DNA Flow Cytometry and Histopathological Grading of Paraffin-embedded Prostate Biopsy Specimens in a Survival Study[1]

Sven Lundberg, John Carstensen, and Ingemar Rundquist[2]

Department of Pathology, Linköping University, S-58185 Linköping [S. L., I. R.]; Department of Cancer Epidemiology, Karolinska Institute, S-10401 Stockholm [J. C.]; and Department of Pathology, University of Lund, General Hospital, Malmö, S-21401 Malmö [S. L.], Sweden

ABSTRACT

Methods to disintegrate old paraffin-embedded tissue blocks for the application of DNA flow cytometry open up new possibilities for retrospective studies on the correlation between tumor cell nuclear DNA pattern and prognosis of the neoplastic disease. In the present work we used such a method to study the relationship between DNA ploidy, histopathological grade, and survival for 50 patients with prostate carcinomas diagnosed 1958–1974. Plugs of histologically identified tissue from benign and tumor areas were sampled from paraffin blocks of prostate biopsy specimens by using a 4-mm skin biopsy punch. Thirty-μm sections were cut from each plug for dewaxing and disintegration. The cell suspensions obtained were stained with 4',6-diamidino-2-phenylindole dihydrochloride and analyzed by flow cytometry. In about one-half of the cases where two or more plugs were analyzed we found a heterogeneous tumor cell nuclear DNA pattern. No apparent correlation was found between the histopathological grade and the DNA ploidy. Using Cox's multiple regression analysis, we found a significant correlation between DNA ploidy and survival of these patients ($P = 0.043$) when we controlled for histopathological grade (Dhom grade), acid phosphatase level, occurrence of metastases, age, year of diagnosis, and type of biopsy. The correlation between DNA ploidy and survival was just above the level of significance ($P = 0.059$) when Gleason grade was substituted for Dhom grade in the regression model.

INTRODUCTION

Carcinoma of the prostate is a tumor that varies greatly in biological behavior, ranging from slowly progressing tumors to highly aggressive metastasizing carcinoma. This wide clinical range is at least partly reflected in the morphological features. Histopathological grade is considered one of the most important factors indicating prognosis, and several grading methods (1–4) are available. Although a certain tumor may contain areas with different histopathological grades, which causes difficulties in obtaining a representative sample from the tumor, histopathological grading combined with information on tumor size, stage, and biopsy sampling factors is at present the primary source of diagnostic information in clinical work.

The distribution of nuclear DNA in tumor cell populations has proved to contain additional information relevant to prognosis (5). The Feulgen method has been used for decades to measure DNA content in archival histopathological and cytodiagnostic material. It has been shown that tumors with abnormal DNA stemlines correlate with worse prognoses than tumors with normal DNA stemlines (5). The use of static methods implies a selection of nuclei suitable for quantitation, with a possibility for an experienced pathologist to choose a certain subpopulation of tumor cells for analysis. However, there is an obvious risk of uncontrolled bias. Such methods also are time consuming and consequently the conclusions are based on a small number of measured nuclei, usually on the order of 100 cells.

Flow cytometry, on the other hand, permits a rapid analysis of a large number of cells, usually 10,000–100,000, and is, therefore, more suitable for routine work (6). This method implies no selection of the cells measured and is therefore objective, but a number of artifacts such as debris and cell doublets are also included. A variable contribution from non-tumor cells in the sample may be another problem. Several fluorescent staining methods are available for fresh material for histopathological as well as cytodiagnostic specimens. Many such studies have been performed on tumor cells for the purpose of relating nuclear DNA pattern to prognosis, but as yet few reports that include disease-free intervals and survival are available.

Cell suspensions prepared by pepsin disintegration of fresh and formalin-fixed biopsy specimens have been used for DNA flow cytometry (7). Hedley *et al.* (8) developed a method to disintegrate paraffin-embedded archival material for flow cytometry. This method may be used in retrospective studies of tumors from patients whose clinical outcome is already known, and it is possibly sufficient for a crude comparison of the DNA ploidy and prognosis despite recent observations regarding the relatively poor quality of the DNA histograms compared to corresponding histograms obtained from fresh material (9).

Several studies comparing DNA ploidy with histopathological or cytodiagnostic grade have been performed on carcinomas of the prostate (10–13). Single cell cytophotometry has been used to correlate DNA ploidy with prognosis (14–17). DNA ploidy has been related to stage using flow cytometry (13, 18), but data on the value of DNA flow cytometry in predicting survival probabilities for patients with prostatic carcinoma are still sparse.

The histopathological heterogeneity within a certain tumor creates severe problems in obtaining representative material for grading. The same problem will affect the results of DNA flow cytometry if a tumor has different DNA patterns in different areas. We have not found any reports concerning a possible variation of DNA ploidy within a carcinoma of the prostate.

We have designed a retrospective study relating DNA ploidy to patient survival using biopsy material from patients with prostatic carcinomas diagnosed 1958–1974. DNA ploidy was also correlated to histopathological grade, and the variation of the tumor cell nuclear DNA pattern within certain tumors was examined.

MATERIALS AND METHODS

Prostate Biopsy Specimens. Biopsy specimens from 71 patients with prostatic carcinoma diagnosed between 1958–1974 were selected from the files at the Department of Pathology, University of Lund, Malmö General Hospital. None of these patients had been treated with estrogen prior to the biopsy. Transurethral resections were performed in 52 cases and transvesical enucleations in 19 cases. They were initially graded, based on all histopathological slides available, according to Gleason (1966) by one of us (S. L.) and according to Dhom (1975) by Dr. Dhom himself.

Dr. Dhom's method separates prostate carcinoma into three groups

based on the percentage of sampled tissue involved by carcinoma. Group I has cancer in less than 10% of the specimen. Group II cases have carcinoma in more than 10% of the specimen, and in group III the specimen shows virtual total involvement with neoplasm. He has shown (2) that an overwhelming majority of group I tumors are histopathologically well differentiated and concurrently that most group III tumors have moderate to poorly differentiated histology.

The Gleason grading system (1) is based on the low power histological growth pattern of the tumor. Five patterns are recognized, and a total score is assigned by adding the two most predominant patterns. The system is based on the gland pattern and does not consider cytological criteria.

From the collection of slides for each case, one representative slide with tumor was chosen for this study and the tumor area was marked. Plugs were taken from the corresponding paraffin blocks with a 4-mm skin biopsy punch. One plug was taken from each tumor area with a certain histopathological appearance, resulting in from one to four plugs representing cancer for each case. If nonneoplastic tissue was found, one additional plug was taken from that area. Each plug was reembedded in paraffin, 30-μm sections were cut for flow cytometry as described below (plug sections), and a 5-μm section was cut for a second histopathological grading according to the Gleason method. The remainder of the paraffin block was also sectioned (30 μm) for flow cytometry (block sections).

Clinical Information and Follow-up. Information on acid phosphatases and the occurrence of metastases at diagnosis was obtained from clinical records. The patients were followed to the 31st of December 1979, resulting in a minimum of 5 years' follow-up time. Tumor-related causes of death were established from death certificates.

Preparation of Cell Suspensions from Paraffin-embedded Tissue. The method described by Hedley *et al.* (8) was used with some modifications. Ten discs, each 30 μm thick, were cut from each reembedded plug using a microtome. Two sections, also 30 μm thick, were cut from the remainder of the original paraffin block. The discs from each plug were enclosed in a separate piece of nylon gauze which was sealed with a clamp marked with the preparation number. The same procedure was applied to the sections from each preparation. The gauze bags then were placed together in a container of an Autotechnicon (Model 24; Technicon Co, New York, NY). The overnight program for dewaxing and rehydration consisted of 2 h in xylene, 1 h in each of 100, 96, 70, and 50% ethanol, and 1 h in distilled water. The specimens were then placed in glass tubes containing 2 ml of 0.5% pepsin (0.3 mkatal/kg, No. 7190; Merck Chemicals, Darmstadt, Federal Republic of Germany) dissolved in 152 mM NaCl solution, adjusted to pH 1.5 with 4 N HCl. The tubes were placed on a shaker board in a chamber at 37°C for 30 min. The cell suspensions were washed once in distilled water before staining.

DNA Flow Cytofluorometry. The cells were stained by suspending them in 5 ml of DAPI[3] (Sigma, St. Louis, MO) (19), 1 μg/ml in phosphate buffer (1.47 mM KH_2PO_4, 2.68 mM KCl, 8.1 mM Na_2HPO_4, and 137 mM NaCl) for 1 h at room temperature. The cell concentration was then increased by centrifugation (1000 rpm for 10 min) and discarding 4 ml of the staining solution before measurement. Nuclear fluorescence was measured using a flow cytometer (Leitz, Wetzlar, Federal Republic of Germany) (20). The DNA flow histograms were obtained with a resolution of 256 channels and transferred to an ABC 800 microcomputer (Luxor AB, Motala, Sweden) for analysis. About 50,000 CRBC were mixed with the sample and used as an internal standard for instrument adjustment. They were also used to estimate the number of cells in each sample. The optical conditions were as follows. UV excitation light from an HBO 100-W mercury arc lamp was selected by a bandpass filter (340–380 nm). The dichroic beam splitter had 50% reflection and transmission at 400 nm. Fluorescent light passed through a barrier filter with its edge at 430 nm. A Leitz oil immersion objective (×40; numeric aperture = 1.30) was used.

DNA Flow Histogram Analysis. The DNA distributions were analyzed by first marking the left and right channels delimiting each peak in the histogram and then calculating their arithmetic means and

[3] The abbreviations used are: DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; CRBC, chicken RBC; CV, coefficient of variation.

coefficients of variation. The first peak after the CRBC peak was considered DNA diploid and was given a DNA index of 1.0. Samples where the DNA diploid peak had a CV above 13% were not accepted. DNA indices for the following peaks were calculated accordingly. The number of cells in each peak was expressed as a percentage of the total number of cells, defined as fluorescence signals above the left channel of the diploid peak, thus excluding CRBC and most of the debris. Samples were considered DNA tetraploid when an additional peak with a DNA index between 1.9 and 2.1 contained more than 7% of the total number of cells (12). Samples with additional peaks located outside this region were defined as DNA aneuploid. Tumors were considered DNA aneuploid when at least one sample showed DNA aneuploidy, DNA diploid when all samples were DNA diploid, and DNA tetraploid otherwise.

Survival Data Analysis. The survival data were analyzed using Cox's proportional hazard regression model (21). In the analysis the patients dying from causes other than prostate cancer or still alive at January 1, 1980, were considered as censored observations. The significance of the prognostic variables were tested by the likelihood ratio test statistic which is approximately x^2 distributed (21).

RESULTS

Seventy-one tumors were originally processed for analysis. Fourteen of these, 12 from transurethral resections and 2 from transvesical enucleations, were excluded from further analysis because none of the DNA histograms from plug sections or block sections showed acceptable quality. Repetitions of the process gave the same results, and the failure could not be explained by any mistakes in the processing.

DNA Ploidy and Histopathological Grade for Tumors. The DNA ploidy for each tumor was related to the initial histopathological grade both according to Gleason (Table 1) and Dhom (Table 2). Although the frequency of DNA aneuploid tumors was somewhat higher among less differentiated tumors, the correlation was far from significant ($P > 0.20$). The correlation coefficient between the two histopathology scoring methods was estimated to 0.68 ($P < 0.001$).

DNA Ploidy and Histopathological Grade for Individual Plugs. For three patients, acceptable DNA histograms were obtained only from block sections. From the remaining 54 patients, a total number of 127 histograms from plugs were obtained. Examples of DNA histograms with good quality are shown in Fig. 1. The number of cells yielded, estimated by comparison with the number of reference cells, varied between 10^4 and 10^6 cells/ml. The background level, measured as the remaining number of cells outside identified peaks (debris, cells in S phase), was always less than 25% of the total. The CV of the diploid peak varied between 2.6 and 12.5% (mean 6.6%). It was

Table 1 *Histopathological grade (Gleason score) in 57 prostate carcinomas related to DNA ploidy*

Gleason score	No. of tumors			
	Total	Diploid	Tetraploid	Aneuploid
3–8	34	9 (26)[a]	18 (53)	7 (21)
9–10	23	10 (43)	5 (22)	8 (35)

[a] Numbers in parentheses, percentage of total.

Table 2 *Histopathological grade (Dhom grade) in 57 prostate carcinomas related to DNA ploidy*

Dhom grade	No. of tumors			
	Total	Diploid	Tetraploid	Aneuploid
I–II	26	9 (35)[a]	12 (46)	5 (19)
III	31	10 (32)	11 (35)	10 (32)

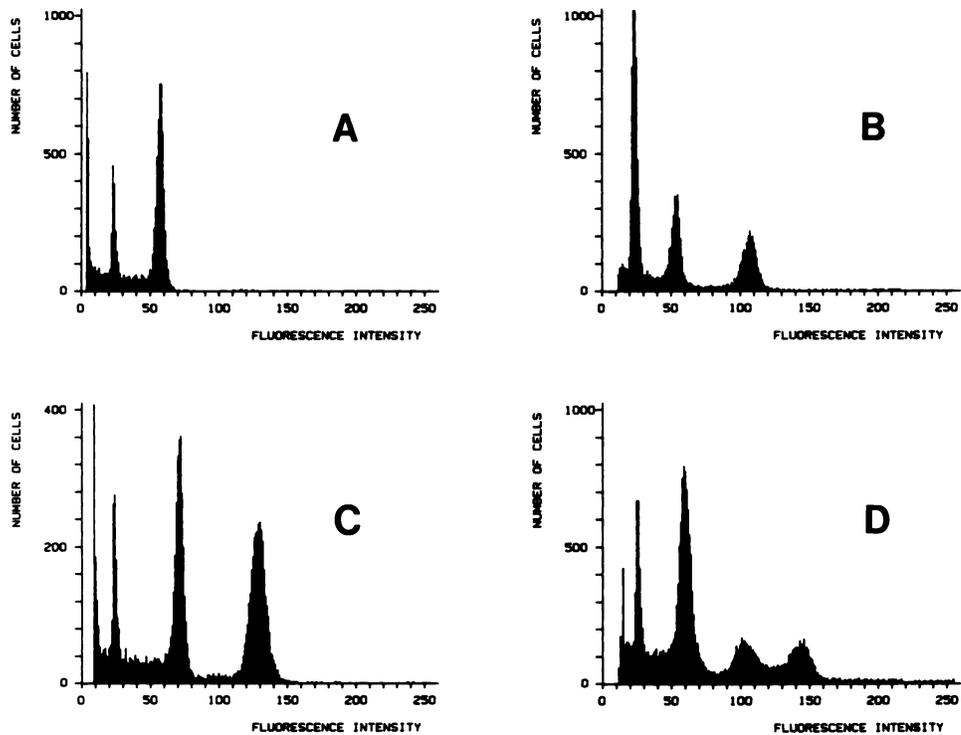[a] Numbers in parentheses, percentage of total.

Fig. 1. DNA histograms for different prostate carcinomas. A, DNA diploid tumor; B, DNA tetraploid tumor; C, DNA aneuploid tumor; D, DNA aneuploid tumor with two abnormal stemlines.

Table 3 *Histopathological grade (Gleason score) related to DNA ploidy for the 24 plugs of the 9 tumors that showed heterogeneity with respect to these 2 variables*

| Tumor | Gleason score | | |
	Diploid	Tetraploid	Aneuploid
1	6		6, 7
2	5, 10		5
3	7	5	
4	6	7	
5	9, 10, 10	10	
6	9		8
7		7	9
8		8	7
9	4, 4	6	8

Table 4 *Standardized relative prostate cancer-related death rate according to DNA ploidy and other characteristics at time of diagnosis*

Characteristics category	No. of patients	No. of cancer deaths	Relative death rate[a]	Test of significance[b] (P)
Metastases				0.028
No	38	24	1.0	
Yes	12	12	2.7	
Acid phosphatase				>0.20
Normal	37	25	1.0	
Elevated	13	11	1.2	
Type of biopsy specimen				0.047
Transvesical enucleation	13	7	1.0	
Transurethral resection	37	29	2.8	
Histopathological grade (Gleason score)				>0.20
2–5	5	2	1.0	
6–8	23	15	1.5	
9–10	22	19	1.9	
DNA ploidy				0.059
Diploid	16	10	1.0	
Tetraploid	22	17	1.6	
Aneuploid	12	9	2.8	

[a] Ratio of the death rate in a given category to that of the reference category (the first group for each characteristic), standardized for the other characteristics listed as well as age at diagnosis and year of diagnosis.

[b] Test for heterogeneity for dichotomous variables and test of trend for trichotomous variable (with the categories coded 1, 2, and 3, respectively).

not correlated to histopathological grade, and there was no difference in CV between plugs taken from transurethral resections (mean 6.6%; $n = 78$) and plugs taken from transvesical enucleations (mean 6.6%; $n = 49$). All plugs from non-tumor areas ($n = 35$) showed a diploid DNA distribution with a maximum of 6% cells, probably doublets, in the tetraploid region.

The paraffin blocks from 31 tumors had at least two plugs from histopathologically identified areas of the carcinoma. The DNA ploidy varied within 8 of 24 tumors where 2 plugs were obtained. Of those five tumors with three plugs, four were DNA heterogeneous and both tumors with four plugs showed DNA heterogeneity.

Of the 14 tumors with varying DNA ploidy in different areas, 9 also showed histopathological heterogeneity when the corresponding individual plugs were graded. As shown in Table 3, no apparent correlation was found between the histopathological malignancy grade and DNA ploidy of the plugs.

Survival Analysis. Seven cases provided no information on clinical status and were not included in the survival study. Of the remaining 50 patients in this study, 36 had died from carcinoma of the prostate before January 1, 1980. The results of the multivariate survival analysis are shown in Tables 4 and 5. In the regression model of Table 4 the histopathological grading is represented by the Gleason system and in Table 5 by that of Dhom. The regression model that contains Dhom

grading produces significant additive prognostic values for both grading and DNA ploidy (Table 5) while the model with the Gleason grade shows nonsignificant results for both grade and DNA ploidy (Table 4), although P for DNA ploidy was just above the level of significance.

DISCUSSION

The method described by Hedley et al. (8) improved the possibility of studying the prognostic significance of DNA ploidy of tumors retrospectively. They reported DNA profiles of acceptable quality and a reasonable output in terms of time needed for preparation and analysis of a large number of cells. We started our work in 1984 and can verify some of the problems that this same group has recently described (9). As

Table 5 *Standardized relative prostate cancer-related death rate according to DNA ploidy and other characteristics at time of diagnosis*

Characteristics category	No. of patients	No. of cancer deaths	Relative death rate[a]	Test of significance[b] (P)
Metastases				0.042
No	38	24	1.0	
Yes	12	12	2.4	
Acid phosphatase				>0.20
Normal	37	25	1.0	
Elevated	13	11	1.3	
Type of biopsy specimen				0.005
Transvesical enucleation	13	7	1.0	
Transurethral resection	37	29	4.3	
Histopathological grade (Dhom grade)				0.002
I	3	1	1.0	
II	18	10	1.8	
III	29	25	5.2	
DNA ploidy				0.043
Diploid	16	10	1.0	
Tetraploid	22	17	1.9	
Aneuploid	12	9	3.1	

[a] Ratio of the death rate in a given category to that of the reference category (the first group for each characteristic), standardized for the other characteristics listed as well as age at diagnosis and year of diagnosis.

[b] Test for heterogeneity for dichotomous variables and test of trend for trichotomous variable (with the categories coded 1, 2, and 3, respectively).

they have indicated, paraffin-embedded tissue is not ideal for use in flow cytometry. We corroborate their most recent findings that its main disadvantages are the time needed to prepare the samples, the relatively poor quality of the DNA histograms compared to fresh material, and the lack of a reliable standard by which hypodiploid cell lines can be identified. Furthermore, we needed a longer time for dewaxing, up to 2 h, compared to the 20 min described in the original report (8). Also, we had substantial problems with samples that did not yield enough cells or did not stain properly with DAPI. These samples sometimes included the total material from a certain case, but in other instances, only certain samples among those of otherwise acceptable quality were unusable. While Hedley et al. (9) suggested improper formalin fixation as an explanation, our study revealed that material from transvesical enucleations (large pieces) with poor possibilties for quick formalin fixation reacted no differently to disintegration and staining than did the small chips from transurethral resections. However, the age of our material, ranging from 10 to 26 years, may be one reason for our high failure rate. Although it was reported that DAPI gave the best results for paraffin-embedded tissue (8), it is certainly possible that other DNA fluorochromes would have been better suited for our material.

As mentioned in the introduction, carcinoma of the prostate is known to be a histopathologically heterogeneous tumor and therefore tissue sampling is an important factor in using histopathological grade as a predictor for prognosis. We have not found any reports on a possible corresponding variation of DNA ploidy within a certain tumor, but a large variability between individual cells as measured by light scatter was reported for rat prostatic adenocarcinoma (22). However, in our study, samples taken from different parts of a tumor revealed different DNA histograms, suggesting that sampling is equally important when DNA ploidy is used as a prognostic predictor.

Several authors report a positive correlation between DNA ploidy based on flow cytometry and histopathological or cyto-diagnostic grade for carcinoma of the prostate (12, 13, 18). Our study did not reveal a significant correlation between DNA ploidy and histopathological grade at the case level, but we noticed a slightly increased percentage of DNA aneuploid tumors in the highest Gleason and Dhom grades (Tables 1 and

2). Although we failed to demonstrate a correlation between DNA ploidy and histopathological grade within tumors (Table 3), such a result can perhaps be explained by the limited number of observations.

Many factors determine the prognosis for prostate carcinoma patients, namely histological grade, nuclear roundness factor (23), DNA ploidy, size of the tumor, tumor stage at diagnosis, tumor progression, and treatment given. Frankfurt et al. (13) demonstrated a significant relationship between DNA ploidy, determined by flow cytometry, and survival, but this positive correlation could be explained by a high death rate among patients having distant metastases. The frequency of DNA aneuploidy increased with advancing clinical stage of the neoplastic disease, and most tumors with distant metastases were aneuploid. In spite of the methodological problems in performing flow cytometry on archival material, our results suggest a correlation between DNA ploidy and survival in a Cox's multiple regression analysis where we also included histopathological grade, acid phosphatase level, occurrence of metastases, age, year of diagnosis, and type of biopsy specimen investigated.

We have restricted our analysis to relative death rates. This is a retrospective study, including patients diagnosed mainly in the sixties and therefore not suitable for estimating "absolute" survival probabilities for the prostate cancer patients diagnosed in the eighties. Prognostic data applicable to today's patients with present day treatment protocols can only be obtained with a prospective study.

ACKNOWLEDGMENTS

REFERENCES

1. Gleason, D. F. Classification of prostatic carcinoma. Cancer Chemother. Rep., 50: 125–128, 1966.
2. Dhom, G., and Hautumm, B. Die Morphologie des klinischen Stadiums O des Prostatacarcinoms (incidental carcinoma). Urologe A, Ausg. 14: 105–111, 1975.
3. Mostofi, F. K. Grading of prostatic carcinoma. Cancer Chemother. Rep., 59: 111–117, 1975.
4. Gaeta, Y. F., Asizwatham, Y. E., Miller, G., and Murphy, G. P. Histologic grading of primary prostatic cancer: a new approach to an old problem. J. Urol., 123: 684–693, 1980.
5. Friedlander, M. L., Hedley, D. W., and Taylor, I. W. Clinical and biological significance of aneuploidy in human tumours. J. Clin. Pathol., 37: 961–974, 1984.
6. Melamed, M. R., Mullaney, P. F., and Mendelsohn, M. L. Flow cytometry and Sorting. New York: John Wiley & Sons, 1979.
7. Haag, D. Flow microfluorometric deoxyribonucleic acid (DNA) analysis supplementing routine histopathologic diagnosis of biopsy specimens. Lab. Invest., 42: 85–90, 1980.
8. Hedley, D. W., Friedlander, M. L., Taylor, I. W., Rugg, C. A., and Musgrove, E. A. Method for analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. J. Histochem. Cytochem., 31: 1333–1335, 1983.
9. Hedley, D. W., Friedlander, M. L., and Taylor, I. W. Application of DNA flow cytometry to paraffin-embedded archival material for the study of aneuploidy and its clinical significance. Cytometry, 6: 327–333, 1985.
10. Zetterberg, A., and Esposti, P. L. Cytophotometric DNA-analysis of aspirated cells from prostatic carcinoma. Acta Cytol., 20: 46–57, 1976.
11. Roters, M., Laemmel, A., Kastendieck, H., and Becker, H. DNA distribution pattern of prostatic lesions measured by flow cytometry. In: O. D. Laerum, T. Lindmo, and E. Thorud (eds.), Flow Cytometry IV, pp. 431–435. Oslo: Universitetsförlaget, 1980.
12. Tribukait, B., Rönström, L., and Esposti, P. L. Quantitative and qualitative aspects of flow DNA measurements related to the cytologic grade in prostatic carcinoma. Anal. Quant. Cytol., 5: 107–111, 1983.
13. Frankfurt, O. S., Chin, J. L., Englander, L. S., Greco, W. R., Pontes, J. E., and Rustum, Y. M. Relationship between DNA ploidy, glandular differentia-

tion, and tumor spread in human prostate cancer. Cancer Res., *45:* 1418–1423, 1985.

14. Tavares, A. S., Costa, J., de Carvalho, A., and Reis, M. Tumor ploidy and prognosis in carcinomas of the bladder and prostate. Br. J. Cancer, *20:* 438–441, 1966.

15. Tavares, A. S., Costa, J., and Maia, J. C. Correlation between ploidy and prognosis in prostatic carcinoma. J. Urol., *109:* 676–679, 1973.

16. Zetterberg, A., and Esposti, P. L. Prognostic significance of nuclear DNA levels in prostatic carcinoma. Scand. J. Urol. Nephrol. Suppl., *55:* 53–58, 1980.

17. Auer, G., and Zetterberg, A. The prognostic significance of nuclear DNA content in malignant tumors of breast, prostate, and cartilage. *In:* L. G. Koss and D. V. Coleman (eds.), Advances in Clinical Cytology, Vol. 2, pp. 123–134. New York: Masson Publishing USA, Inc., 1984.

18. Tribukait, B. Flow cytometry in surgical pathology and cytology of tumors of the genito-urinary tract. *In:* L. G. Koss and D. V. Coleman (eds.), Advances in Clinical Cytology, Vol. 2, pp. 163–189. New York: Masson Publishing USA, Inc., 1984.

19. Göhde, W., Schumann, J., and Zante, J. The use of DAPI in pulse cytophotometry. *In:* Pulse-Cytophotometry, Part 3, pp. 229–232. Ghent, Belgium: European Press, 1978.

20. Steen, H. B. A microscope-based flow cytophotometer. Histochem. J., *15:* 147–160, 1983.

21. Cox, D. R., and Oakes, D. Analysis of Survival Data. London: Chapman and Hall, 1984.

22. Benson, M. C., McDougal, D. C., and Coffey, D. S. The use of multiparameter flow cytometry to assess tumor cell heterogeneity and grade prostate cancer. The Prostate, *5:* 27–45, 1984.

23. Diamond, D. A., Berry, S. J., Umbricht, C., Jewett, H. J., and Coffey, D. S. Computerized image analysis of nuclear shape as a prognostic factor for prostatic cancer. The Prostate, *3:* 321–332, 1982.