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Representativity of Human Mammary Tumor Cell Cultures: DNA-Cytophotometry as a Method for Checking Tumour Cell Characteristics

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Abstract—*Short term cultures (3–6 days) of 40 primary human mammary carcinomas were prepared and compared with the original tumours from which they were derived. As a criterion the nuclear DNA Frequency Distribution Pattern (FDP), cytophotometrically measured, was used. Comparisons were made between the FDPs of smears of freshly-cut tumour surfaces and their cultures.*

Twenty-nine (73%) cultures showed FDPs identical with the smears. Eleven cultures (27%) showed gross shifts in ratios between different peaks or showed a complete loss of one or more peaks in the FDP and were classified as not representative.

Our results show that it is necessary to check primary mammary tumour cultures to determine whether or not they are representative of the original tumour. This is especially so if conclusions are to be drawn from the cultures about the original tumour.

Analysis of FDPs in the cell islands of the cultures (migration of cells from attached clumps) resulted in a better understanding of the FDPs found in the smears. We showed that cultures of human mammary tumours either can be composed of cell islands with identical FDPs (diploid or aneuploid) or may show heterogeneity between different cell islands within one tumour.

INTRODUCTION

THE CELLULAR composition of a carcinoma is often far from homogeneous; various subpopulations of tumour cells with different properties may be present [1–4]. If tumour cell cultures are to be used for "predictive" testing of sensitivity of hormones or chemotherapeutic drugs, it is of the utmost importance to know whether all different subpopulations present in the original tumour are also represented in the cultures. Generally, authors just assume, without checking, that the cultures do represent the original tumour.

As a parameter to recognize subpopulations, the nuclear DNA content of the tumour cells can be used. It is known that in tumours the nuclear DNA content is often abnormal [5]. Investigations have usually been done on smears or imprints of tumour cells made directly from the tumour or using suspensions of cells after dissociation of the tumour [6, 7]. Sometimes measurements were done on histological sections [8, 9]. In many reports the abnormal DNA distribution pattern is correlated with prognosis [7, 10–14].

Human mammary carcinomas have been shown to be diploid in around 25% of cases; the remaining 75% show abnormal nuclear DNA frequency distribution histograms. These abnormal histograms can be classified into three types [6, 7] and often also within the limits of these types there are features in the histograms that characterize individual tumours. Thus histograms may be considered as "fingerprints" of these tumours.

To check whether cell cultures are representative of the original tumours we compared at random measured nuclear DNA frequency distribution patterns of human mammary carcinomas with those of the cultures derived from them. As will be shown, cell islands as are found in the cultures using our method often represent rather pure subpopulations of tumour cells.

MATERIALS AND METHODS

Patient's material

Mammary carcinomas were obtained, sterile, immediately after operation and after parts of them

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had been taken for histopathological diagnosis and hormone receptor determinations. Thus the material was obtained only if tumour size exceeded 1 cm in diameter. The tumour fragment was immersed and stored in tissue culture medium at 4° C and transported to the tissue culture laboratory as soon as possible. The interval between operation and dissection in the tissue culture laboratory was mostly less than 4 hr.

Smears

The tumour fragment was cut into two halves through its largest diameter under sterile conditions. Tumour cells were scraped off the freshly-cut surface with a surgical blade and suspended in a drop of hypotonic solution (1 vol. culture medium RPMI-1640 + 6.5 vol. of a 0.1% BSA solution in aquadest). This causes the nuclei to swell slightly, which ensures optimal DNA measurement afterwards. After 10 min the drop with the cells was smeared onto a microscope slide, warm-air-dried and fixed in Holt's fixative (a buffered formalin fixative containing 7.5% sucrose) for 2 hr. The slides were kept overnight in ethanol 70%.

Histology

The tumour part adjacent to the surface from which the smear was taken, was fixed, embedded and sectioned for histological examination. The sections were examined microscopically to make sure that the material was indeed tumour and to check the state of the material regarding necrosis and cellularity. Material insufficient in these respects was not used in this investigation.

Tissue culture

For culturing, the tumour was subjected to enzymatic digestion by a combination of the methods of of Lasfargues [15] and Hiratsuka [16]. The tumour material was cut into pieces of around 1 mm dia. The minced material was transferred into an Erlenmeyer flask containing collagenase CLS III (Worthington) 200 IU/ml and hyaluronidase (Sigma) 0.4 mg/ml dissolved in tissue culture medium RPMI 1640 with 10% Newborn Calf Serum (Flow-Laboratories), 5 μg/ml insulin and 500 IU/ml penicillin–Na. Overnight incubation at 37° C for 16–18 hr generally weakens the tumour-stroma sufficiently so that subsequent pipetting up and down a Pasteur pipette frees tumour cells and cell clumps. The resulting suspension was centrifuged for 5 min at 1000 rpm. The supernatant containing only blood elements and debris was discarded and the pellet was resuspended in 10 ml culture medium (RPMI 1640 + 20% NCS) and left standing for 15 min. The supernatant, carefully pipetted off, containing mainly debris and single cells of negligible viability was discarded. The sediment was suspended in tissue culture medium and seeded into Falcon T-flasks. The cultures were gassed with air + 5% CO_2. They were kept undisturbed for 48 hr at 37° C. After that period the medium was renewed every second day. At the end of the culture period (in these experiments generally after 3–6 days) the cultures were rinsed with Ringer solution at 37° C to remove the serum of the medium, and fixed in Holt's fixative.

DNA-Cytophotometry

Feulgen-staining with pararosanilin was carried out using the method indicated by James [17] in which hydrolysis is performed at room temperature. This method guarantees uniform results in overall staining on each slide and generally in staining intensities amongst preparations fixed on different days.

DNA measurements were made at 456 nM using a scanning cytophotometer of a type designed by Jansen [18] and modified by us to meet our requirements for speed, ease of handling, sensitivity and stability. The DNA content of 100 nuclei were measured at random (excluding blood cells and recognisable fibroblastic cells). Cells that, due to overlapping of nuclei, could not be measured correctly and cells with damaged nuclei were also left out.

The measurements were expressed in arbitrary units (AU) and compiled in frequency distribution histograms. As a standard for normal diploid values in the cultures, fibroblastic cells were used; for the hypotonic smears, imprints of mouse liver on the same slides were used. Subpopulations of cells belonging to separate DNA-"families" were recognised visually as peaks in the histograms.

The Frequency Distribution Patterns (FDP) of nuclear DNA of human mammary carcinomas were classified into four types earlier described [6,7]. Characteristic examples of these are shown in Figs 1 (a–d).

DNA-Cytophotometry of the individual islands

During culturing individual cell islands (migrations of cells from the clumps attached to the bottom of the culture vessel) were marked under an inverted microscope in phase contrast on the outer surface of the bottom by scratching a square around the area to be marked using the point of a scalpel. Each square was numbered. The cell islands within the squares were photographed. After Feulgen-staining the nuclear DNA was measured at random within the marked cell islands.

RESULTS

The great majority (over 90%) of the explanted cell clumps attached to the vessel bottom, their

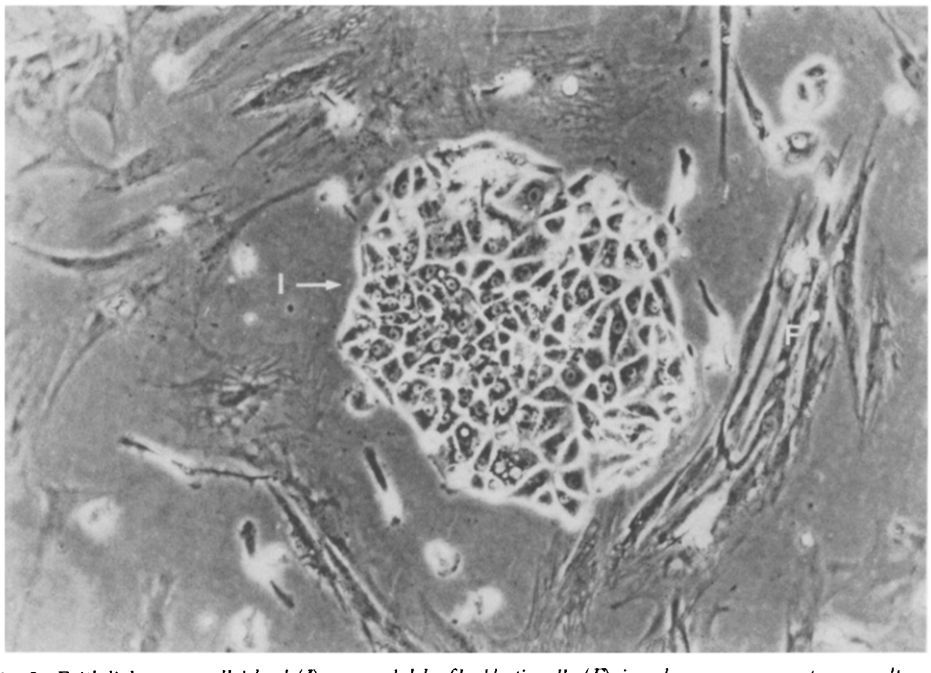

Fig. 2. Epithelial tumour cell island (I) surrounded by fibroblastic cells (F) in a human mammary tumour culture.

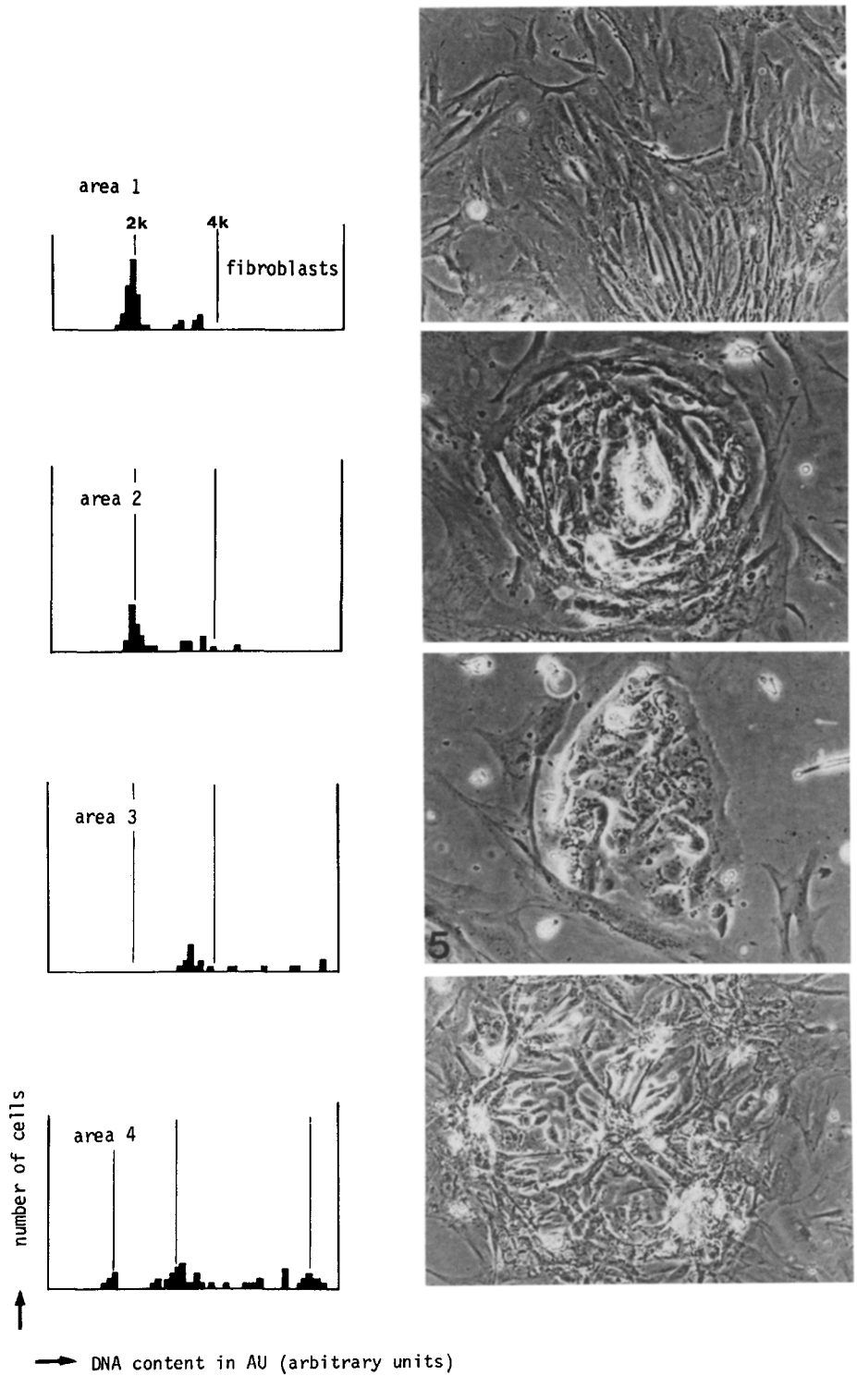

Fig. 5. DNA frequency distribution patterns and the corresponding photographs of the measured cell islands within one tumour. The vertical lines indicate the 2k, 4k and 8k regions.

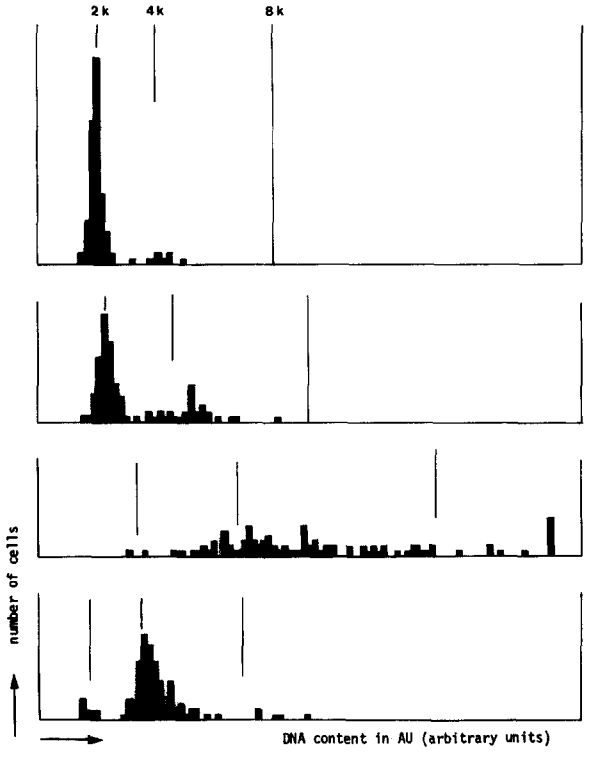

Fig. 1. *Classification of DNA frequency distribution patterns of smears made of human mammary tumours according to Prop* et al. (1978). *All four histograms are characteristic examples of the described types selected from our results. The different diploid values shown in the various types were due to differences in staining intensities of different preparations made at different times.*

cells spreading as monolayered epithelial cell islands (Fig. 2).

Comparing the DNA distribution patterns in the tumours of 40 patients with the patterns in their cultures, 29 (73%) showed identical patterns indicating a good representativity of the cultures for the *in vivo* situation (Fig. 3). Eleven cases (27%) showed deviations indicative of gross shifts in ratios between different subpopulations or a complete loss of one or more subpopulations in the culture (Fig. 4). In Table 1 the results of this representativity check are subdivided over the four types DNA distribution patterns of the human mammary tumours (see Material and Methods, Fig. 1). Cultures of tumours of type 1 were always representative. Tumours of type 2 had in 2/8 no representative cultures (fewer than 20% of the cells were hyperdiploid in the culture). Cultures of tumours of type 3 were in 2/4 not representative in that only a diploid peak could be detected in the culture. Tumours of type 4 showed in 7/19 discrepancy resulting from a complete or almost complete loss of one or more aneuploid subpopulation(s).

Within a mammary tumour culture the cell islands may show similar or different DNA frequency distribution patterns when measured separately. When all islands had similar FDPs this could be either diploid or aneuploid. Tumours could also be composed of different populations (cell islands) with different FDPs. An example of this heterogeneity is given in Fig. 5.

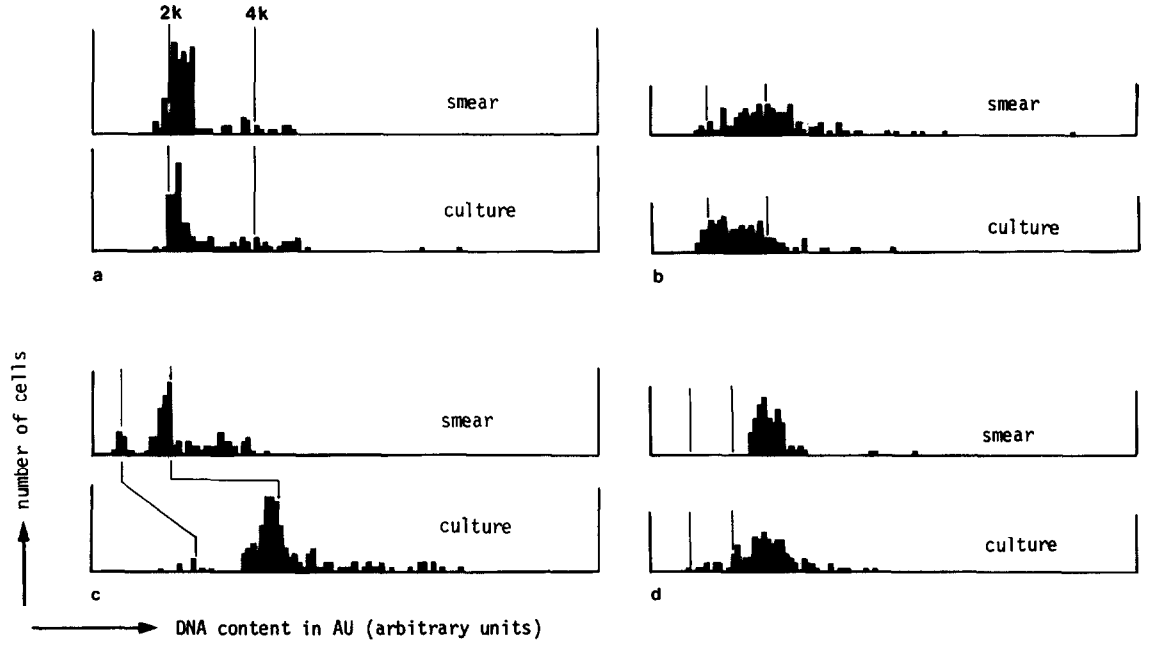

Fig. 3. *Four examples of the comparison of DNA distribution patterns of smear and culture of human mammary tumours showing a good representativity.* (2*k* = *diploid*; 4*k* = *tetraploid*).

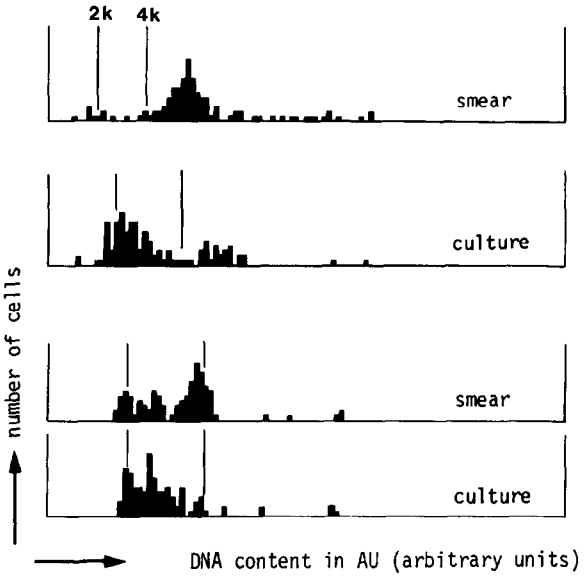

Fig. 4. Two examples of the comparison of DNA distribution patterns of smear and culture of human mammary tumours showing a representativity described as "not representative". (2k = diploid; 4k = tetraploid).

Photographs of four marked cell islands all in the same culture are shown, together with their corresponding FDP. Except for the diploid standard using fibroblasts (area 1) only cells with epithelial aspect were measured in the islands. In area 2 and area 3 two different islands showed different FDPs. The first had a normal diploid FDP comparable with that of the fibroblasts, the other area had an FDP with a small peak near the tetraploid region. In the last area tiny peaks could be observed in the FDP of this island in the diploid, tetraploid and octaploid region.

DISCUSSION

For many types of investigations it is imperative that observations made on cultured tumour cells should, as much as possible, reflect the situation in the tumour from which the cells were taken. Too often it is just assumed that the cells in the culture represent all the tumour cells.

Our results show that it is possible and worthwhile to check routinely the representativity of the cultures. In a majority of cases the nuclear DNA distribution patterns found in the cultures did not deviate from those of the original tumour; at least the same subpopulations were recovered, though the ratios may be somewhat different. The more the histograms of the cultures deviate from those of the original tumour the more the certainty of representativity diminishes. Sometimes a subpopulation will not even be found at all in the culture. The question may arise as to whether or not the cells scraped off the cut surface of the tumour do represent the whole tumour. Sub-

Table 1. Representativity of human mammary carcinoma cultures in relation to their DNA type

DNA-type*	Total	(†)+	−
1	9	9/9	—
2	8	6/8	2/8
3	4	2/4	2/4
4	19	12/19	7/19

*: DNA typing as described (see Material and Methods Fig. 1).
†: + = representative (good correlation);
− = not-representative (one or more subpopulation(s) missing in the culture).

populations may be missed by this procedure. The fact, however, that in none of our cases "new" aneuploid subpopulations were found in the culture, indicates that selection is unlikely. Our culture technique is aimed at obtaining as high as possible "plating efficiency" in order to avoid disappearance of certain subpopulations of the tumour. Thus most tumour cells are separated from the stroma but retain part of their intercellular bonds. From these cell clumps, cells emigrate and form cell islands. Such islands often show particular patterns of DNA distribution. The reason for this is that the DNA pattern in a smear of a freshly-cut mammary tumour shows all cell populations contained in the cut merged into one histogram; in the cultures the different cell islands represent groups of cells that belong together and that may be derived from one stem cell, thus representing one subpopulation (it should be noted that in our cultures of very short duration, cell islands are not representing clonogenic subpopulations).

Some tumours were found to be homogeneous, showing identical DNA patterns in all islands; others were heterogeneous as shown in Fig. 5. By this method, a mapping of the subpopulations of the tumour can be done. Minor subpopulations of cells that did not show clearly in the frequency distribution patterns of the smear sometimes were discovered in the cell islands in the culture.

A randomly-measured FDP of a smear of a mammary tumour indicates roughly whether the tumour had a homogeneous or a heterogeneous composition with respect to its DNA content. Using our parameter, the nuclear DNA, for "representativity checking" only makes sense if the FDP's show abnormal, i.e "aneuploid" DNA patterns (type 2, 3 or 4).

Our method of comparing the smear of a tumour with the culture showed that when there is a heterogeneity present in the FDP of the smear one can analyse how this composition is expressed in the different islands (outgrowth of organoid struc-

tures). Thus even very small subpopulations could be detected. These little, but still not insignificant, subpopulations may give an extra contribution to the prognostic value of the different DNA distribution patterns derived from randomly-measured nuclei in the smear or imprint [19, 20]. The prognostic value of the proportion of hyperdiploid cells in distribution patterns measured in imprints of tumours by image cytophotometry [21, 22] could also be further analysed in the cultures of these tumours by measuring the different cell islands. Our approach of image cytophotometry in tumour cultures by measuring marked cell islands can also give additive information to the results obtained by flow cytophotometry [23]. We are aware that more parameters (e.g. various monoclonal antibody markers) will be needed to fully define subpopulations in a tumour.

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