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Measurement of Cellular DNA Content as an Adjunct to Diagnostic Cytology in Malignant Effusions

DAVID W. HEDLEY,*† JEANETTE PHILIPS,‡ CATHERINE A. RUGG* and IAN W. TAYLOR*

*Ludwig Institute for Cancer Research (Sydney Branch), Blackburn Building, University of Sydney, Sydney, N.S.W. 2006, Australia and ‡Department of Anatomical Pathology, Royal Prince Alfred Hospital, Missenden Road, Camperdown, N.S.W. 2050, Australia

Abstract—*We reviewed the final diagnosis and outcome of 119 patients who developed serous effusions. In addition to routine cytological examination, the cellular DNA content of fluid samples aspirated from the effusions was measured using flow cytometry in order to determine whether the detection of aneuploid cells could aid in diagnosis or serve as a guide to prognosis. The final diagnosis of 35 patients was non-malignant and a further 40 patients with biopsy-proven cancer had cytologically negative effusions. In all of these cases flow cytometry revealed the presence of diploid cells only. The effusions from 36 cancer patients were reported by cytology to contain a variable proportion of malignant cells, and aneuploid cells were detected in 23 of these samples, the remainder containing only diploid cells. Of 8 effusions where cytology was equivocal, one contained aneuploid cells and clinical outcome subsequently showed that all 8 were malignant. Median survival of patients with cancer was 3 months, and a positive cytology had no influence on survival. However, of the patients with positive cytology, those whose effusions contained aneuploid cells had a poorer short-term prognosis than those cases where only diploid cells could be detected (median survival 1.5 vs 4 months). Measurement of cellular DNA content using flow cytometry can occasionally confirm cancer in a cytologically equivocal effusion, but the negative results in 13 out of 36 (36.1%) effusions where cytology was reported as positive suggests that it has only a limited role in this clinical setting, using currently available techniques.*

INTRODUCTION

AN IMPORTANT investigation in the diagnostic work-up of patients presenting with serous effusions of unknown aetiology is cytological examination of the fluid. Although an unequivocal cytological diagnosis of cancer nearly always proves to be correct, conventional cytology carries a high false-negative rate, either because the cancer is not shedding cells into the effusion or because the cancer cells cannot be distinguished with confidence from the normal cellular components which are invariably present, such as inflammatory cells and mesothelial cells. In the latter case a diagnostic test which could be used in addition to cytomorphology would be very helpful.

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†To whom correspondence and requests for reprints should be addressed.

Measurement of cellular DNA content using flow cytometry is rapid and reliable. Recent studies of human tumour biopsies have shown that between 60 and 100% [1, 2] contain detectable aneuploid cell populations, and aneuploidy appears to be a highly specific marker for cancer, with the exception of a few occasionally tetraploid normal tissues such as liver and myocardium. We therefore measured the cellular DNA content of serous effusions obtained from patients with known or suspected cancer in order to assess its potential role in routine diagnostic cytology.

MATERIALS AND METHODS

Patients

Samples of fluid were obtained by paracentesis from 119 patients. Details of final diagnosis and outcome were obtained from the hospital records.

In 35 patients the cause of the effusion was non-malignant, and the remainder had a diagnosis of cancer confirmed by biopsy at the time of presentation or at subsequent follow-up.

Cytology

Fresh specimens of 20 ml of aspirated fluid with no anticoagulant added were divided into two aliquots of 10 ml each and then centrifuged at 2000 rev/min for 5 min. The supernatant was discarded and smears were made from the sediment. Earlier in the series four smears fixed in 95% ethanol and stained by a modified Papanicolaou method were prepared. In the latter part of the series three smears were prepared as described and one smear was air-dried, fixed in methanol and stained by the May-Grünwald-Giemsa technique. Any residual sediment was then fixed in formal/acetic acid/alcohol and prepared as a cell block.

The smears and sections of the cell blocks were examined for the identification of malignant cells.

Flow cytometry

Between 1 and 5 ml of fluid, depending on cellularity, were centrifuged and the cell pellet resuspended in 2 ml RPMI 1640 tissue culture medium containing 10% foetal calf serum. Chicken red blood cells (CRBC) were added as an internal DNA standard, and the cells stained with ethidium bromide and mithramycin in detergent as previously described [3]. Immediately before analysis RNase was added and cellular DNA content was measured using a standard ICP 22 flow cytometer (Ortho Instruments, Westwood, MA). The results obtained from at least 1×10^4 cells were examined and displayed as frequency distribution histograms of DNA content. The peak corresponding to the G_1 phase of normal diploid cells (e.g. inflammatory or mesothelial cells) could be identified by reference to the CRBC peak, since the ratio of these two fluorescences is highly reproducible. Populations of cells with an abnormal DNA content (i.e. aneuploid cells) produced a second G_1 peak and their relative DNA content was expressed as the DNA index, i.e. the ratio of their DNA content to that of diploid cells. If the sample appeared to contain only cells with a DNA content corresponding to normal diploid, great care was taken to examine the histogram for possible small aneuploid G_1 peaks superimposed on the area corresponding to the S phase of the diploid cells. In order to provide an estimate of proliferative activity, the percentage of cells in S phase was estimated using a computer program [4].

RESULTS

Out of 119 patients with serous effusions, 35 were subsequently shown to have non-malignant disease, the commonest conditions being infection, pulmonary embolus, cardiac failure and cirrhosis. Neither cytology nor flow cytometry produced a false-positive diagnosis of cancer in any of these patients. The remaining 84 developed effusions during the course of histologically proven cancer, and histogenesis and the type of effusion (i.e. pleural, ascitic or pericardial) are shown in Table 1. Cytology showed a variable (less than 1% to nearly 100%) content of malignant cells in 36 cases, in a further 40 the appearances were of normal host cells only and in 8 the findings were reported as equivocal. Analysis of cellular DNA content showed that none of the cytologically negative samples contained detectable populations of aneuploid cells. Thirteen of the cytologically positive samples were also negative using flow cytometry, and there were 23 which contained aneuploid cells. Table 2 breaks these results down according to histogenesis, and Table 3 gives a similar analysis according to the type of effusion. Of 8

Table 1. Histogenesis and site of effusions in cancer patients

Tumour	Pleural	Ascitic	Pericardial	Total
Ovary	6	9	1	16
Breast	15	4		19
Lung	19			19
Lymphoma	8	1		9
Unknown primary	4	1		5
Colon	1	3		4
Cervix	1	1	1	3
Mesothelioma	2			2
Others	4	2	1	7
Total	60	21	3	84

Table 2. Histogenesis, cytological results and DNA content

	Cytology-positive		Cytology-negative
	Diploid	Aneuploid	or equivocal
Ovary	4	8	4
Breast	3	3	13
Lung	3	4	12
Lymphoma	0	1	8
Unknown primary	0	3	2
Colon		1	3
Cervix	1		2
Mesothelioma	1	1	
Others	1	2	4
Total	13	23	48

N.B. All patients with negative or equivocal cytology had diploid cells only, with the exception of one patient with breast cancer, whose cytology was equivocal.

samples which were cytologically equivocal, one contained aneuploid cells (the patient had breast cancer) and the remaining 7 contained diploid cells only.

The coefficient of variance (c.v.) of diploid G_1 peak, which is a measure of resolving power, was calculated for all samples. The mean c.v. for the effusions from patients with non-malignant disease was 2.7 ± 0.7% S.D. and for the malignant effusions it was 2.8 ± 0.8% S.D. The DNA indices for the aneuploid effusions are shown in Table 4. Because of the possibility that malignant effusions might be characterised by abnormal proliferative activity, we calculated the percentages of cells in S phase for the cytology-positive diploid effusions, the cytology-negative effusions from patients with known malignancy and the effusions from patients with non-malignant disease. The mean values were 7.1 ± 5.8% S.D., 5.1 ± 2.8% S.D. and 5.2 ± 5.7% S.D. respectively.

Survival of the cancer patients from the time of paracentesis is shown in Fig. 1. Median survival was 3 months, irrespective of cytological appearances. Of those patients with positive cytology, the aneuploid group had a median survival which was markedly shorter than the diploid group (1.5 vs 4 months, Fig. 2), but this difference is not statistically significant using the log-rank test [5].

DISCUSSION

Examination for malignant cells in a sample of the fluid is an important part of cytopathological practice, and fresh samples from patients who have not received prior cytotoxic chemotherapy usually contain cells whose appearances are sufficiently well preserved to make the distinction between benign and malignant cells straightforward, although this is not invariably the case. Karyotypic examination of malignant effusions has shown that chromosomal abnormalities are common, and although this may be technically difficult to perform on all samples, the method is being evaluated as a diagnostic test [6]. Measurement of cellular DNA content using flow cytometry allows several thousand cells in a sample to be examined and is rapid, reliable and technically simple. Its application to solid tumour biopsies has shown that the majority are aneuploid, and although the sort of information it provides may be less detailed than cytogenetic studies, it would probably lend itself more readily to routine clinical practice.

In the present series only a single cytologically equivocal effusion contained detectable aneuploid cells. This disappointing result reflects the low rate of positivity for the samples that contained obvious malignant cells, where only 23

Table 3. Type of effusion, cytological results and DNA content

Tumour	Cytology-positive Diploid	Cytology-positive Aneuploid	Cytology-negative or equivocal
Pleural	9	13	38
Ascitic	3	8	10
Pericardial	1	2	
Total	13	23	48

Table 4. DNA indices of aneuploid effusions

Tumour	DNA indices
Ovary	1.2, 1.3, 1.4, 1.4, 1.7, 1.8, 1.8, 1.8
Breast	1.6, 1.7, 2.3
Lung	0.9, 1.7, 3.0, 3.6
Lymphoma	1.1
Unknown primary	1.6. 1.6, 1.6
Colon	1.1
Mesothelioma	1.9
Others	1.1, 1.5

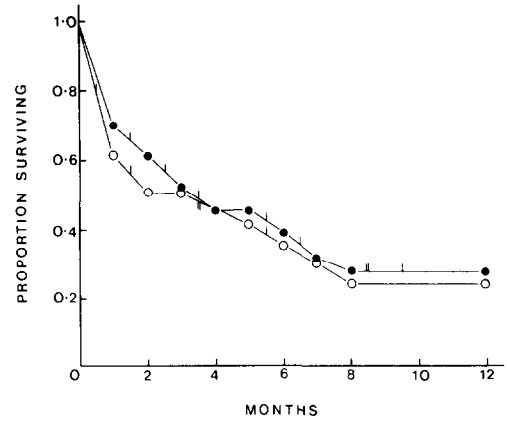

Fig. 1. Survival of cancer patients from the time of paracentesis; (●——●) cytology positive, (○——○) cytology negative.

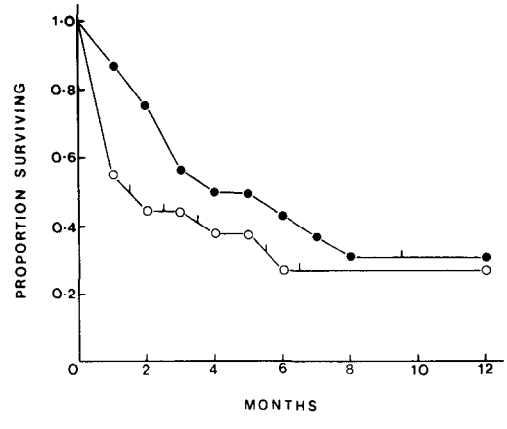

Fig. 2. Survival of patients with positive cytology; (●——●) diploid, (○——○) aneuploid.

out of 36 samples were positive using flow cytometry. We have now examined several hundred biopsies of breast and ovarian cancer [7–9], which are the commonest tumour types, in the present series. Nearly 80% of these samples were aneuploid but in the remainder we were only able to detect diploid cells, and it should be noted that several other groups have shown similar results [1, 2]. Some of our flow-cytometry-negative samples in this study might therefore have contained diploid tumour cells, which could not be distinguished from normal cells by cellular DNA content alone.

The most likely cause of flow-cytometry-negative results, however, is the low proportion of malignant cells in some samples. In all aneuploid effusions they comprised a substantial percentage of the total cell population, whereas some diploid effusions contained fewer than 1% malignant cells. Using mixtures of aneuploid tumour cells grown in tissue culture and normal peripheral blood lymphocytes, it has been found in this laboratory that the present technique has a lower limit of detection for tumour cells of approximately 1% [10]. The threshold for detection of tumour cells in clinical samples is probably higher, due to the presence of cellular debris and proliferating normal cells, whose S phase can obscure small hyperdiploid G_1 peaks.

The percentage of cells in S phase was similar for the non-malignant and the cytology-negative effusions of cancer patients (mean of 5.2 vs 5.1%), whereas for those effusions which were cytology-positive but contained only diploid cells this value was 7.1%. Although this trend suggests that malignant effusions contain on average a higher proportion of actively proliferating cells, the range of percentage in S phase was too wide in all subgroups for it to have any diagnostic significance.

The survival curves for the cytology-positive patients suggest that the diploid group live longer, but we cannot exclude the possibility that during the course of their terminal illness these patients had on average more samples sent for cytological examination, and hence a greater chance of being included in the study.

We have shown in this study that in many cases malignant cells cannot be distinguished from normal host cells in malignant effusions on the basis of a simple single parameter measurement of DNA content, and this method is therefore unlikely to yield a significant number of positive results from samples which are cytologically equivocal. Nevertheless, the application of automated cytology to serous effusions remains a worthwhile goal, and improved tumour-population identification may be achieved with multiparameter analysis (DNA–RNA OR DNA–narrow-angle light scatter) or by flow cytometric detection of cells labelled with fluoresceinated immunoglobulins or lectins.

REFERENCES

1. Barlogie B, Drewinko B, Schumann J *et al.* Cellular DNA content as a marker of neoplasia in man. *Am J Med* 1980, **69**, 195–203.
2. Laerum OD, Farsund T. Clinical applications of flow cytometry: a review. *Cytometry* 1981, **2**, 1–13.
3. Taylor IW. A rapid single step staining technique for DNA analysis by flow microfluorimetry. *J Histochem Cytochem* 1980, **28**, 1021–1024.
4. Milthorpe BK. FMFPAK1: a program package analysis of single parameter flow microfluorimetric data on a low-cost minicomputer. *Comput Biomed Res* 1980, **13**, 417–429.
5. Peto R, Pike MC, Armitage P *et al.* Design and analysis of randomised clinical trials requiring prolonged observation of each patient. II. Analysis and examples. *Br J Cancer* 1977, **35**, 1–39.
6. Watts KC, Boyo-Ekwueme H, To A, Posnansky M, Coleman DU. Chromosome studies on cells cultured from serous effusions. Use in routine cytologic practice. *Acta Cytol* 1983, **27**, 38–44.
7. Taylor IW, Musgrove EA, Friedlander ML, Foo MS, Hedley DW. The influence of age on the DNA ploidy levels of breast tumours. *Eur J Cancer Clin Oncol* 1983, **19**, 623–628.
8. Friedlander ML, Taylor IW, Russell P, Musgrove EA, Hedley DW, Tattersall MHN. Ploidy as a prognostic factor in ovarian cancer. *Int J Gynecol Pathol* 1983, **2**, 55–63.
9. Friedlander ML, Hedley DW, Taylor IW, Russell P, Coates AS, Tattersall MHN. The influence of cellular DNA content on survival in advanced ovarian cancer. *Cancer Res* 1984, **44**, 397–400.
10. Friedlander ML, Taylor IW, Russell P, Tattersall MHN. Cellular DNA content—a stable feature in epithelial ovarian cancer. *Br J Cancer* In press.