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Improved Application of Impulse Cytophotometry for the Diagnosis of Urinary Bladder Carcinoma*

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Summary. Using pulse cytophotometry, almost quantitative separation of the leucocyte fraction from DNA histograms was possible by means of an anticoincidence discrimination device. This modified technique was employed for biparametric DNA/protein measurements of voided urine samples, bladder washings, and tumour tissues. The results show a high degree of correlation between these samples so that, for tumour diagnosis from DNA histograms, voided urine specimens can be used rather than bladder washings. The criteria for the bladder tumour diagnosis are derived from DNA measurements of 32 controls and 35 tumour patients. The diagnostic sensitivity of this method is 0.91 and the specifity 0.75.

Key words: Impulse cytophotometry, Urinary bladder tumour, Leucocyte elimination, Anti-coincidence discrimination, Diagnostic criteria.

Introduction

It is well known that significant differences in the DNA content occur between normal and malignant cells [2, 7, 18]. Flow methods are particularly convenient for measuring the ploidy of cells and their quantitative distribution [1–3, 13, 19, 16]. Impulse cytophotometric studies have been employed using both bladder washing and tumour tissues [4, 8, 12, 14, 15, 17, 22–24], so that this method has already been evaluated for diagnosing urinary bladder tumours [4, 8, 12, 17], as well as for interpreting the biological behaviour of tumour cells [14, 15, 22–24]. However, some investigators have not considered the problem of possible leucocyte contamination of voided urine and bladder washings and its significance on the DNA histograms resulting in possible false negative readings [12, 23]. Petersen et al. [17] met this difficulty by correction of DNA histograms by parallel microscopic determination of the leucocyte count. Collste et al. [5] were able to eliminate leucocytes by detergent treatment and biparametric measurement of cells for DNA and RNA fluorescence.

This paper shows that a reliable distinction between leucocytes and urothelial cells can be accomplished by means of an electronic separation method and biparametric fluorescent staining of both protein and DNA. Results obtained by this new method are presented with emphasis on the reliability of leucocyte elimination, comparative cytophotometric analysis of benign and malignant urothelial cells in voided urine specimen, irrigation fluid, and tumour tissue. The criteria for tumour diagnosis by this technique are outlined.

Materials and Methods

For pube-cytophotometry, 67 urine samples (32 from tumour-free patients with benign urological disease-controls, 35 from patients with urinary bladder carcinoma), 20 bladder washings from tumour patients, and 10 tumour cell suspensions were used. The tumour stage was assessed according to the UICC [25] and the grade of malignancy was determined using the criteria of Mostofi et al. [16].

Urine samples and bladder washings were immediately fixed with Esposti-fixative [6] in a ratio of 1:1. Samples prepared in this way could be mailed or stored at 4 °C for at least 10 days without any noticeable cytomorphological alteration.

Approximately 100 ml of the prefixed material were spun down at 2,000 CPM (600 g) for 15 min. The pellet was resuspended with 10 ml of a fixative consisting of 20% methanol, 0.9% NaCl and 0.1% thymol [9, 21]. After this fixation, the cell suspension could be kept at +4 °C for at least 3 more weeks without any effect on the microfluorometric analysis. For further processing, the cell suspension was centrifuged at 2,000 CPM (600 g) for 15 min and the pellet was resuspended in 10 ml of the protein fluorescence staining solution (3 mg Sulforhodamine SR-101 obtained from Eastman Comp. No. 14,318 dissolved in 100 ml Tris-buffer) for 30 min at room temperature. After centrifugation at 2,000 CPM (600 g) for 15 min, the pellet was resuspended in 1.5 ml Tris-buffer and aspirated 20 times by an injection syringe with a 23 gauge needle. Then the suspension was filtered through a 75 μm nylon mesh and mixed with 50 μl

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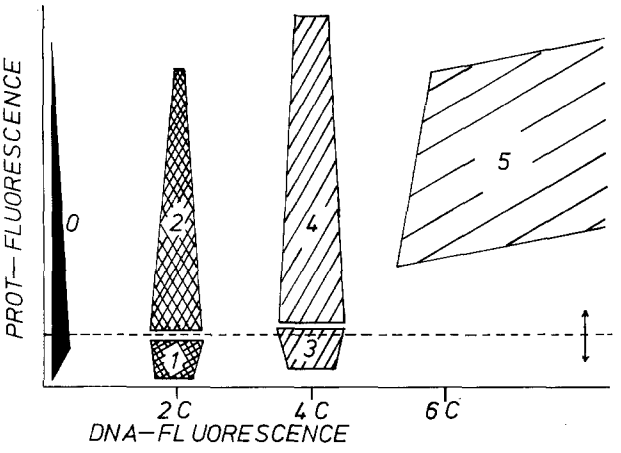

Fig. 1. Schematic drawing of identified cell fractions on the oscilloscope screen. *0* = cell debris of different seize (100%); *1* = leucocytes (> 95%); *2* = 2c-fraction of urothelial and squamous cells (> 95%); *3* = leucocytes doublets (> 95%); *4* = 4c-fraction of urothelial and squamous cells plus approximately 5–10% urothelial and squamous cell doublets and binucleated urothelial cells; *5* = 6c- and higher-ploide urothelial and squamous cells (60%), as well as clumped 2c urothelial and squamous cells (40%); – – – – = variable threshold of the anticoincidence discriminator

DNA fluorescence staining solution (10 mg DAPI obtained from Serva Comp. No. 18,860 dissolved in 100 ml Tris-buffer). Then the cells were analysed in the impulse cytophotometer (ICP). Parallel to this microfluorometric analysis, one drop of the cell suspension was examined for the presence of cell-doublets and/or for complexes of clumped cells under the fluorescence microscope.

Fresh or ethanol fixed tumour tissues were minced with scissors and passed through a 75 μm nylon mesh together with Tris-buffer. The cell suspension was then processed as described above for urine and irrigation fluid samples.

Biparametric measurement of the cellular protein (red fluorescence) and DNA (blue fluorescence) content was performed with an impulse cytophotometer (ICP-22, Phywe Comp.). DNA histograms were obtained by a free programmable multichannel analyser IN96B1 (German Intertechnique Comp.). Biparametric analysis of the 67 urine samples was followed on-line by means of an oscilloscope. For evaluation of the distribution patterns of the different cells in each sample, Polaroid pictures were taken from the oscilloscope screen. Ten representative urines (five from tumour patients and five from tumour-free controls) were analysed by a cell sorter[1]. The different cells in each specimen were evaluated by biparametric protein and DNA analysis as outlined above. The purity of each fraction was controlled microscopically by identifying the predominant cell group and determining its percentage. This experimental design allowed a clear distinction between leucocytes and urothelial cells. In this way, by means of an anticoincidence discrimination switch (German Intertechnique Comp.), the contaminating leucocytes could be eliminated from the DNA histograms, during the flow analysis. Setting and control of the threshold were faciliated by the oscillographic on-line picture. The DNA histograms of urothelial cells were background corrected by an exponential fit [10]. The peaks of the corrected functions were measured by adapting ideal Gaussian curves and thus calculating the approximate integral function. Comparisons between the area integrals of the 2c and 4c peaks were performed by expressing the percentage of the 4c peak relative to the 2c peak and the upper limit of the normal value was determined. The positions of the peaks (stem lines) were derived from the 2c point which represented the dividing point of the first peak. Deviations of the stem lines in normal and tumour patients were calculated by the mean values of the deviations relative to the predetermined stem lines, using urine, irrigation fluid, and tumour tissue. The range of the normal deviation of stem lines was outlined.

Criteria for tumour diagnosis from DNA histograms were the position of stem lines and the relation of integral squares of the measured peaks. For control of the impulse cytophotometric measurements, Papanicolaou-stained cytological routine preparations were obtained from all urines and irrigation fluids. Sensitivity and specifity of the DNA analysis were calculated according to Imich [11].

Results

A. Elimination of Leucocytes from the DNA Histograms

Figure 1 represent a schematic re-drawing of the principal features of 67 Polaroid pictures obtained from the biparametric on-line analysis of urines from controls and bladder tumour patients. Further differentiation and identification of the cell fractions revealed that in one specific field more than 95% leucocytes were plotted, whereas the other areas almost exclusively contained urothelial and squamous cells of various degrees of ploidy. In cell suspensions with not more than 95% leucocytes, the fractions could clearly be distinguished. Therefore, leucocytes were separated from the impulses of urothelial and squamous cells by an anticoincidence discrimination switch, which could be individually set and controlled on the oscilloscope. The advantage of this modification which permitted almost quantiative separation of leucocytes from urothelial cells is exemplified in Fig. 2. Depending on the relative leucocyte count in the individual cell suspension to be measured, extreme changes of the 2c/4c peak ratio could be anticipated so that the interpretation of these measurements was greatly influenced.

B. Comparative DNA Analysis of Urothelial Cells from Urines, Bladder Washings, and Tumour Tissues

DNA histograms of urines, bladder washings, and tumour tissues showed a very close similarity as far as the variation of the position of the 2c peak was concerned. This applied for both tumour patients and controls (Table 2). The area integrals of the first and second peaks did not show a defined relation except for the fact that in tumour patients the second peak always exceeded 5% of the first peak. The individual data are shown in Table 1; three examples are demonstrated in Fig. 3.

1 This part of the work was performed in the Institute of Experimental Pathology, German Cancer Research Center, Heidelberg (Director: Prof. Dr. Goerttler)

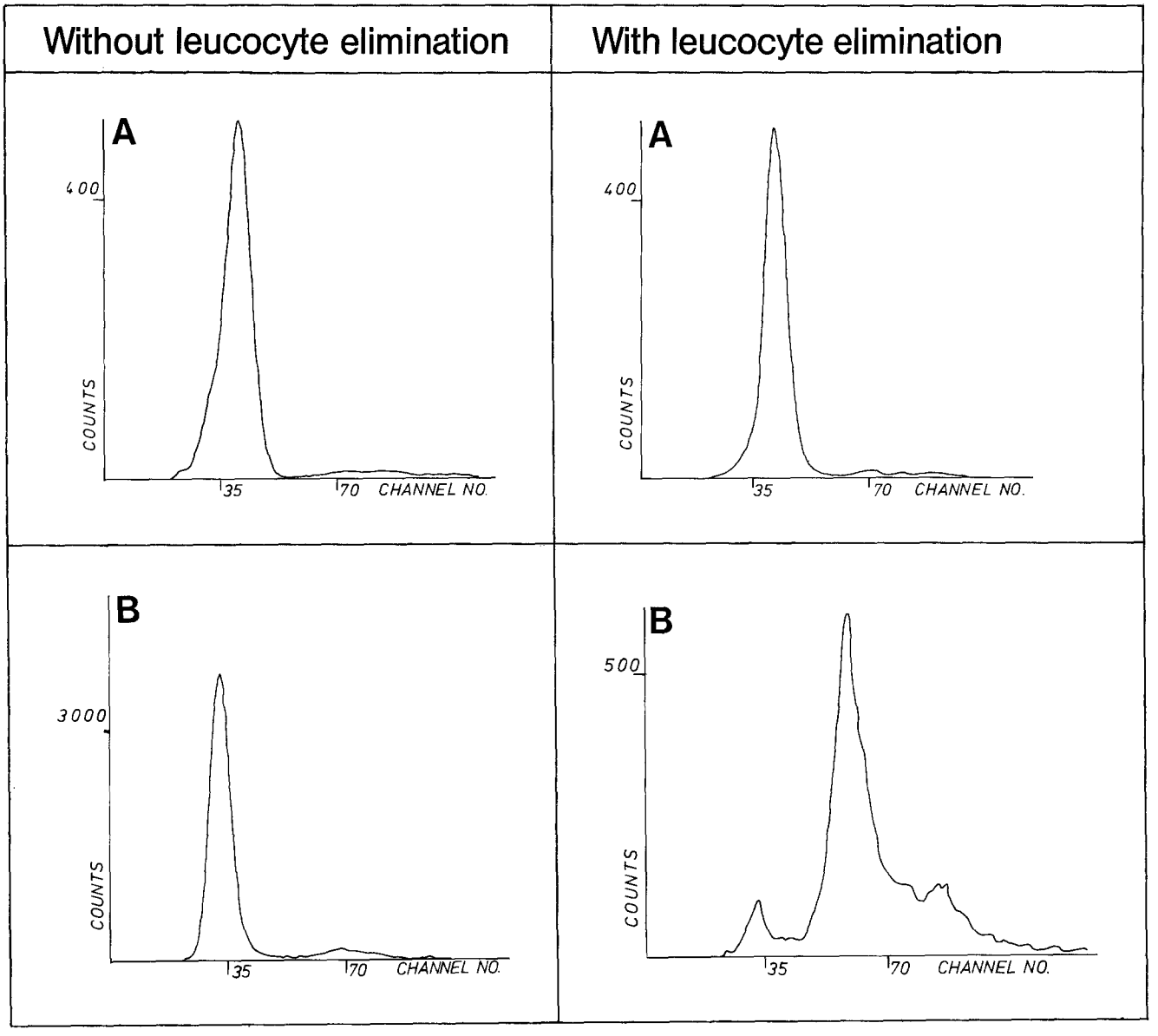

Fig. 2. DNA histograms from urine samples of control and tumour patients with and without leucocyte elimination. **A** = tumour-free patient; urine sample containing approximately 80% leucocytes, with and without leucocyte elimination. **B** = tumour patient; urine sample containing approximately 80% leucocytes, with and without leucocyte elimination

C. Criteria for the Diagnosis of Bladder Tumours by Our Modified ICP DNA Analysis from Urine Samples

In our series with 32 tumour-free patients, the 2nd peak was found at 3.93 ∓ 0.24 C, so that any deviation of more than 0.5 C was defined as aneuploidy. In the group of tumour free patients, the relative square integral of the 2nd peak in relation to the first one was 2.5% ∓ 1.3%. Therefore, second peaks of more than 5% were defined as positive. The results of our ICP analysis in 24 tumour-free patients revealed a second peak smaller than 5% of the first one as well as euploidy. 33 tumour patients showed a positive second peak (> 5%), whereas only 11 tumour patients (= 32%) exhibited criteria of aneuploidy as defined above. In two cases the ICP diagnosis was falsely negative; both patients had T1G1 and T1G2 tumours, respectively. In these two patients, however, ICP analysis of bladder washings and tumour tissues revealed negative results. In eight tumour-free patients, ICP measurements gave false positive results; the referring diagnoses were multifocal dysplasia (one case), urolithiasis (one case), bacterial cystitis (two cases) or benign prostatic hyperplasia (two cases). From our results, the sensitivity of the modified DNA analysis for tumour diagnosis was 0.94 and the specifity was calculated to be 0.75.

A relatively small number of patients were evaluated according to histopathological tumour stage and grade of malignancy. The results are presented in Table 3.

Table 1. Comparative DNA analysis from urines, bladder washings, and tumour tissues of 20 tumour patients with regard to cytological and histological diagnosis

Urine			Bladder washings		Tumor tissue		
Cytology (Pap.)	4c/2c peak area [%]	E = euploidy A = aneuploidy	4c/2c peak area [%]	E = euploidy A = aneuploidy	4c/2c peak area [%]	E = euploidy A = aneuploidy	Histological tumor grade
V	11.4	E	35.2	E	20.9	E	II–III
II	8.6	E	34.5	E	–	–	III
II	3.1	E	2.2	E	–	–	II
V	7.4	E	7.0	E	–	–	I
III	9.6	E	6.4	E	–	–	I
III	3.6	E	4.0	E	–	–	I
V	13.3	E	9.0	E	14.8	E	I
V	47.2	E	35.5	E	25.2	E	II
V	19.9	E	146.4	E	17.8	E	II
–	874.0	A	747.0	A	–	–	II–III
IV	21.2	A	119.0	A	162.0	A	III
IV	23.3	A	16.0	A	–	–	I
–	67.2	E	36.4	E	–	–	III
V	28.9	A	25.8	A	–	–	III
V	58.8	A	16.1	E	8.2	E	III
V	78.3	A	67.7	A	–	–	II
–	96.4	E	83.8	E	7.1	E	I–II
–	19.9	E	161.5	E	10.1	E	I–II
III	4.0	E	3.5	E	–	–	I–II
–	18;2	A	56.8	A	23.7	A	III

Table 2. Stem line deviations of the 2nd peak in tumour patients and controls with reference to the examined material

Material	*n*	Stem line deviation of 2nd peak
Tumour patients		
urine/bladder washing	20	0.26 C ∓ 0.28 C
urine/tumor tissue	10	0.33 C ∓ 0.33 C
bladder washing/tumour tissue	10	0.26 C ∓ 0.37 C
Controls (urine)	32	0.13 C ∓ 0.15 C

Table 3. Correlation of tumour stage and grade with the ploidy analysis

Stage/grade		Euploidy (*n*)	Aneuploidy (*n*)
Stage	T1	10	2
	T2	–	–
	T3	2	1
	T4	1	2
Grade	G1	7	2
	G2	3	1
	G3	6	3

Discussion

The need to eliminate the influence of leucocyte contamination for pulse cytophotometric measurement of urine and bladder washings has been regularly described in the literature [4, 5, 8, 17]. In addition, an example of the possible extreme influence of the leucocyte portion on DNA analysis is given in Fig. 2. Some clear advantages of our method of anticoincidence discrimination are obvious in comparison with the separation procedures which have been employed before. Thus, our method is much less laborious than parallel microscopic examination as suggested by Pedersen et al. [17]. Furthermore, additional preparatory steps with potential damage to the cells are avoided, e.g. treatment with detergents [5]. Control of the discrimination threshold is always possible during the on-line oscillographic monitoring. Even samples with high leucocyte portions are easily measured. Since leucocyte impulses do not enter the DNA histogram they cannot alter its pattern. Employing our method, the usefulness of DNA spectra depends only on the absolute amount of urothelial cells rather than on their relative portion. False results from clumping may occur with every flow method, independent of the prepared material. Therefore, controls of the samples for the number of cell complexes are mandatory by fluorescent microscopy. Samples which have to be discarded for that reason can be re-used by aspiration through a needle and/or short ultrasonography. Despite this, a low percentage of cell doublets and binucleated cells is supposed to be present in each sample [9]. This almost constant error may lead to misinterpretations in cases in which the DNA histograms do not allow a definite diagnosis. In those situations accounting for approximately

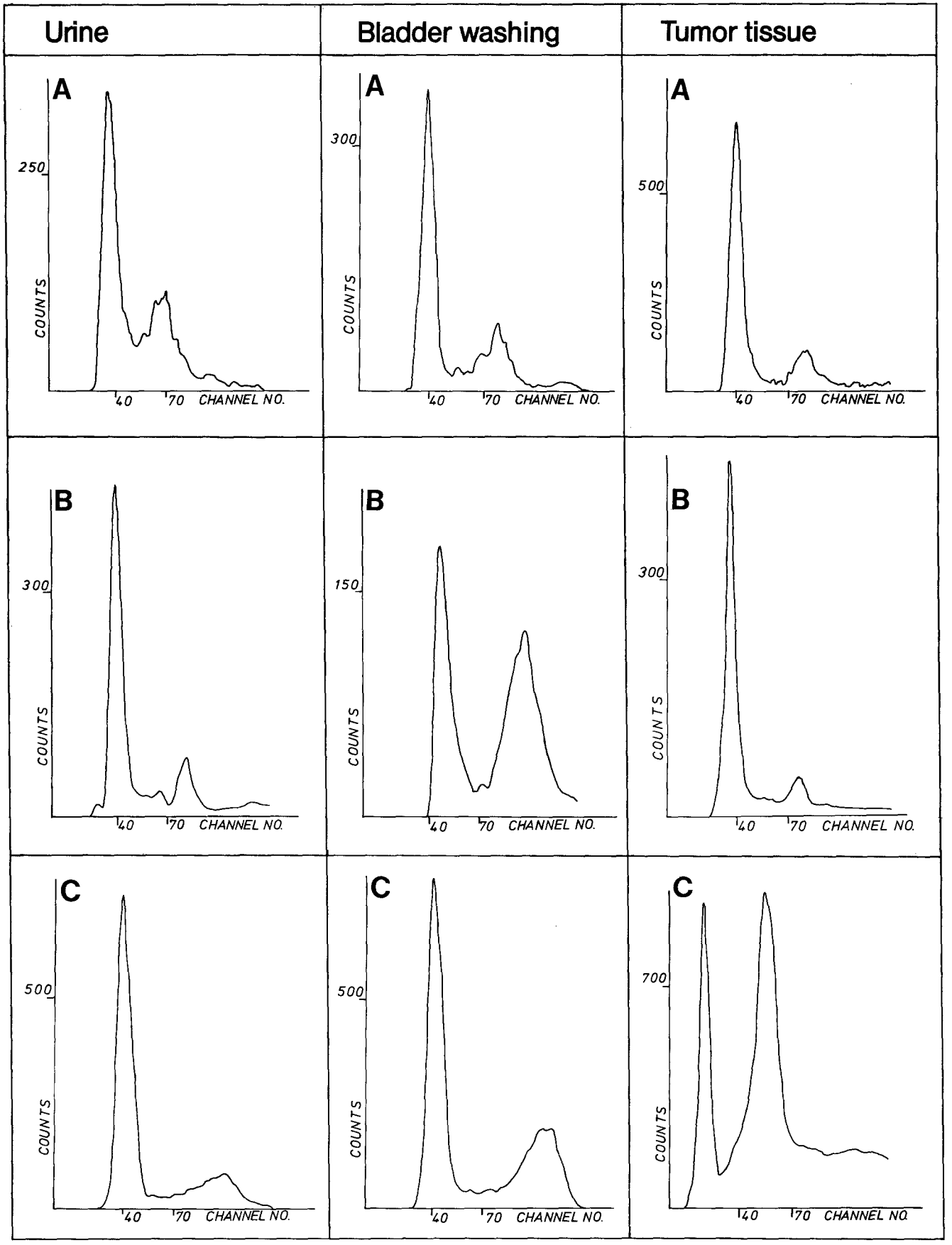

Fig. 3. Comparisons between ICP measurements of urines, bladder washings, and tumour tissues in three tumour patients (A, B, C): only slight deviations of the stem lines and considerable variability of the relative area integrals of the peaks occur with all 4c-peaks > 5% of the 2c-peaks

10% of all measurements, a repeat examination with new material should be performed.

As shown in Table 1, ploidy DNA analysis of voided urine and simultaneously performed bladder washings exhibit almost identical results which, in all cases, are compatible with the results obtained from cell suspensions of tumour tissue. The relative peak areas, however, vary considerably, a result which might be related to many individual factors not examined in the context of this investigation.

Since, in our series, 32% of the tumour patients exhibited aneuploidy, ploidy analysis seem to offer a possibility to differentiate between tumours of different biological behaviour. In a small number of patients, we tried to correlate both the tumour stage and grade with the ploidy analysis as determined by our method. Our results (Table 3) seem to be in agreement with the data published by Tribukait et al. [23, 24] and Lederer et al. [15] indicating a relationship between both tumour stage and grade and the percentage of aneuploidy of urothelial cells.

With reference to the criteria for tumour diagnosis from DNA analysis, it is evident that voided urine specimens yield as reliable results for the definite diagnosis as bladder washings. Considering the disadvantages of irrigating the urinary bladder just for diagnostic purpose, it seems reasonable to prefer a non-invasive method with voided urine samples. The overall error rate of this method accounts for 15%. The degree of diagnostic sensitivity is remarkably high with 0.91, whereas the specificity is slightly lower with 0.75. These figures match favourably with other diagnostic methods so that DNA analysis by flow cytophotometry can be considered as a valuable diagnostic tool in addition to other methods.

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