Ultrastructural Pathology, 30:379–385, 2006

ISSN: 0191-3123 print/1521-0758 online
DOI: 10.1080/01913120600854269

informa
healthcare

Value of the Innovated Technique Agarose Cell Block in Improving the Sensitivity of Urine Cytology in Cases of Bladder Carcinoma

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ABSTRACT Proper handling and processing of urine sample can greatly improve diagnostic sensitivity. This work investigates the value of agarose cell block technique in processing urine samples simultaneously for light and electron microscopic examination, with the prospect to enhance the quality of diagnosis. The material of this study consisted of 45 voided urine samples, processed for the performance of Papanicolaou-stained urine smears, agarose cell blocks paraffin sections stained with hematoxylin & eosin, and electron microscopy-contrasted ultrathin sections. The studied technique increases the sensitivity of urine cytology and opens a new prospect for cytomorphological study.

KEYWORDS agarose cell block technique, electron microscopy, urine cytology

Bladder cancer is the most common urologic malignancy [1]. Its proper diagnosis and long-term management represent a major challenge for most urologists. Many urine-based tests for the detection of urothelial cell carcinoma (UCC) have been developed and tested in different populations. Nevertheless, clinical evidence is insufficient to warrant the substitution of the cystoscopic follow-up scheme by any of the currently available urine marker tests [2]. So, urine cytology with confirmatory cystoscopy and biopsy of suspicious bladder lesions still forms the cornerstone of diagnosis and follow-up of patients with bladder neoplasm [1]. Cystoscopy and biopsy are reliable but invasive [3]. Urine cytology has the advantage of being noninvasive, fast, and inexpensive, but is of limited value because of its low sensitivity in low-grade UCC [4, 5]. Finding new techniques that may improve urine sample processing becomes an important task. Initially, cytological diagnosis was assessed on smears made from the sediment of centrifuged urine samples [6]. Subsequently, new methods included thin membrane filtration, cytocentrifugation, and monolayer technology [7]. Recently, Mansy [8] innovated a technique for processing urine samples in a block manner using agarose gel as the embedding media. The processed

Received 28 December 2005; accepted 9 February 2006.

This work was financially supported by the Theodor Bilharz Research Institute in the frame of the project ''71D.''

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sample can be simultaneously examined for light and electron microscopy. In the present work we have studied the effectiveness of this technique in increasing the sensitivity of urine cytology.

MATERIAL AND METHODS

Forty-five voided urine samples were subjected to this study. They were collected from the outpatient clinic and urology department of the Theodor Bilharz Research Institute hospital. Out of them, 30 urine samples were collected from patients with bladder carcinoma. They were diagnosed histopathologically by the examination of cystoscopic or postoperative bladder biopsies. Fourteen urine samples were collected from patients with acute cystitis. They were diagnosed clinically by the presence of suprapubic pain, frequency, dysuria, urgency, and urine analysis with a minimum of 100 pus cells per high-power field. Moreover, a urine sample from a patient subjected to adjuvant Bacille Calmette Guerin (BCG) treatment, after transurethral resection of primary tumor (TUR-T) for superficial bladder cancer and with no cystoscopic apparent bladder mass, was included in this study.

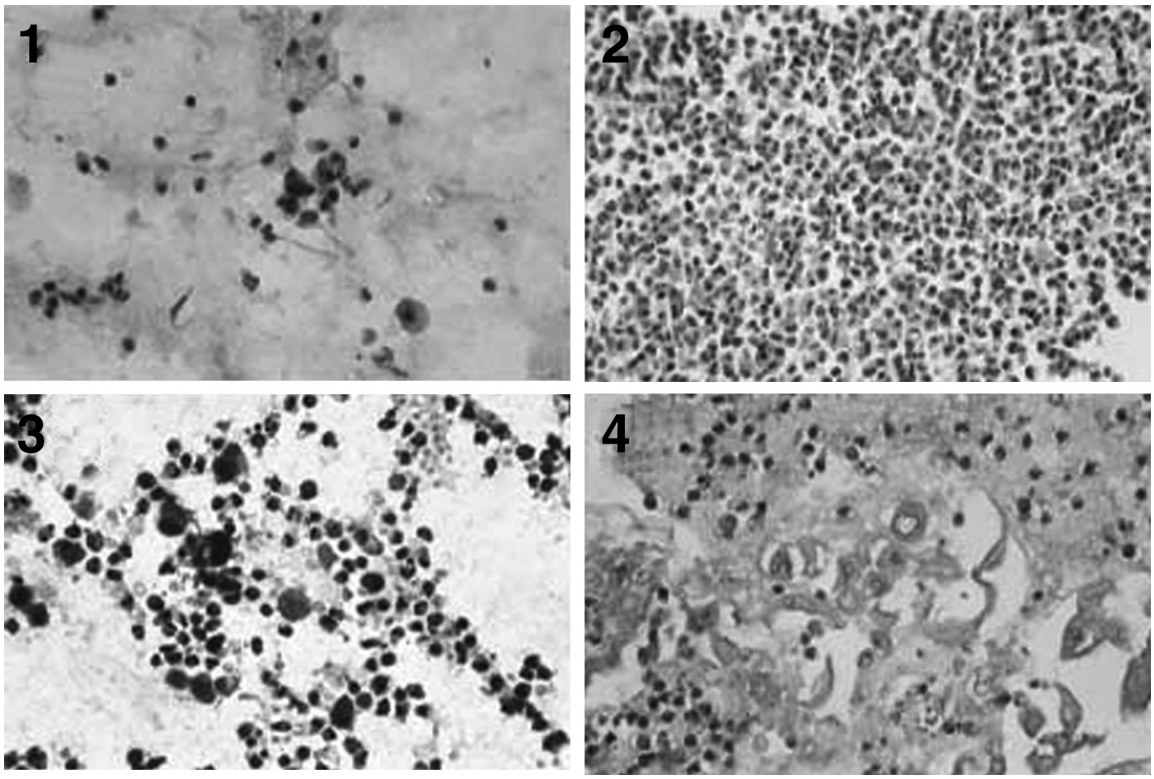
Two centrifuge tubes each containing 10 mL of the collected urine from each case were centrifuged at 1500 rpm for 10 min. The supernatant was poured off and the deposit from the first tube was smeared on a slide and processed for Papanicolaou (Pap) stain. The deposit of the second tube was processed according to the agarose cell block technique of Mansy [8] for light and electron microscopic (EM) examination. The deposit was fixed in buffered 4% glutaraldehyde for 1 h. The fixed cells were recentrifuged with melted agarose (molecular biology grade, Promega, USA) for 7 min at 1500 rpm. The solidified agarose cell block was then divided into halves. One half was fixed in buffered formalin and embedded in paraffin. Then 4-μm-thick sections were prepared and stained with hematoxylin and eosin stain. The other half was divided into tiny pieces and refixed in buffered 4% glutaraldehyde for 2 h, then washed, postfixed in 2% osmium tetroxide for 1 h, dehydrated in ascending alcohol, then infiltrated and embedded in epoxy resin. Ultrathin sections were cut using Leica Ultracut R. The sections were stained with uranyl acetate and lead citrate and examined under a Philips EM 208S. Blind examination of the processed urine samples by light and electron microscopes was done without knowledge about the clinical and histopathological diagnoses.

In this study, UCC was classified into low- and high-grade lesions. Low-grade neoplastic bladder lesions included grade I and grade II (Ta-T1) histopathologically diagnosed malignant lesions. High-grade lesions involved grade II (T2-T4) and grade III neoplasms. This division was based on the classification reported by Baithum et al. [9] and the 1999 World Health Organization/International Society of Urological Pathology Classification System cited by Epstein et al. [10]. The criteria of malignancy and cellular dysplasia were interpreted at the level of light microscope according to Brown [7] and Renshaw [11]. Discrimination of low-grade transitional cell carcinoma (TCC) from nonneoplastic urothelial cells depended on the detection of irregular nuclear membrane and the increase in nuclear to cytoplasmic ratio (greater than 1–3). Diagnosis of high-grade TCC relied on nuclear pleomorphism, nuclear hyperchromasia, coarse irregularly distributed chromatin, irregular nuclear membrane, often prominent nucleoli, and nuclear overlap.

Statistical analyses of the obtained results were compared using the student t test. Sensitivity, specificity, positive and negative predictive values of Pap smear and agarose cell block tests, together with their 95% confidence intervals were calculated using histopathological results as gold standard. Comparisons between the two tests were done according the MacNemar's chi-square test [12]. p values $< .05$ were considered significant.

RESULTS

The examination of paraffin-prepared sections from the fixed urine samples revealed an increase in the number of separated cells in comparison with the corresponding Pap-stained smears (Figures 1,2). The mean number of separated cells per high-power field in Pap-stained smear was 4.1 ± 1.42 and 16.7 ± 7.9 in cases of acute cystitis and malignant cases, respectively, versus 7.3 ± 1.6 ($p < .05$) and 22.8 ± 9.7 in the corresponding cases prepared by agarose cell block technique, paraffin embedded, and stained with hematoxylin and eosin. Pap-stained urine smears of cases diagnosed clinically as acute cystitis were inconclusive in 2 samples due to poor



FIGURES 1–4 **(1) A case of acute cystitis. The smear shows few cell sediment. Pap-stained urine smear, ×400). (2) The same case processed using agarose cell block technique. The smear shows increased number of sedimented inflammatory and urothelial cells. H & E-stained section, ×400. (3) A case of squamous cell carcinoma, showing neoplastic squamous cells with keratin production. Pap-stained urine smear, ×400. (4) The same case done using agarose cell block technique, the squamous pearls and malignant keratinized cells can clearly be identified. H & E-stained paraffin section, ×400.**

cellularity. Dysplastic cellular changes were evident in 6 cases of acute cystitis. The case that was under BCG treatment revealed severe dysplasia in the Pap-stained urine smear and in hematoxylin and eosin-stained paraffin sections. Ultrastructural examination of the corresponding prepared samples for EM displayed transitional cells with criteria of malignancy in association with dysplastic cells in the case under BCG treatment and in a case of acute cystitis showing dysplastic cellular changes at the level of light microscopy. Multiple punch bladder biopsies were performed in these cases. One of the punch biopsies taken from the patient under BCG treatment revealed a TCC.

The 30 malignant bladder lesions subjected to this study were classified histopathologically into 26 cases of transitional cell carcinoma TCC (2 grade I Ta, 1 grade I T1, 2 grade II Ta, 11 grade II T1, 5 grade II T2, 1 grade III T2, 2 grade III T3a, 2 grade III T3b) and four cases squamous cell carcinoma (1 grade III T2, 1 grade III T3a, 2 grade III T3b). Thus, this study included 16 cases of low-grade malignant lesions and 14 cases of high-grade lesions.

In this study evaluation of the diagnostic sensitivity of Pap-stained urine smears and agarose cell block paraffin sections in relation to histopathological diagnosis of malignant bladder lesions was done. The sensitivity of Pap-stained urine smears

TABLE 1 **Sensitivity of Tests According to Histopathological Diagnosis**

Measurement	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	+ve Predictive value (%) (95% CI)	−ve Predictive value (%) (95% CI)
Pap smear	70.0 (50.4–84.6)	100.0 (73.2–100.0)	100 (80.8–100.0)	57.1 (34.4–77.4)
Agarose cell block	90.0 (72.3–97.4)	100.0 (73.2–100.0)	100.0 (84.5–100.0)	82.4 (55.8–95.3)

CI: Confidence interval.

TABLE 2 Sensitivity of Tests According to Grade

Lesions grade	Total number	Pap smear n (%) (95% CI)	Agarose cell block n (%) (95% CI)	P-value*
Low	16	10 (62.5) (35.9–83.7)	14 (87.5) (60.4–97.8)	0.134
High	14	11 (78.6) (48.8–94.3)	13 (92.9) (64.2–99.6)	0.480
Total	30	21 (70.0) (50.4–84.6)	27 (90.0) (72.3–97.4)	0.041

*P-value $\leq$ 0.05 is considered significant.

was 70% (21/30) versus 90% (27/30 cases) in agarose cell block prepared samples and examined with light microscopy (Table 1). On the other hand, malignant cells were detected in 62.5% of the low-grade lesions (10/16 cases) in Pap-stained smears versus 87.5% (14/16 cases) in agarose cell block paraffin-embedded hematoxylin and eosin-stained sections (Table 2). Moreover, the type of malignant lesion was more easily identified in the hematoxylin and eosin-stained agarose cell block paraffin-prepared samples (Figures 3, 4) as well as in the sections examined using EM (Figure 5).

In the present work, electron microscopic examination of urine samples of the 30 malignant cases subjected to this study revealed urothelial cells with malignant criteria in all the cases examined. Discrimination between the normal, dysplastic, and malignant urothelial cells at the ultrastructural level was based mainly on the specific cellular criteria that are used to identify each lesion at the level of light microscopy. Moreover, some important observations were noticed in this study concerning each lesion.

The dysplastic urothelial cell differed from low-grade UCC by the characteristic picture of its nucleus. The nucleus of dysplastic cell retained the oval or round shape with smooth uniform regular nuclear membrane of the normal urothelial cell in spite of the increased nuclear size in some cases. Moreover, the nuclear chromatin was formed exclusively with fine electron-dense heterochromatin, which appeared uniformly distributed with no prominent nucleoli (Figure 6). Dysplastic cells may show intracytoplasmic vacuoles, cell membrane processes, and microvilli, which didn't involve all the cell circumference. The group of dysplastic cells retained intercellular cohesiveness or even intercellular contact through cell membrane blebbing and microvilli. On the other hand, low-grade malignant urothelial cells (Figures 7, 8) displayed cellular and nuclear pleomorphism, often increased in nuclear size, which may fill more than two-thirds of the cell, irregular nuclear membrane, and increased density of heterochromatin, corresponding to the hyperchromasia seen at the light microscopic level. The

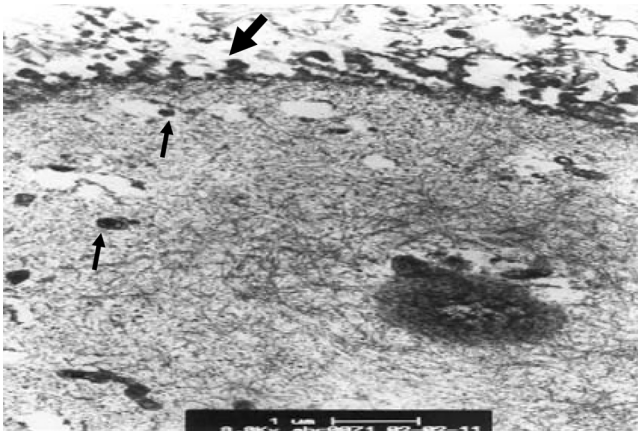


FIGURE 5 Electron micrograph of a case of squamous cell carcinoma, showing cytopasmic tonofilaments, keratohyaline granules (arrows), many cellular membrane projections showing fibrillar and beaded appearance (thick arrow), ×8000.

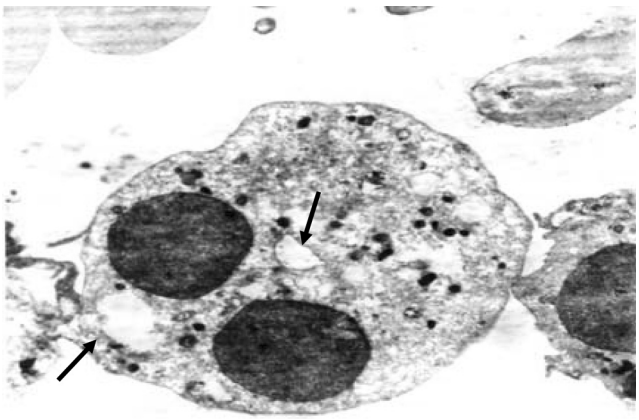


FIGURE 6 Electron micrograph of binucleated urothelial cell in a case of acute cystitis showing mild dysplastic changes. The nucleus shows mild increase in size, electron-dense heterochromatine is homogeneously distributed, and note the uniform contour of nuclear membrane and evident cytoplamic vacuoles (arrow), ×5000.

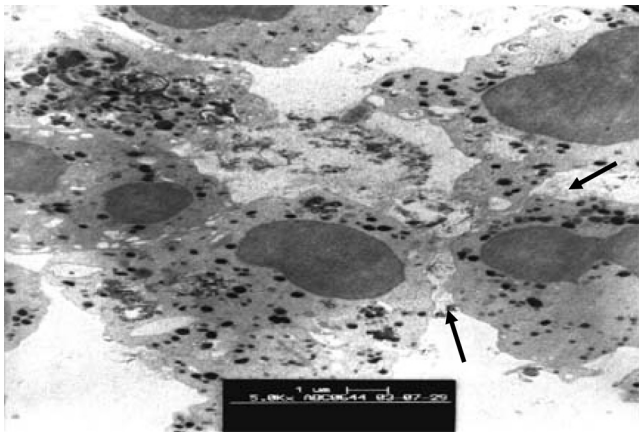


FIGURE 7 **Electron micrograph of a case of grade I TCC showing group of malignant cells with apically situated voluminous nucleus with dense heterochromatin. The nuclei appear irregular in size and shape with irregular nuclear membrane. An increase in cytoplasmic lysosomes is evident. Projection of membraneous microvilli (arrow) extending between neighboring cells with loose intercellular cohesiveness is remarkable, ×5000.**

nucleus of low-grade UCC had a characteristic voluminous appearance. This criterion was considered by this study another point of differentiation between dysplastic cells and low-grade malignant lesions (Figures 7, 8). In addition, low-grade malignant lesions showed cellular processes and microvilli extending nearly from all the cellular circumference, a decrease in cytoplasmic vacuoles, and an increase in cytoplasmic lysosomes. The group of low-grade malignant cells showed loose intercellular cohesiveness with loss of intercellular contacts in many places.

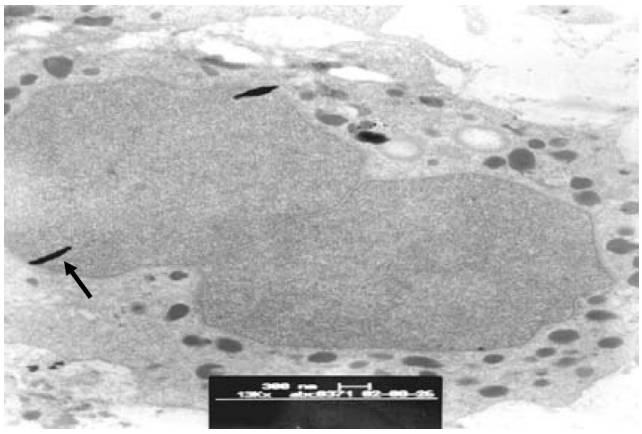


FIGURE 8 **Electron micrograph of binucleated malignant urothelial cell from a case of grade II TCC showing large voluminous nuclei filling nearly all the cytoplasm; electron-dense heterochromatin, nuclear indentation (arrow), cytoplasmic lysosomes, and cell membrane projections are apparent, ×13,000.**

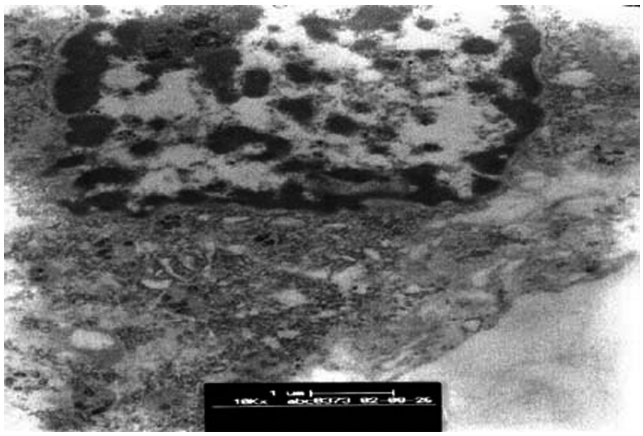


FIGURE 9 **A case of transitional cell carcinoma grade III, showing a large nucleus with coarse chromatin, abnormal configuration of rough endoplasmic reticulum, increased free ribosomes, and cytoplasmic projections, ×10,000.**

High-grade TCC revealed pleomorphic cells with atypical nuclei, coarse chromatin or dense heterochromatin with condensation along the nuclear membrane, and disarrayed membraneous microvilli extending from the all circumference of the malignant cell (Figure 9). Distended rough endoplasmic reticulum with granular material was sometimes detected. Small cells with tail-like process showing vacuolated cytoplasm with a large bizarre-shaped or degenerated nucleus were often encountered in high-grade lesions. The group of malignant cells lacked normal cohesiveness between them.

Small anucleated urothelial cells with cytoplasmic cellular processes and many intracytoplasmic lysosomes were frequently encountered in the malignant cases. They were most probably the result of

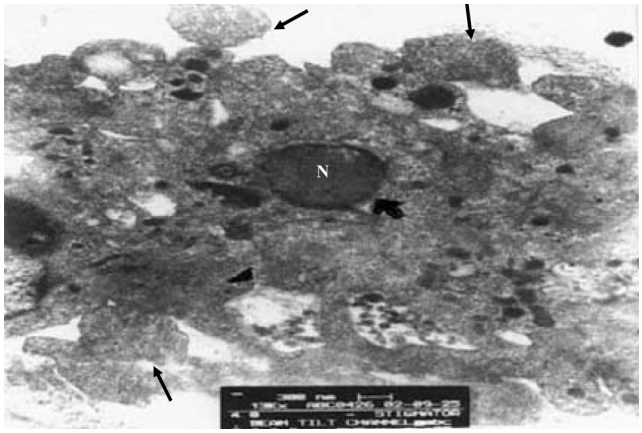


FIGURE 10 **Case of TCC grade III. The cell shows a Pyknotic nucleus (N) with many detached fragments of cancerous urothelium (arrow), ×13,000.**

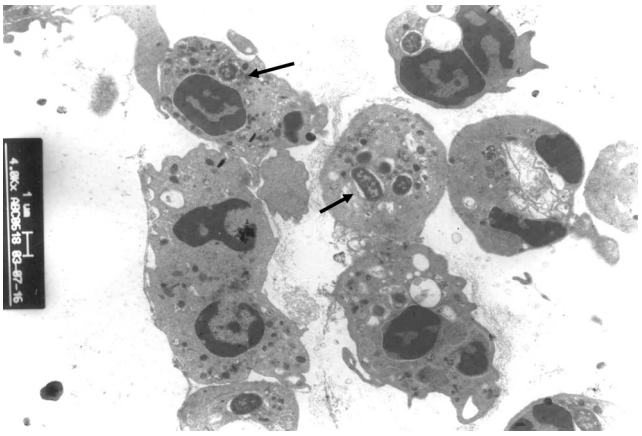


FIGURE 11 **Electron micrograph of phagocytozed bacilli (arrow) seen in a group of inflammatory cells, ×13,000.**

detached cellular processes from an original apoptotic nucleated urothelial cells (Figure 10). Electron microscopy was very useful in the identification of the infective organisms and the visualization of the interaction between inflammatory cells and these microorganisms in the examined samples (Figure 11).

DISCUSSION

The previous reports about the diagnostic sensitivity of urine cytology for low-grade neoplastic bladder lesions show a range between 40 and 60% [5, 7, 13–15]. In the present work, application of agarose cell block technique in the processing of urine samples proved to be effective in increasing the sensitivity of urine cytology. It reached 87.5% in the diagnosis of low-grade malignant lesions versus 62.5% in the corresponding Pap-stained smears. These results can be referred mainly to the proper cell sedimentation and the increased cellularity in the examined sections prepared by the agarose cell block technique versus the conventional methods.

In the present study the increased sensitivity of urine cytology obtained by the application of agarose cell block technique was comparable to the results of more expensive immunocytological techniques used for the same purpose. Pfister et al. [16] assessed the clinical performance of immunocytological (DiagnoCure, Saint-Foy, Canada) in the detection of bladder cancer in 10 French centers. The sensitivity of the combined tests (conventional urine cytology and immunocytology) was 66.7, 78, and 87% in G1, G2, and G3 respectively.

In the present study, simultaneous examination of the same urine sample by light and electron microscopy allowed a subjective cytomorphological study of benign and malignant urothelial cells. Discrimination between the ultrastructural appearance of normal, dysplastic, low-grade and high-grade malignant urothelial cells were contented with the previously settled features seen at the level of light microscopy by Brown [7] and Renshaw [11]. Moreover, characteristic ultrastructural features for each lesion were highlighted in this work, such as the maintained regular nuclear membrane with homogeneously distributed heterochromatin in dysplastic cases, the voluminous nucleus in low-grade malignant lesion, the coarse nuclear chromatin in high-grade lesion. On the other hand, specific morphological criteria that can indicate the turnover of a dysplastic cell to a nonmalignant behavior need further investigation.

The few reported ultrastructure studies of urine cytology such as those of Mansy et al. [15] and Logothetou-Rella et al. [17], were concentrated only on the study of neoplastic cellular criteria. Their reported findings of nuclear features, increased lysosomes, and dilated rough endoplasmic reticulum with granular material were in agreement with our observations.

In the present work, the detection of many anucleated cells with cell membrane processes or microvilli or small rounded cellular parts forming apoptotic bodies were seen only in malignant cases. We suggest that these anucleated cellular particles are a main contributing factor to the unclean background and necrosis seen in malignant cases at the level of light microscopy. Raab et al. [18], Potts et al. [19], and Renshaw [11] reported that the finding of necrosis and anisonucleosis consistently is found to be a very specific marker (approaching 100%) of neoplasia.

In the present study, malignant cells were exclusively determined using EM in 2 cases, which were misdiagnosed at the level of light microscopy as acute cystitis with cellular dysplasia. This must focus attention on the importance of EM as a diagnostic tool in controversial cases and in predicting tumor recurrence in those patients under a postoperative regimen of treatment such as chemotherapy or immunotherapy, since negative preselected site biopsies from those patients are not always reliable [20]. It was reported by Grossman [13] that a positive

cytologic diagnosis is highly predictive of TCC, even in the presence of normal cystoscopy. This was also confirmed by Mckee [21], who reported that positive cytology may precede clinically obvious bladder tumors by a considerable interval. This was confirmed in this study by the determination of TCC in one of the punch biopsies taken from the patient under the BCG treatment. Also, malignant cells may appear in the urine long before any cystoscopically detectable lesion emerges [22], leading to a seemingly inflated rate of false positive results [7]. Moreover, EM allowed the visualization of the infective microorganism in acute cystitis and in malignant cases, which is not seen by conventional cytology, since the causative organism cannot be identified reliably by cytology [21].

In conclusion, the application of the agarose cell block technique in processing urine samples proved to be effective in increasing sensitivity of urine cytology. Its application in other centers is recommended to test the reproducibility of the obtained results.

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