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Bladder wash flow cytometry in transitional cell carcinoma: useful or misleading?

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Abstract To evaluate the practical usefulness of flow cytometry (FC) applied to bladder wash specimens for the diagnosis of transitional cell carcinoma (TCC), a study was conducted on a series of 101 cases comprising 60 patients with tumor or with past history of TCC, and a control group of 41 patients undergoing cystoscopy for causes other than TCC in which the absence of tumor was confirmed after 1 year of follow-up. When results of the 33 patients with tumor were compared with those of the control group, FC gave low specificity and positive predictive values (54% and 58%, respectively). Conventional cytologic study was superior to FC in this setting. Although the combination of both techniques increased the sensitivity for low-grade tumors, specificity remained lower than that of cytologic study alone. Otherwise, when considering only the cases with a past history of TCC, results of FC were superior to those of cytologic examination, and the combination of both techniques gave high sensitivity and negative predictive values (94% and 93%, respectively). In conclusion, the use of FC in a general diagnostic setting could be misleading, whereas in the follow-up of patients with a history of TCC it becomes a useful adjunct to cytologic study in order to obtain a high diagnostic performance that could allow cystoscopies to be spaced out in these patients.

Key words Bladder washing · Cytologic study
Flow cytometry · Transitional cell carcinoma

Bladder wash flow cytometry has been repeatedly claimed as a valuable adjunct to cytologic study for the diagnosis of transitional cell carcinoma (TCC) [1, 2, 5, 8, 10, 11, 16]. What has not been sufficiently stressed is that many of the previous studies had some major limitations, i.e., exclusion of low-grade papillary tumors, lack of a control group, or high rate of false-positive results when controls were used [2, 4, 8, 10, 11, 16]. Furthermore, criteria for the interpretation of flow cytometric data are variable and sometimes poorly defined [19, 20]. To clarify this topic from a practical viewpoint, we have conducted a comparative study of cytologic and flow cytometric analyses in a series of consecutive bladder wash specimens, considering two different settings: diagnosis of TCC on a general basis, and follow-up of patients with known previous TCC.

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Material and methods

From October 1990 to May 1991, 205 consecutive bladder washings were performed in patients with clinical suspicion or history of TCC of the urinary bladder, and in patients undergoing cystoscopy for miscellaneous benign causes to be used as controls. All the specimens were obtained during cystoscopy; thus gross abnormalities were recorded and all suspicious lesions were biopsied. Bladders were washed through the cystoscope sheath with 50 ml saline, and the resulting specimens were mixed thoroughly and divided into aliquots for urinary cytologic examination and flow cytometry.

Aliquots for urinary cytologic examination were cytocentrifuged for 10 min at 700 rpm using a Shandon Cytospin 2 and stained with the Papanicolaou technique. The cytologic diagnoses were categorized into positive, atypical, and negative. A positive diagnosis was made when finding malignant cells or obvious tumoral fragments in the smear. Atypical results referred to smears containing few abnormal cells on an otherwise benign background, or irregular clusters of slightly atypical cells suggestive of low-grade tumor.

Aliquots for flow cytometry were centrifuged for 10 min at 1800 rpm, resuspended in PBS and recentrifuged for 5 min at 1800 rpm. Pellets were treated with 1 ml of a stock solution of propidium iodide (250 ml distilled water, 250 mg sodium citrate, 15 mg trizma hydrochloride, 250 µl Nonidet P-40 (Sigma), 104 mg propidium iodide, 420 mg spermine tetrahydrochloride, and 7.5 mg ribonuclease B). Cell clusters were removed by filtering through a 73-µm nylon mesh. Peripheral blood lymphocytes were systematically used as controls for flow cytometric analysis. This was performed using a Epics II Profile cytometer (Coulter). Specimens with less than 3000 cells or a coefficient of variation (CV) higher than 7 were considered unsatisfactory for evaluation. The average CV of satisfactory cases was 4.8 (5.1 for diploid histograms and 4.4 for aneuploid ones). The average CV of lymphocyte controls was 2.8. Aneuploidy was

considered when a distinct peak with a DNA index higher than 1.1 was observed, according to the guidelines of the Committee of Nomenclature of the Society for Analytical Cytology [9]. S-phase fraction and proliferative index (PI=S-phase+G_2M) were calculated in diploid histograms using the Cytologic software (Coulter). Both cytologic smears and flow cytometric histograms were evaluated without knowledge of the cystoscopic and biopsy findings.

With the aim of obtaining the most significant results, 101 patients were selected for this study using the following criteria: All the patients had a definite diagnosis of the current tumoral status of the urinary bladder at the time of collection of the specimen, obtained by cystoscopic and histopathologic study; follow-up data were available for all patients; all the specimens had enough cellularity and adequate preservation for both cytologic and flow cytometric studies. Fifty-two patients were eliminated due to low cellularity for flow cytometry, 15 due to CV>7, 5 for technical problems in the flow cytometric analysis, 17 for inadequate material for cytologic evaluation, 5 for lack of definite knowledge of the current tumoral status, and 10 for lack of adequate follow-up. Of the 101 selected patients, 33 were patients with histologically confirmed TCC of the urinary bladder at the time of collection of the specimen. Of these, 16 had a previous history of TCC. Of the 68 patients without tumor, 27 were under follow-up for previous TCC. The rest (41 patients) had undergone cystoscopy for causes other than TCC and, after completion of 1 year of follow-up without evidence of bladder neoplasm, were considered the control group.

Twenty-two patients had been treated with topical therapy within the previous 12 months. Fifteen of these had no TCC at the time of collection; the therapy used in this group was bacillus Calmette-Guérin (BCG) (11 patients) and mitomycin combined with thiotepa (M+T)(4 patients). Data analysis was performed comparing the results of cytologic examination and flow cytometry in two different settings: a general diagnostic one, in which the 33 patients with histologically confirmed TCC were compared with the control group (41 patients); and a follow-up setting, in which only the patients with a previous history of TCC (16 patients with current tumor and 27 patients without tumor) were considered.

Diagnostic accuracy was determined by calculating the rates of sensitivity (likelihood that a test will be positive when the patient has the disease), specificity (probability that the test will be negative when the patient does not have the disease), positive predictive value (PPV: probability that the patient will have the disease when the test is positive), negative predictive value (NPV: probability that the patient will not have the disease when the test is negative), and efficiency (frequency with which all patients are correctly classified as having or not having the disease). Data were compared using the chi-square test.

Results

General diagnostic setting

Results of cytologic and flow cytometric analyses of the 33 specimens from patients with histologically confirmed TCC, compared with those of the control group, can be seen in Table 1. A positive cytologic result and an aneuploid histogram were specific for tumor, although they were present in only 48% and 24% of the patients, respectively. In diploid cases, however, the average PI was clearly elevated in grade II and III tumors. After several cut-offs at different levels, a PI equal or higher than 20% was found to be more discriminant, so histograms with that feature were considered suspicious for tumor. In this way, all but one tumor of grade II and III could be diagnosed or suspected by cytologic analysis, and all but two by flow cytometry. All bladder washings from grade I tumors were diploid, and cytologic analysis was superior to flow cytometry in their detection. High proliferative indices in flow cytometry were frequently observed in the control group, in contrast with the lower rate of atypia in the cytologic analysis. Two instances of incidental prostatic carcinoma were detected in the control group; both shed aneuploid cells in the bladder washings, but in only one of them were malignant cells recognized by cytologic evaluation.

For the calculation of the diagnostic rates, suspicious results (cytologic atypia or PI≥20) were considered together with positive ones (positive cytologic findings and aneuploidy, respectively) (Table 2). Combining cytologic analysis and flow cytometry, sensitivity increased for the detection of grade I carcinomas, although differences were not significant ($P>0.05$). Specificity, positive predictive value, and efficiency remained lower than that of cytologic examination alone ($P<0.05$).

Follow-up setting

Table 3 compares the results in the groups with a previous history of TCC. All missed tumors were grade I carcinomas except one carcinoma in situ. Combining both techniques, this last tumor was the only one that could not be detected. Eight patients in the group without tumor showed high PI by flow cytometry, all of them also showing cytologic atypia. In this group, four patients had demonstrated moderate dysplasia in random biopsies; three showed cytologic atypia, two of them also showing high PI. The proliferative index was elevated in 6 of 15 treated patients, and in only 2 of 12 untreated patients. By contrast, cytologic atypia was observed in a similar proportion of treated patients (8 out of 15) as in untreated patients (5 out of 12). Table 4 shows how the results of flow cytometry were superior to those of cytologic analysis in this setting, although the differences were statistically nonsignificant. Specificity and positive predictive values were still low but, considering both techniques together, sensitivity and negative predictive values were very good.

Six patients (22%) in the group without tumor under follow-up for previous TCC experienced recurrences after a period ranging from 3 to 25 months from the collection of the specimen (mean 13 months). The mean follow-up in patients without recurrences was 17 months (range 5–24 months). Only one of the patients with further recurrences had high PI and cytologic atypia; with this patient, the recurrence occurred within 3 months, in spite of negative cystoscopic findings and random biopsies at the time of collection.

Discussion

The sensitivity of cytologic analysis for the detection of TCC varies according to the tumor grade. Even using highly cellular specimens such as bladder washings, most grade I TCCs are missed. Considering positive and atypi-

Table 1 Results of cytologic examination and flow cytometry in patients with tumor compared with controls (*PI*, proliferative index)

Tumor grade	Cytological examination			Flow cytometry				Combined	
	Positive	Atypical	Negative	DNA ploidy		PI (diploid)		+	−
				Diploid	Aneuploid	Average	PI≥20		
I (n=13)	4	4	5	13	0	19%	5	10	3
II (n=9)	5	3	1	6	3	31%	5	8	1
III (n=8)	5	3	0	5	3	32%	4	8	0
CIS (n=3)	2	0	1	1	2	11%	–	2	1
Total (n=33)	16	10	7	25	8	25%	14	28	5
Control (n=41)	1[a]	5	35	39	2[a]	20%	16	20	21

[a] There were two cases of incidental prostatic carcinoma in the control group. Both were aneuploid, and one had positive cytologic findings

Table 2 Diagnostic rates in the general setting. Cases with prostatic carcinoma in the control group are excluded (*PI*, proliferative index; *PPV*, positive predictive value; *NPV*, negative predictive value)

	Cytological examination Positive + Atypia	Flow cytometry Aneuploid + PI≥20	Combined
Sensitivity			
Overall	79%	66%	85%
GI	62%	38%	77%
GII	89%	89%	89%
GIII	100%	87%	100%
Specificity	87%	59%	54%
PPV	84%	58%	50%
NPV	83%	68%	81%
Efficiency	83%	62%	68%

cal cytologic findings together, as in our study, the detection rate increases, but causes the appearance of false positives or, more properly, "false suspicious". This is especially likely to occur when faced with patients with urologic diseases disturbing in some way the vesical mucosa. We expected that flow cytometry could aid us in improving the sensitivity and in better discriminating better such "false suspicious" cases.

A critical analysis of some previous reports on the usefulness of flow cytometry for the diagnosis of TCC reveals some methodologic limitations or intriguing results. For example, Tétu et al. [18] used cytologic evaluation as a diagnostic criterion for grading; they found an unexpectedly high rate of aneuploidy among grade I TCC (26%, whereas it was only 13% among grade II TCC). Murphy et al. [16] worked mostly on high-grade invasive TCC; moreover, a false-positive rate higher than 16% for flow cytometry in their control group was euphemistically described as "a few specimens." Badalament et al. [2], Chin et al. [4], Hermansen et al. [8], and Jitsukawa et al. [10] did not use a control group; therefore specificity was not recorded. Koss et al. [14], in their follow-up study, did not report on the current tumoral status when the specimen was collected, hence the correlation with recurrences is difficult to interpret.

A major shortcoming in the evaluation of the clinical usefulness of flow cytometry is the rate of unsatisfactory specimens. Few reports mention this question, although it is probably a common problem. Depending on the methods of collection and fixation, unsatisfactory samples have been estimated to occur in up to 48% of cases [6, 13]. In our study, reproducing a routine clinical approach, we found 35% of samples were unsuitable for flow cytometric interpretation, most of them due to scant cellularity. Provided that the desirable clinical application of the technique requires the procurement of the samples using urinary catheters instead of cystoscopy, a certain level of skill should be necessary for the correct collection of the specimen [1].

In this study, we selected a well-controlled group of nonneoplastic patients, with no history of TCC, and with a follow-up of at least 1 year in which no urothelial malignancies developed. Criteria for interpretation of histograms were very strict to give objectivity and reproducibility. This probably accounts for the lower rate of aneuploidies we found in comparison with previous works. On the other hand, it is well known that S-phase and PI measurements are poorly reproducible between laboratories [21]; therefore our selected cutoff level (PI≥20) should be adapted according to the experience and software used in each laboratory. However, from our own results and those of previous publications [11, 15, 19], it seems evident that the only specific flow cytometric feature for transitional cell carcinoma is aneuploidy, considered as the presence of one or more distinct peaks outside the G_0–G_1 and G_2M channels. Additional features such as "shoulders", tetraploidy, or increased PI, which might be useful as prognostic indicators when present in a given tumor, are also displayed by benign conditions such as cystitis, urolithiasis, and benign prostatic hypertrophy [11, 12, 16, 21]. In our experience, the differential diagnosis with such conditions is better done with cytologic criteria than with flow cytometric criteria. Therefore, considering a general diagnostic setting, in which high specificity and positive predictive values are needed, the usefulness of flow cytometry would be restricted to the detection of "in situ" and high grade carcinomas, which are also easily detected by cytologic analysis. For the diagnosis of low-grade TCC the use

Table 3 Results of cytologic examination and flow cytometry in patients under follow-up for previous TCC (*PI*, proliferative index)

	Cytological examination			Flow cytometry				Combined	
	Positive	Atypical	Negative	DNA ploidy		PI (diploid)		+	–
				Diploid	Aneuploid	Average	PI≥20		
Tumor (n=16)	8	6	2	11	5	28%	8	15	1
No tumor (n=27)[a]	0	13[a]	14	27	0	18%	8[a]	13	14

[a] Four patients had moderate dysplasia on random biopsies. Three showed atypia on cytologic examination and two had PI≥20

Table 4 Diagnostic rates in the follow-up setting (*PI*, proliferative index; *PPV*, positive predictive value; *NPV*, negative predictive value)

	Cytological examination Positive + Atypia	Flow cytometry Aneuploid + PI≥20	Combined
Sensitivity	87%	81%	94%
Specificity	52%	70%	52%
PPV	52%	62%	54%
NPV	87%	86%	93%
Efficiency	65%	74%	67%

of flow cytometry seems disturbing at least. As stated previously, "flow cytometry probably should not be used to tip the balance between benign and malignant diagnoses in equivocal cases" [15].

The diagnostic strategy in the monitoring of patients with previous resections for TCC is somewhat different, the main goal being to achieve high sensitivity and negative predictive values, in order to safely spare cystoscopies in patients with negative results. Several workers have focused on this problem, concluding that flow cytometry significantly increases the sensitivity of cytologic examination, and it should therefore be used as an additional diagnostic tool [3, 7, 11, 14, 17, 18]. In our series, although sensitivity is only slightly increased by using flow cytometry, the technique has worked better than in the general setting, false positives being clustered mostly in the BCG therapy group. This last point is in disagreement with other reports showing the usefulness of flow cytometry for monitoring BCG treatment [3, 17]. On the other hand, the false-positive rate of cytologic analysis in our study dramatically increased as compared with the general group. This could be attributed, besides the effect of therapy, to the presence of occult dysplastic areas and perhaps to a greater awareness of the cytologists. This last pitfall is bypassed by flow cytometry, thus explaining its better performance. It is noteworthy that, in both the general and the follow-up groups, "false positives" occurred in the categories of cytologic atypia and PI≥20%, not being found among conclusively positive cytologies and aneuploidies. However, false-positive results continue to be a major shortcoming, even in this particular setting [12, 19]. A recent study of patients with a history of TCC found that most false positive cytologic and flow cytometric results were related to recurrences after 4 years of follow-up [7]. Although it would be expected that flow cytometric abnormalities would correlate mainly with early recurrences [14], we have not been able to confirm these results in our follow-up cases. Even so, this could be an additional argument to reinforce the role of flow cytometry as an useful adjunct for cytologic examination.

Patients with a history of TCC are currently monitored by periodical cystoscopies. Our results confirm that bladder wash cytologic examination together with flow cytometry, using wide diagnostic criteria, and in experienced hands for both specimen collection and interpretation, offers a sufficiently high sensitivity to space out cystoscopies in patients with negative results, with the confidence that any eventual missing tumors will correspond to low-grade low-risk tumors.

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