate monoclonal antibodies (Dakopatts a/s, Denmark); 2) antigens identified by LEU-M1 (10, Becton-Dickinson, Mountain View, CA), T16 and 19A211 (8,9), and G4 and E7 (2) murine

monoclonal antibodies (mAbs).

Histopathological controls were studied in all cases. Grading was according to the World Health Organization's classification (16). In addition, whenever a discrepancy was noted between the histopathological grade of a given tumor and the appearance of the mucosa under the SEM, the SEM sample was, after thorough SEM examination, embedded in paraffin and the histopathological grade reconsidered under the light microscope.

## Results

The results dealing with deletion of ABH antigens and those illustrating expression of tumor associated antigens will be presented separately.

### ABH Antigens

A total of 104 biopsies were included in this part of the study. They originated from 11 cases of normal controls, 36 cases with well documented history of bladder cancer but no pathological diagnosis at the time of biopsy (NPD/TH cases), 29 cases of low grade and 28 cases of high grade TCCs.

The labeling patterns were classified into three arbitrarily defined groups: homogeneous labeling of most adjacent cells, phenotypic heterogeneity i.e. "mosaic" labeling pattern, and predominantly or completely deleted expression of the antigen. The results are summarized in Table 1, the labeling patterns being referred to as "homogeneous", "mosaic" or "deletion".

Table 1. Expression of the ABH Antigens on Superficial Cells of Bladder Urothelium

| Clinical data | ABH Expression Homog | Mosaic | Delet |
|---|---|---|---|
| Contr.(n=11) | 10 | 1 | 0 |
| NPD/TH (n=36) | 15 | 19 | 2 |
| Low gr. TCC (n=29) | 7 | 16 | 6 |
| High gr. TCC (n=28) | 4(*) | 4 | 20 |

The secretor status of the patients included in this study was not systematically tested. A small percentage (probably less than 10%) of the deleted expression may, therefore, be accounted for by a hypothetical non-secretor status. Low grade TCC included grade 1 tumors while high grade TCC referred to grade 2 and 3. As already noted in our initial report (6) and confirmed in the present study, no significant difference in labeling pattern was recognized among patients belonging to blood group A, B, AB, or O. Unexpectedly, homogeneous expression of the antigen was observed in 4 cases of high grade TCC(*, in Table 1). Reviewing the histopathological diagnosis in these cases, one case showed a submucosal tumor covered by an apparently normal mucosa. In another case multiple biopsies had been taken for histopathology making it difficult to ascertain which one was equivalent to the sample processed for SEM.

One example of "mosaic" labeling pattern is illustrated in Fig.1 a and b which originates from a patient from the NPD/TH group. Fig. 1a illustrates the surface morphology of representative cells viewed in the SE imaging mode. Fig.1b shows the same field in the BE imaging mode, demonstrating the heterogeneous deletion of the antigen, i.e. the mosaic pattern, clearly delineated along cell borders.

The complete "deletion" could in some cases be interpreted as originating from "non-secretor" patients. Mosaic patterns of ABH labeling were observed in 52% of the NPD/TH cases and in 55% of the low grade TCC cases. The high incidence of "deletion" (20/28) in cases of high grade TCC is probably somewhat in excess due in part to the frequent desquamation of the superficial cells and in part to the defective maturation of the transformed cells.

### Tumor Associated Antigens

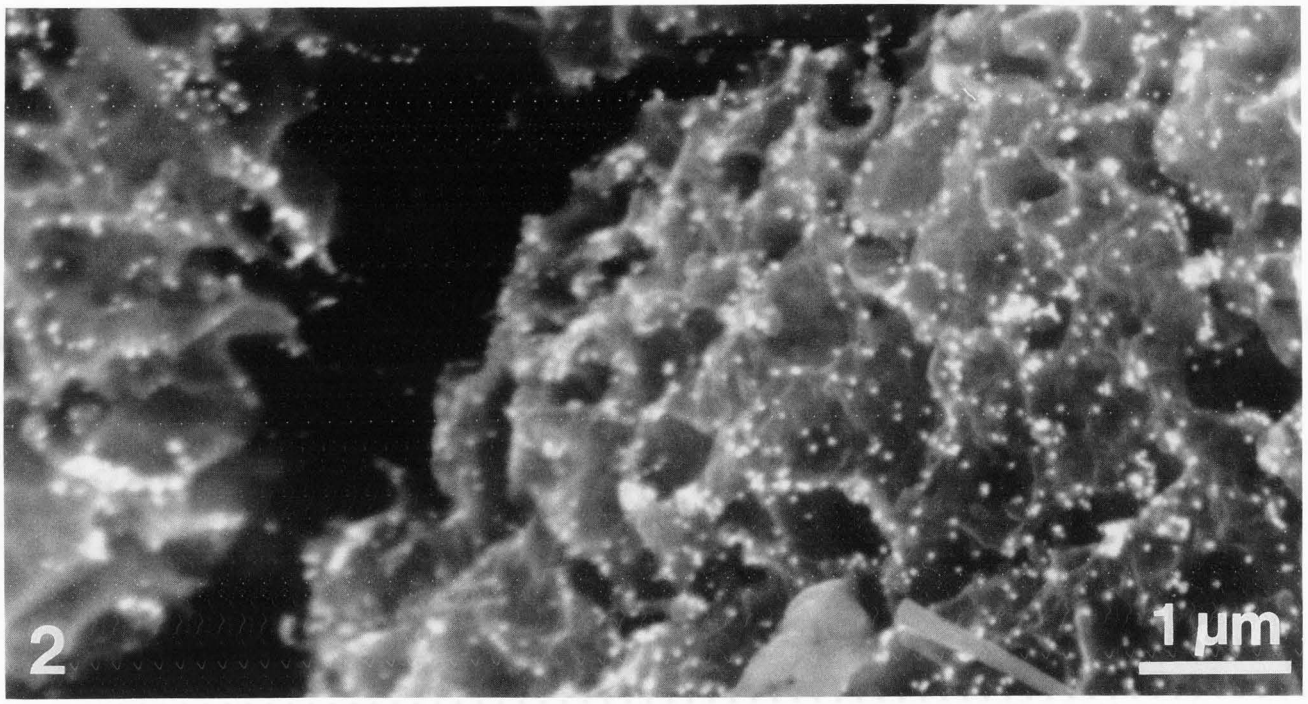
Ninety-seven samples were analyzed in this part of the study. There were 19 normal controls, 41 from the NPD/TH group, 11 from patients with low grade TCC, and 26 from patients with high grade TCC. The results were classified as "positive" or "negative", without specific reference to the homogeneous or heterogeneous labeling pattern. Results are expressed as the number of positive identifications of a given antigen divided by the number of examined samples and are tabulated in Table 2.

Examples of labeling patterns are illustrated as follows. Fig. 2 illustrates positive labeling for the antigen identified by the LEU-M1 mAb in a normal control sample. Fig. 3 documents phenotypic heterogeneity (i.e. "mosaic") in the expression of the LEU-M1 antigen in a NPD/TH case. Fig. 4 a and b illustrate the particular labeling pattern for the T16 antigen observed in a TCC grade 2, where the labeling of intermediate, villous cells is recognized through a window of desquamation of the superficial "umbrella" cells. Fig.5 a and b show, in a NPD/TH case labeled with the G4 mAb, the surface morphology of a superficial cell characterized by plaques and microridges (Fig 5a) while Fig.5b,



Fig. 1a. Bladder biopsy from a patient from the NPD/TH group, viewed in the secondary electron (SE) imaging mode. Large polygonal superficial cells display the plaques and microridges characteristic of the normal urothelial surface. Immunogold labeling for the blood group antigen cannot be recognized in the SE imaging mode.

Fig. 1b. The same field as in Fig.1a, viewed in the backscattered electron (BE) imaging mode. The colloidal gold markers is now clearly recognized on the surface of some of the cells, while sparsely represented or totally absent on others, therefore creating a typical mosaic labeling pattern.



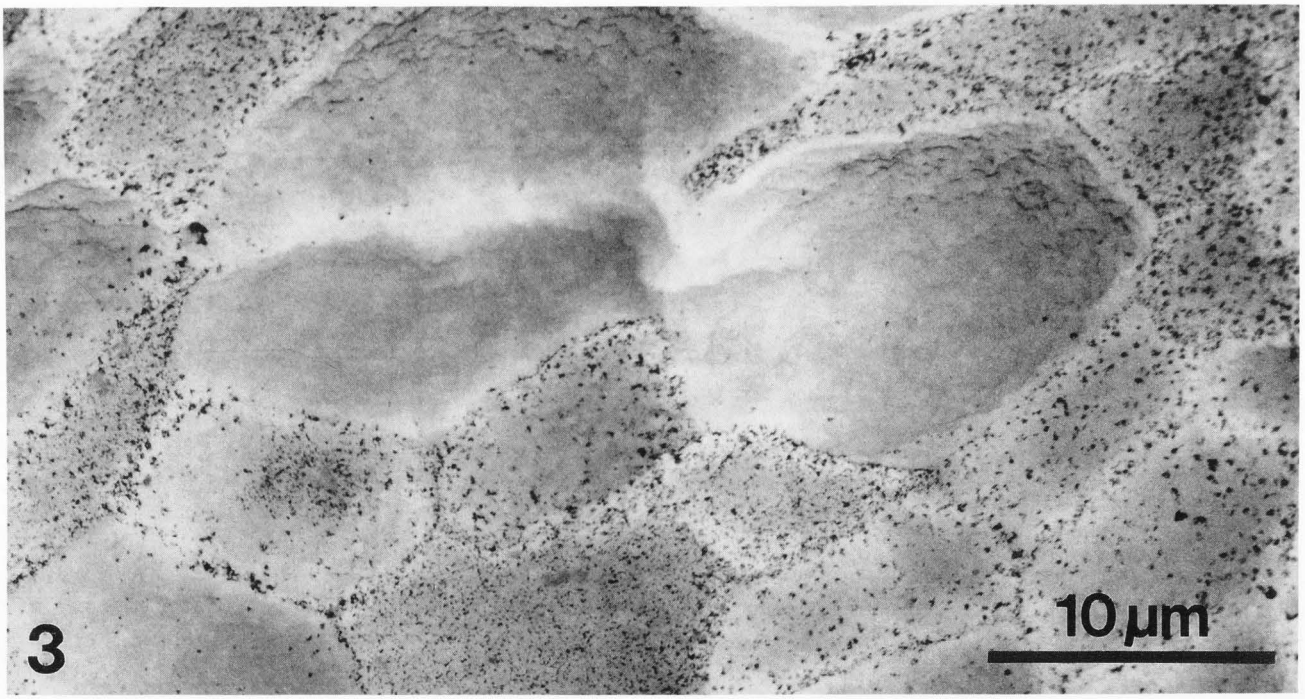
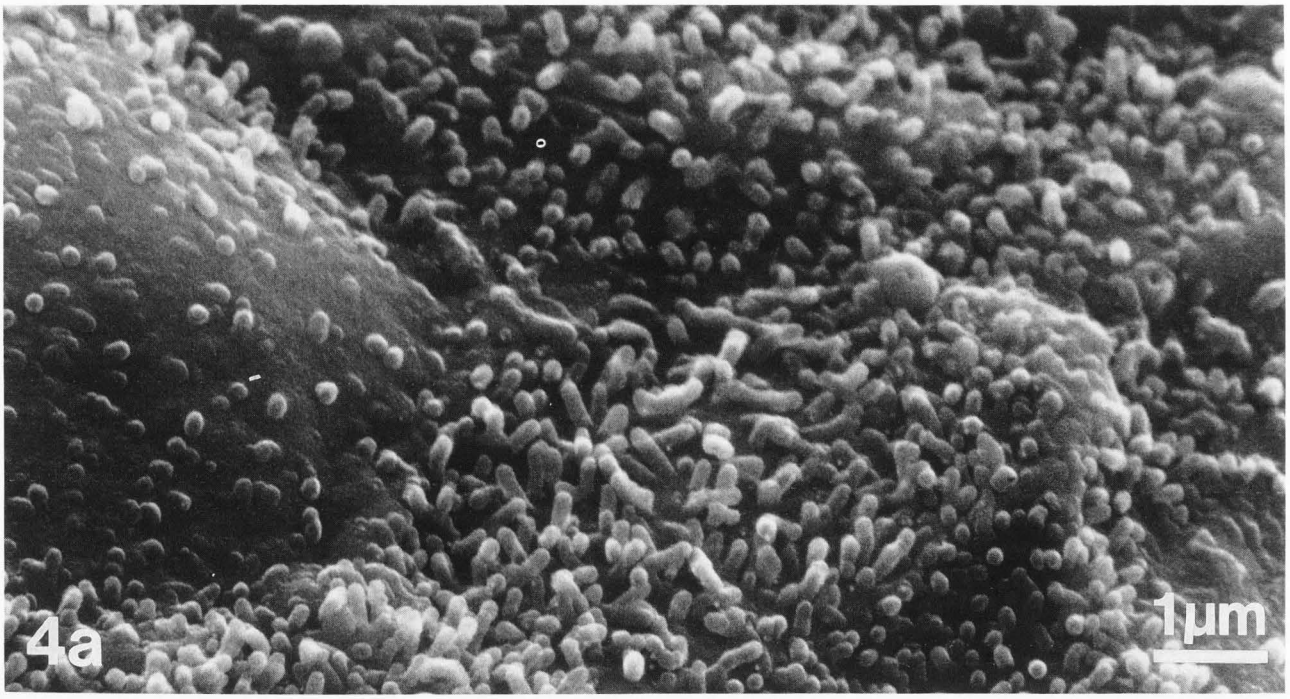




Fig. 2. Bladder biopsy from the normal control group, immuno-gold labeled with the LEU-M1 mAb. Heavy labeling of the superficial cells is observed here in a mixed SE/BE image.

Fig. 3. In a case from the NPD/TH group, also labeled with the LEU-M1 mAb, a characteristic mosaic labeling pattern is observed in the BE imaging mode (reverse polarity).



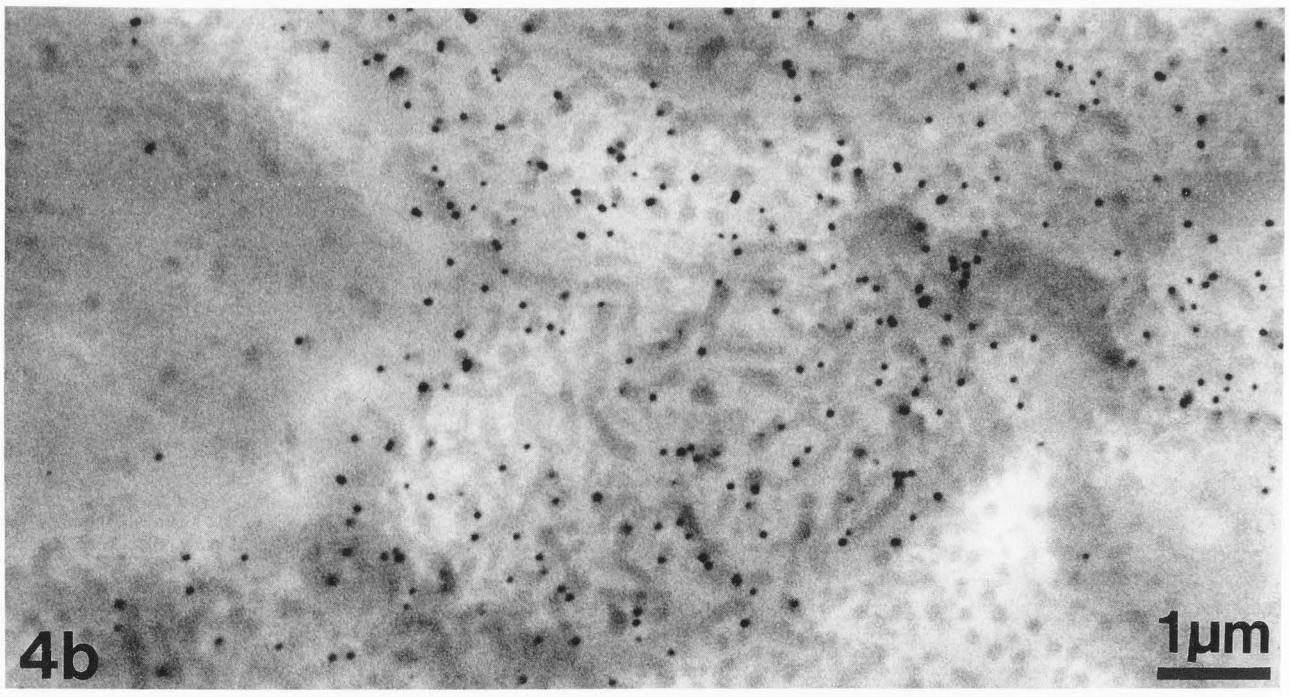
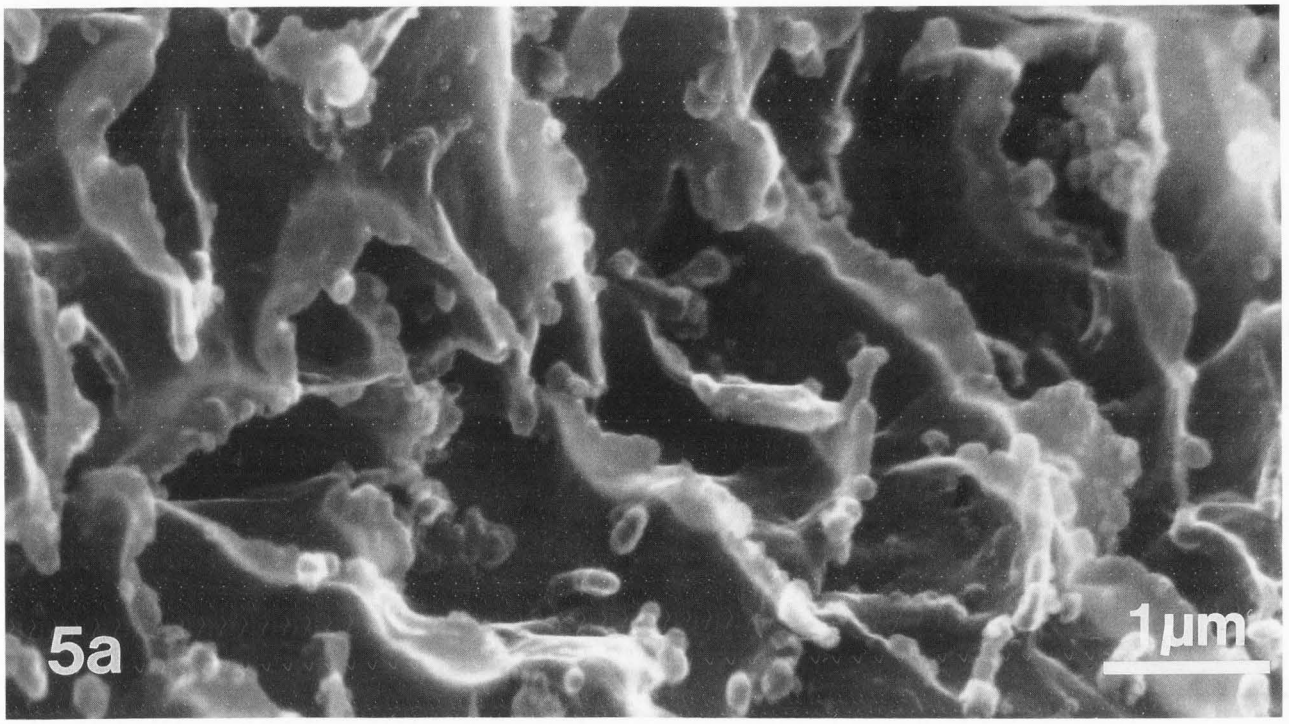




Fig. 4a. Bladder biopsy from a patient with TCC grade II, labeled with the T16 mAb. Through windows of desquamation of the superficial cells, urothelial cells of the intermediate layer are observed, in the SE imaging mode; they display an abundance of surface microvilli.

Fig. 4b. The same field as in Fig. 4a, viewed in the BE imaging mode, demonstrating significant labeling for the antigen recognized by the T16 mAb on the villous cells of the intermediate layer.



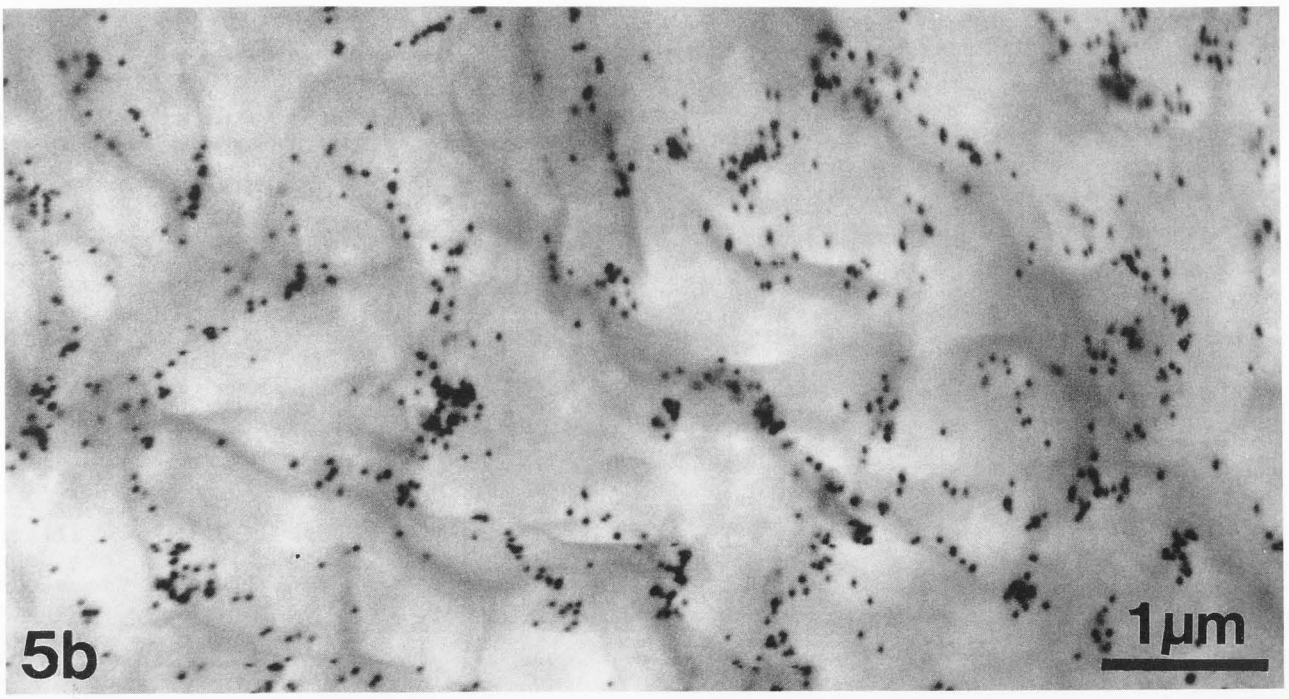




Fig. 5a. Bladder biopsy from a patient from the NPD/TH group, labeled with the G4 mAb. The plaques and microridges are well recognized in the SE imaging mode.

Fig. 5b. The same field as in Fig. 5a, viewed in the BE imaging mode (reverse polarity), clearly demonstrating that most of the 30 nm gold marker particles are located on the microridges, not on the intervening plaques of this superficial urothelial cell.

Table 2. Expression of Tumor Associated Antigens on the Superficial Cells of the Bladder Urothelium

| Clinical data | Antigen identified by monoclonal antibodies: | | | | |
|---|---|---|---|---|---|
| | G4 | E7 | T16 | 19A211 | LEU-M1 |
| Norm. Contr (n=19) | 2/3 | 1/2 | 6/6(*) | 1/2 | 5/6 |
| NPD/TH (n=41) | 11/13 | 7/11 | 8/8 | 5/6 | 2/3 |
| Low gr.TCC (n=11) | 3/3 | 2/3 | 2/2 | 1/1 | 2/2 |
| High gr.TCC (n=26) | 4/5 | 5/6 | 3/4 | 2/2 | 9/9 |

Note that positive labeling with the T16 mAb in normal control(*) was restricted to urothelial cells of the intermediate layer, occasionally observed through little "windows" of desquamation of the superficial cells.

taken in the BE imaging mode of the SEM, clearly indicates that the majority of the gold labeled sites are localized on the crests of the microridges, but not on the plaques of this apparently normal urothelial luminal surface.

Our analysis of antigenic expression has been restricted to mucosal biopsies in normal controls, in NPD/TH cases, and to tumor biopsies in the cases of low and high grade TCCs. It did not involve mucosal biopsies adjacent to tumors and therefore does not illustrate tumor associated "field changes" (14).

## Discussion

Our results indicate that, as far as the deletion of the ABH blood group antigens is concerned, observations cannot be classified in "positive" or "negative" as was frequently done in the past, although zones of heterogeneous expression of the ABH antigens were detected by light microscopy immuno-fluorescence by Vacant et al. (20). When viewed with the SEM after immuno-gold labeling, adjacent superficial cells with very similar ultrastructure (6) express strikingly different amounts of antigen (the "mosaic" pattern). This mosaic pattern of labeling was rarely observed in normal controls but was present in more than 50% of NPD/TH cases and low grade TCCs. In previous studies of cells from the urinary sediment, the "mosaic" type of deletion was never demonstrated (18), because single cell suspensions of mixed anatomical origin were studied. Moreover, in many light microscopy studies of bladder biopsies combined with various approaches to immunolabeling, the "mosaic" also remained undetected, because cross-sections of the mucosal surface were studied. The face-view of relatively large areas of the bladder mucosa offered by the SEM, combined with the high resolution of the instrument are unquestionably needed to observe this particular labeling pattern.

Antigenic expression in bladder neoplasia has to be compared with appropriate normal control samples of bladder mucosa. Our controls consisted of mucosal biopsies collected during surgery for the correction of non-neoplastic and non-inflammatory bladder conditions such as urinary stress incontinence, from female patients belonging to the 40-50 year age group. These controls minimize the effects of age and the interference by inflammatory or neoplastic processes. We have identified the LEU-M1 antigen in most of our control samples, at variance with the results reported by Hoshi et al. (11). One explanation of this discrepancy may reside in our selection of better controls. Another explanation may involve the high sensitivity of the immuno-gold labeling method which detects low levels of antigenic expression in the normal bladder mucosa, undetectable by peroxidase immuno-cytochemistry. It should be emphasized also that the antigen recognized by the LEU-M1 mAb was initially demonstrated on the surface of normal, non-transformed human myelomonocytic cells (10), and cannot, therefore, be regarded as tumor-associated.

All the monoclonal antibodies tested so far gave some positive labeling in control samples. This cannot be explained by non-specific background labeling because control experiments, in which the primary monoclonal antibody was either omitted or substituted by an irrelevant mAb, gave totally negative labeling results. Rather, these findings support a statement recently made, "the search for specific markers of low grade papillary superficial bladder tumors had long been unsuccessful, probably due to the close resemblance of these cells to their normal counterpart" (9). Cancer-progression antigens (9) should be studied by immuno-gold labeling in further attempts to validate this interpretation.

The phenotypic heterogeneity which we initially observed in the deletion of the blood group antigens (6), is also occasionally observed in the expression of other antigens such as that identified by the LEU-M1 mAb (Fig. 3). The significance of these observations is not presently clear. They may, however,

reflect early changes taking place in the basal cell layer of the urothelium. If this would prove to be the case, the phenotype of the superficial urothelium might be regarded as offering a slightly delayed testimony of primary transformation events taking place in the basal cells.

Finally, the immuno-gold labeling of antigens expressed on the luminal surface of the bladder urothelium permits unequivocal correlations between the topographical distribution of certain antigen and ultrastructural features of cell surfaces. This is best illustrated in the case of the G4 mAb (2) which recognizes an antigen only on the microridges of the superficial cells, not on the intervening "plaques" of an asymmetric unit membrane (1,13). Further experiments will be needed, however, to verify that the antigen is not efficiently masked on the plaques, while being readily accessible on the microridges. Whatever final interpretation is reached, the observation could not have been made without the high resolution of the immuno-gold/SEM labeling method.

In conclusion, immuno-gold labeling combined with scanning electron microscopy in the backscattered electron imaging mode has provided a new approach to study the antigenic phenotype of the luminal surface of the human bladder urothelium, normal and transformed. The observations presented in this study could not have been made at the resolution of light microscope immunocytochemistry or by flow cytometry. The immuno-gold/SEM methodology is relatively simple, highly reproducible, and comparatively inexpensive. A one to two year follow-up on all the NPD/TH patients entered in this study is presently in progress and should establish whether or not the observation of a "mosaic" deletion of ABH antigens is relevant to the clinical prognosis of invasive disease of the bladder.

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The G4 and E7 monoclonal antibodies were a generous gift from Dr. Dominique Chopin, Hopital Henri Mondor, Créteil, France. The T16 and 19A211 monoclonal antibodies were graciously sent to us by Dr. Yves Fradet, Laval University, Hotel-Dieu, Québec City, Canada. The present study was supported by grant #MA-9169 from the Medical Research Council of Canada, and grant #1214 from The National Cancer Institute of Canada.

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## Discussion with Reviewers

R.M. Albrecht: Were biopsies that were mosaics for the blood group antigens also mosaics for the Leu-M1 antigen or other antigens?
Authors: Samples labeled for blood group antigens were usually not labeled for tumor associated antigens. We could not, therefore, establish such a correlation.

R.M. Albrecht: Have the authors performed any double labeling studies to determine if the negative cells, in the mosaic pattern, are negative for all surface markers or selectively negative for one or more of the antigens? In the presence of the mosaic pattern can one population of cells be negative for one antigen while a different population of cells is negative for a second antigen and so forth such that mixed populations can exist where one cell may be negative for several or all antigens while another cell may be negative for only one or two surface antigens?
Authors: We have not, so far, performed any significant double labeling experiments on bladder biopsies in attempts to answer these interesting questions.

R. M. Albrecht: Are there any distinguishing morphological, cytological or cytochemical features specific to the negative cells?
Authors: Yes, there is a tendency for the negative cells to exhibit only sparse surface features.

G. M. Hodges: What are the advantages provided by the GW115 Summing Unit for the mixing of secondary and backscattered electron signals over and above that available on the JEOL JSM840 instrument: Please detail the address of GW Electronics.
Authors: The advantage resides in the possibility to control the exact proportion of the SE/BE signals mixing, instead of simply adding them up. The address of the company is: GW Electronics Inc., 6981 Peachtree Industrial Boulevard, Norcross, GA 30092.

G. M. Hodges: What evidence do you have that the cells labeled in Fig 4a and b are "intermediate" cells? Furthermore, it is surely inappropriate to call these cells "villous cells". The term of "villous" is generally applied to finger-like tissue processed such as intestinal/placental/ or chorionic villi. The individual cells that are part of such villi may be themselves patterned by microvilli projecting from the free surface of these cells (as, for example, illustrated in Fig 4a).
Authors: Low magnification observation of that sample clearly demonstrated that the cells represented in Figs 4a and b were seen through a well delineated "window" of desquamation of one or a few superficial cells. We use the term "villous" in the same sense as it is routinely used in the SEM literature on "villous" lymphocytes, although this does not, as you point out, correspond to the traditional, anatomical definition of the word.

S. M. Cohen: Was there any evidence of hyperplasia in the biopsies from controls or NPD/TH by light microscopic histopathology?
Authors: None was reported, in spite of the fact that all the histopathological slides were reviewed prior to insertion in this study.

S. M. Cohen: Was there any relationship of antibody expression to specific surface features such as uniform microvilli, pleomorphic microvilli, spaces between microvilli, microridges, cell junctions, etc.?
Authors: Yes, the most interesting being probably that observed with the G4 antibody which seems to recognize an antigen restricted to microridges, although we did not perform any experiment aimed at possibly unmasking the antigen in other sites. For other antibodies, there is a general tendency to label microvilli more intensely than the space between microvilli. So far, we never observed any specific distribution of the antigens under investigation along cell junctions of the superficial cells.

J.B. Reitan: On fig. 1 a/b a mosaic labeling pattern is demonstrated. The cell without ABH-antigen marking up to the right looks normal in SE-mode to me, whereas those at the bottom are somewhat blurred. Is it an artifact or may be they are not really normal?
Authors: You are right. We do not feel, however, that these cells are out of focus because, in that case the gold would no show as well as it does on the bottom center cell. We are left, therefore, with the possibility that the morphology of these few cells has been poorly preserved.