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Aneuploid Subpopulations in Tumour-invaded Lymph Nodes from Breast Cancer Patients

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Fresh, paired primary tumours and lymph node metastases from breast cancer patients were compared by DNA flow cytometry. Although 65% of primary tumours were aneuploid, the detection of aneuploid peaks in corresponding nodal metastases was rare (only 6 cases out of 25) in single-parameter DNA analysis. Detection of aneuploid subpopulations in lymph nodes was greatly improved in dual-parameter DNA analysis using an anti-cytokeratin (CK) antibody which allowed ploidy determination on CK$^+$ epithelial cells alone. Examination of 12 lymph nodes for CK$^+$ cells revealed the presence of both diploid and aneuploid tumour cells in tumour invaded nodes. In patients with multiploid primary tumours, a subpopulation of the primary aneuploid cells was dominant in the nodal metastases. This suggests that aneuploidy is an integral property of metastatic cells and that within a primary tumour a subpopulation may have a higher metastatic potential.
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INTRODUCTION

FLOW CYTOMETRIC (FCM) DNA analysis of primary tumour cells gives information on both the nuclear DNA content (DNA ploidy), which is frequently abnormal in primary tumours, and the S phase fraction (SPF), which is a measure of the proliferative rate of a growing cell population. A number of studies have now shown that DNA ploidy and SPF are of prognostic relevance and predictive value in patients with breast cancer. By correlation with histopathological factors, these studies found DNA aneuploidy to be significantly associated with axillary lymph node involvement [1, 2, 3], histological grade [2, 4–10, 13], and steroid receptor status [6, 11, 12, 13]. A few studies, however, failed to observe any correlation of DNA aneuploidy with lymph node involvement and histological grade [3, 10, 14], while others found that the association with steroid receptors did not reach statistical significance [4, 7, 10, 14]. Retrospective and long term follow-up studies in some of these reports have revealed more consistent results. By assessment of both recurrences and overall survival, it has been found that patients with diploid, low S-phase tumour DNA profiles have a good prognosis [1, 2, 8, 15, 16, 17]. A universal finding in these studies on frozen and paraffin embedded tissues from breast cancers has been the presence of a substantial proportion of aneuploid cells in a high percentage (mean 63%) of patients [18]. The ploidy pattern of tumours, however, differs from patient to patient.

The above studies were confined to only the DNA ploidy and SPF of the primary tumours and were carried out principally to assess the potential of DNA flow cytometry as a prognostic indicator. Very little attention has so far been directed towards comparing the ploidy in metastases with that of the primary tumour. The clinical outcome of patients with breast cancer depends largely on the tendency of the tumour to produce distant metastases and thus the presence of axillary lymph node metastases still remains the most important indicator of the tumour's biological aggressiveness. The treatment of breast cancer usually involves the excision of the ipsilateral axillary nodes to determine whether they have been invaded by tumour. The availability of invaded lymph nodes together with the primary tumour from the same patient makes it possible to conduct a simultaneous comparative DNA analysis of the two tissues.

A comparative study was made between the DNA profiles of fresh primary tumours and fresh metastatic tumour cells obtained at the same time from the axillary nodes of the same patients. In the past, detection of the tumour DNA profiles in the node has been difficult because of the comparatively small number of metastatic tumour cells present. However, in the present study, the availability of an antibody against cytokeratin made it possible for the profile of tumour cells to be distinguished from the lymphoid cells in the node where the abundant lymphocyte population is cytokeratin negative. The data show that both diploid and aneuploid tumour cells appear in a lymph node following metastasis. However, aneuploid cells can preferentially dominate the nodal population in a patient whose primary tumour contains both diploid and aneuploid cells. In cases where a tumour has two aneuploid populations, or has presented as a double primary, a definite subset of the primary tumour cells predominate in the metastatic deposit.

PATIENTS AND METHODS

Patients

Fresh, primary tumour and axillary lymph node samples were obtained from 55 consecutive patients undergoing definitive surgery for breast carcinoma in the Western Infirmary, Glasgow. Subsequent histological examination of the primary tumours showed that all samples were from patients with invasive ductal carcinoma of the breast. Involvement of lymph nodes was confirmed by histological examination of matching halves of nodes sent to pathology.

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Tumour dissociation

Both tumour and lymph node samples were disaggregated immediately after resection into single cell suspensions by mechanical mincing with scalpel blades in a petri dish containing RPMI 1640 medium. The cells were then centrifuged for 5 min at 300 *g* and resuspended in citrate buffer (sucrose 250 mmol/l; trisodium citrate, 40 mmol/l; dimethylsulphoxide (DMSO), 5% (v/v), pH 7.6). Three tumour samples were treated ezymatically with collagenase (200 U/ml in 7 ml tissue culture medium) overnight in addition to being mechanically disaggregated. No differences in the DNA index were found in all 3 cases and in 2 of the 3, a greater proportion of aneuploid cells was observed in samples obtained by mechanical disaggregation. All subsequent samples were disaggregated mechanically.

Preparation of nuclei and flow cytometric analysis (FCM)

Nuclei for flow cytometric DNA analysis were prepared and stained according to the method of Vindelov *et al* [19]. Briefly, the cell suspension was treated with trypsin for 10 min at room temperature followed by a further 10 min incubation with trypsin inhibitor and RNAase. Finally, the cells were stained with a solution containing propidium iodide (PI) as the DNA fluorochrome and spermine tetrachloride and incubated in the dark at 0°C until analysed 15–60 min later. Chicken red blood cells (CRBC) were stained identically in a separate tube and then added to serve as internal standard to each sample to ≤10% of the total acquisition. 10000 nuclei were acquired per sample on a FACScan flow cytometer (Becton Dickinson). Human peripheral-blood lymphocytes (PBL) were stained and analysed separately with each sample being evaluated for DNA content. DNA content was assessed with reference to the CRBC peak and further confirmed to be diploid if the Go/G1 peak of blood lymphocytes was superimposable with that of the tumour sample. A primary tumour was considered aneuploid only if the suspected aneuploid Go/G1 peak contained at least 20% of the total sample events and the corresponding G2/M peak was also detectable. The DNA index (DI) is calculated as the ratio of the peak channel number of the aneuploid Go/G1 peak to the peak channel number of the diploid Go/G1 peak.

Tumour grade was assessed by the method of Bloom and Richardson [20] and the oestrogen receptor (ER) status by the ligand binding method [21].

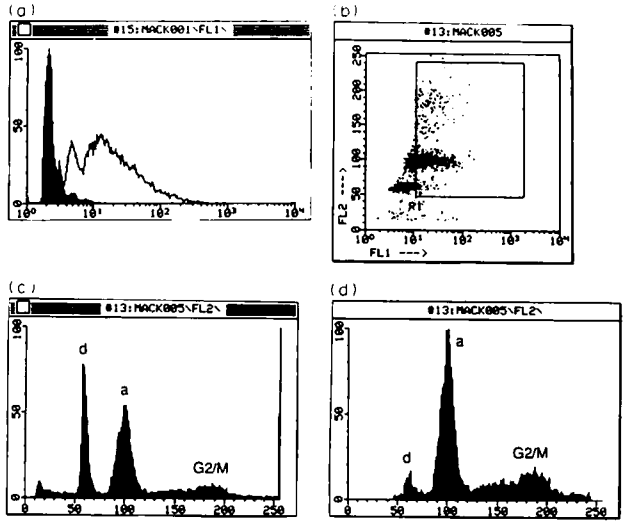

Fig. 1. Flow cytometric gating for CK⁺ cells. (a) The reaction of the anti-CK antibody with tumour cells (unshaded area) overlaid on the reaction with lymphocytes (shaded area). (b) A flow cytometric "gate" (area within box) drawn on the plot of Fl 1 (green—CK⁺) and Fl 2 (red—PI) to analyse the ploidy status of CK+ cells only. (c) An ungated DNA histogram of a primary tumour showing a diploid population (d, Go/G1 peak at channel 55–58) and an aneuploid Go/G1 population (a, Go/G1 peak at channel 93) and its corresponding G2/M peak. (d) A CK gated DNA histogram of the same tumour showing CK⁺ epithelial cells. The diploid peak (d), representing non-epithelial cells, has been largely gated out. d = diploid; a = aneuploid; G2/M = G2/M peak of the aneuploid population.

Table 1. DNA ploidy of primary tumours and nodal status of 55 patients with breast cancer.

Histopathology of node	FCM DNA analysis Diploid	Aneuploid
Involved	4	25
Non-involved	15	11
	19	36

Two colour DNA flow cytometry with propidium iodide (PI) and cytokeratin (CK) staining

When analysing paired primary tumour and lymph node metastases it was necessary to exclude non-epithelial cell components including leukocytes, stromal fibroblasts and endothelial cells. This was achieved in a multiparametric 2-colour FCM analysis using a fluorescein isothiocyanate (FITC) labeled antibody to CK (CAM 5.2-FITC). The CAM 5.2 monoclonal antibody detects CK 8, CK 18 and CK 19, which are CK peptides consistently present in all layers of normal and malignant mammary epithelium [22–24]. Gating the CK positivity thus allowed DNA analysis to be restricted to epithelial cells. Cell suspensions from both tumour and lymph node were fixed in ethanol and stained according to the method of Zarbo *et al.*[25] except that a directly labeled anti-CK-FITC antibody (Becton Dickinson) was used. CK-positive (CK⁺) cells were identified during analysis by comparing single-parameter FL1 (green) intensity to negative controls. Cells stained with FITC-mouse IgG of irrelevant specificity served as a green fluorescence negative control, while those treated with phosphate buffered saline was used as an autofluorescence control. Next a gate was drawn on FL1 (CK-FITC stain) versus FL2 (PI stain) to include only the cells positive for CK (CK⁺) (Fig. 1). DNA profile analysis (FL2 parameter) was performed as described above.

RESULTS

Ploidy studies in the absence of CK antibody on primary tumour and invaded lymph node samples from the same patient

In order to select matched aneuploid primary tumours with involved lymph nodes, we screened 55 primary tumours and corresponding lymph nodes for DNA aneuploidy and correlated the data with histopathological examination for nodal involvement. 65% (36) of the tumours were aneuploid, and of these 25 were histopathologically defined as invaded (Table 1). The distribution of DNA indices of all primary tumours with involved nodes (29/55) ranged from 0.8 to 2.9. The distribution of DI (DNA index) over such a wide range suggests that the metastatic capability is not restricted to an aneuploid cell population with a specific DI. On routine analysis we could detect no aneuploid cells in lymph nodes, whether involved or

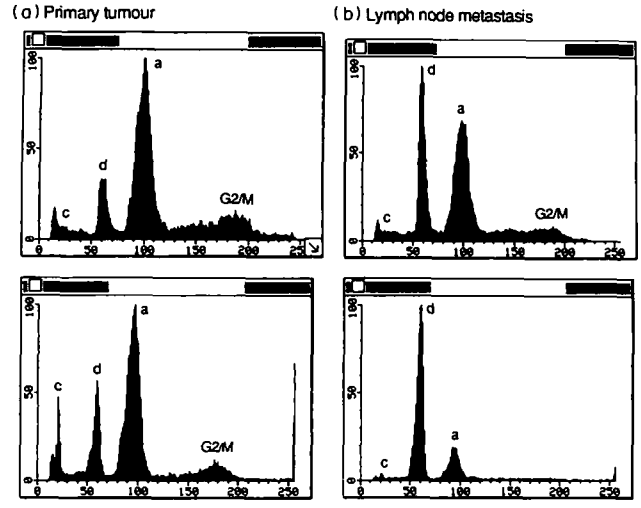

Fig. 2. DNA content of primary tumours (a) and associated heavily involved lymph node metastasis (b) in 2 patients without use of the CK^+ gating. The two examples cited here represent cases where the lymph nodes were grossly involved and aneuploid peaks (a) were detected without CK^+ gating. c = Chicken red blood cells; d = Diploid Go/G1 peak; a = Aneuploid Go/G1 peak; G2/M = G2/M peak of the aneuploid population.

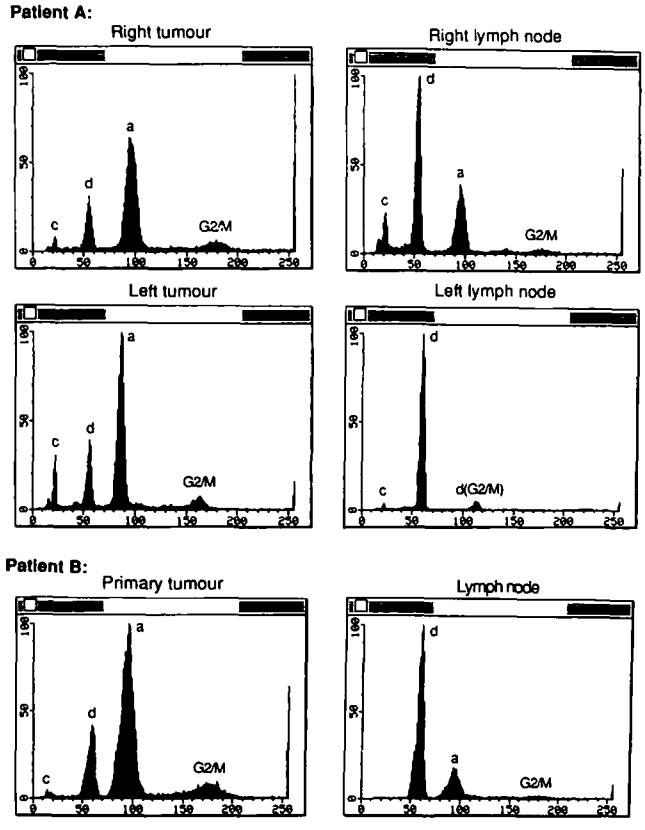

Fig. 3. Single parameter DNA profiles without CK staining of 2 patients presenting with double primary tumours. In patient A (bilateral) the aneuploid population in the invaded right node has a DNA index of 1.75, identical to that in the right primary tumour. The left tumour has a different aneuploid population (DNA index 1.6). No aneuploid population can be detected in the left uninvolved node. Patient B with both primaries on the same side, and a diploid tumour (data not shown) and an aneuploid tumour as shown (DNA index = 1.6). The aneuploid peak can be seen in the tumour invaded node. c = Chicken red blood cells; d = Diploid Go/G1 peak; a = Aneuploid Go/G1 peak; d(G2/M) = diploid G2/M; G2/M = aneuploid G2/M.

not, from patients with diploid tumours. Within the 25 involved lymph nodes from patients with aneuploid primary tumours, an aneuploid peak was detected in 6 cases. The DI of the aneuploid peaks in all these lymph node metastases were identical to the DI of the corresponding primary tumours. Figure 2 shows two representative cases.

In patients with double primary tumours, the two have different ploidy and only one can be detected in metastatic tissue

In the course of this study 2 patients presented with rare double primary tumours. In both cases, only one of the two tumours appeared to have metastasised. In the case of Patient A, who had bilateral carcinoma, both right and left tumour samples and the corresponding lymph nodes were available for DNA analysis. Histopathology revealed both tumours to be poorly differentiated (grade III) infiltrating carcinomas. Single parameter DNA analysis revealed both tumours to be aneuploid but with differing DI (patient A, Fig. 3). The DNA histogram of lymph node cells from the right node, which was involved, showed aneuploid cells with DI identical to the right primary tumour. Although the left tumour was aneuploid, no aneuploid population was detected in the left lymph node, which on histopathological examination was found to be tumour free. Comparison of the DNA histograms of the two tumours, suggests that the left tumour does not represent a secondary deposit of the right primary tumour but rather is a second distinct primary.

The second double primary, Patient B, presented with two distinct primary tumours on the same side. Tumour specimens

Table 2. Results from dual-parameter DNA analysis of CK-gated paired primary tumour and lymph node from the same patient

Patient No.	Histopathology of lymph node	Ploidy and DI of CK + ve cell population	
		Tumour	Lymph node
1	Involved	Diploid & Aneuploid DI = 1.0 + 1.7	Aneuploid DI = 1.7
2	Involved	Diploid & Aneuploid DI = 1.0 + 1.45 + 1.8	Aneuploid DI = 1.8
3	Involved	Diploid & Aneuploid DI = 1.0 + 1.6 + 1.8	Aneuploid DI = 1.85 + 2.7
4	Involved	Diploid DI = 1.0	Diploid DI = 1.0
5	Involved	Aneuploid DI = 1.3	Aneuploid DI = 1.3
6	Involved	Diploid & Aneuploid DI = 1.0 + 1.4	Aneuploid DI = 1.4
7	Involved	Diploid DI = 1.0	Diploid DI = 1.0
8	Non-involved	Diploid DI = 1.0	None
9	Non-involved	Aneuploid DI = 2.2	None
10	Non-involved	Aneuploid DI = 1.6	None
11	Non-involved	Aneuploid DI = 1.8	None
12	Non-involved	Diploid DI = 1.0	None

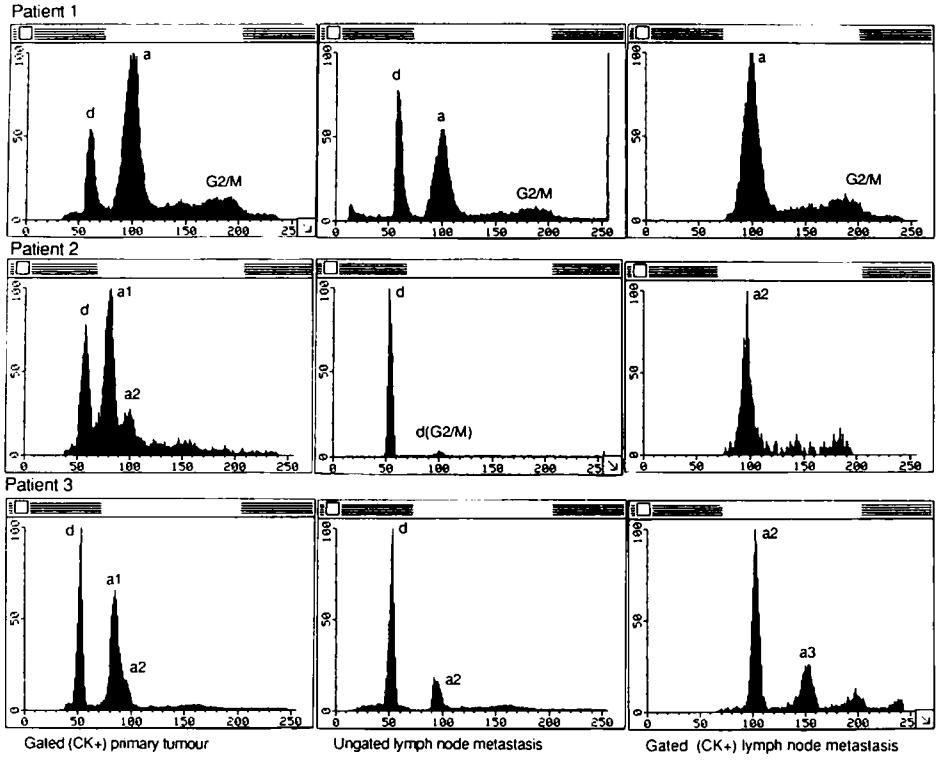

Fig. 4. CK⁺ gated and ungated DNA profiles of three lymph node metastases together with their primary tumours. In patient 1 the diploid peak of the primary tumour was not represented in the lymph node following CK⁺ gating and the aneuploid population in the node matches the DNA index (1.7) of the aneuploid population in the tumour. In patient 2 the aneuploid peak in the node is detected only after CK⁺ gating and represents the smaller of the two aneuploid peaks (a2, DI = 1.8) seen in the tumour. In patient 3 two aneuploid peaks appear after CK⁺ gating. Peak a2 (DI = 1.85) has a DI close to but not identical to the a2 peak in the primary tumour (DI = 1.8), whereas peak a3 (DI = 2.7) was not detected either in the ungated node or in the primary tumour. c = Chicken red blood cells; d = Diploid Go/G1 peak; a = Aneuploid Go/G1 peak

from the two regions and lymph nodes were analysed (patient B, Fig. 3). Histological evaluation, which revealed infiltrating carcinoma (grade II) was possible in only the larger of the two tumours. The smaller tumour was diploid whereas the involved lymph node had a detectable aneuploid peak with DI identical to the larger primary tumour (Fig. 3).

Two colour staining shows that a subpopulation of cells in the primary tumour has metastatic properties

The above results indicate that invaded lymph nodes harbour aneuploid tumour cells which appear identical to a subpopulation of cells within the corresponding primary tumour(s) with respect to their DNA content. However, aneuploid cells were detected in only 24% (6/25) of the analysed lymph nodes from patients with aneuploid primary tumours. This could reflect the low sensitivity of detection of DNA peaks from small numbers of highly aggressive aneuploid cells, effectively swamped by the large dilution effect caused by the inevitable presence of lymphocytes in the node preparation. However, it could also reflect the presence of a substantial malignant diploid population from which terminally differentiated and essentially harmless aneuploid cells evolve. The distinction has obvious significance. To resolve this matter it was necessary to exclude lymphocytes from lymph node samples and, for primary tumours, it was necessary correctly to establish if the diploid peak was contributed by epithelial tumour cells and not by contaminating stromal and inflammatory cells. We, therefore, undertook dual-parameter DNA analysis of primary tumours and lymph nodes using cytokeratin (CK) staining. When primary tumours were analysed for both CK and PI staining, diploid peaks were always reduced, sometimes to a negligible level compared with PI staining alone (Fig. 1). In the lymph node, gating for CK positive cells improved detection of aneuploid peaks which had been undetectable in single parameter DNA analyses. The sensitivity of CK⁺ gating was high enough to detect as low as 5% of tumour cells in involved lymph nodes, allowing a 20-fold enrichment.

Twelve paired primary tumours and lymph nodes, i.e. from the same patients were selected to determine the ploidy of the CK positive primary tumour cells and their corresponding lymph nodes were analysed to detect the ploidy of the invading tumour cell population (CK⁺). The results are summarised in Table 2. In lymph node samples, CK⁺ cells were absent in normal, non-involved lymph nodes, but where the nodes were involved, invading CK⁺ tumour cells were always detected.

Where a tumour was multiploid, there was evidence to suggest that the corresponding lymph node revealed only one aneuploid peak, suggesting that aneuploid tumour cells are capable of selective metastasis. 3 such cases are detailed further in Fig. 4. In patient 1, although both aneuploid and diploid cells were present in the primary tumour, only aneuploid cells were detected in the lymph node metastasis and they had a DI identical to the aneuploid cells in the primary tumour. The invaded lymph node in patient 2 revealed a single aneuploid peak with a DI identical to peak 2 of the primary tumour. In patient 3 an aneuploid peak with DI similar to the shoulder (a2 in Fig. 4) in the main aneuploid peak of the primary tumour was detected in the lymph node. An additional peak (a3, DI = 2.7) which was not detectable in the tumour also appeared in the lymph node histogram.

DISCUSSION

In agreement with all previous reports [18], we find that 65% (36/55) of breast tumours are aneuploid. It is generally assumed that only a subpopulation of tumour cells have the capacity to metastasise. As a result of the surgical treatment of breast cancer, investigators have access to untreated lymph nodes. In addition to any previous study such as those involving fine-needle aspirates [26], we employed a directly labelled antibody to cytokeratin, which enabled us to detect subpopulations of aneuploid cells. Our aim was to investigate if the metastatic population in invaded nodes included the cells with grossly abnormal DNA. Our findings suggest that these cells are capable of metastasis and indeed that within these cells, certain further sub-populations are capable of preferential metastasis (Fig. 4).

The use of a CK antibody to differentiate epithelial cells from other cell types in both tumour and node samples confers a significant advantage. It is possible to analyse the ploidy status of the tumour cells alone and this becomes important when they form a small percentage of the cells under analysis. In the node where there are extensive numbers of lymphocytes, this is of particular value. However the tumour may also contain as much as 50% lymphocytes [27] in addition to stromal and other cell types and therefore this antibody will be of use in the flow cytometric analysis of tumour markers.

Do the aneuploid cells represent a distinct tumour population with a higher metastatic potential? Although the question is difficult to answer, it is possible that if only a single clonal population appears in a metastasis, this population has a higher metastatic potential than any of the other aneuploid, if present, and/or diploid populations in the associated primary tumour. On the other hand one cannot exclude the possibility that a diploid clonogenic cell may invade the lymph node, divide and evolve in a similar fashion as in the primary tumour and thus give rise to both diploid and aneuploid cells. In lymph node metastases, several observations argue that if this second mechanism is operating, it probably does so in a restricted manner. Firstly, in 4 cases (Table 2) diploid peaks, although present in the primary tumour, were absent in nodes. Secondly, in cases of multiploid primary tumours, preferential appearance of a single aneuploid peak of higher DI was observed. Thirdly, there are the examples of the bilateral carcinoma patient and the patient with two tumours on the same breast. They show that even in the same patient two tumours may have different ploidy and since one of the bilateral tumours metastasised and the other did not, this again raises the question of the relationship between metastatic potential and aneuploidy. Further studies with more refined methodology will perhaps lead to a better understanding of the true biology of aneuploid cells in solid tumours.

The fact that most of the cells in many primary epithelial tumours have a grossly abnormal DNA content has interested clinicians seeking for prognostic indicators for breast cancer for several years. It is at first surprising that, in the context of such major changes in the primary genome involving the addition or deletions in the range of 10^9 base pairs, researchers have tended to concentrate their interests on small stretches of DNA. The restricted ability of current tissue culture methodology to propagate primary aneuploid tumour cells [28, 29] means that only rarely can a primary tumour or metastasis with abnormal ploidy be subjected to conventional cytogenetic analysis, internal labelling with isotope, or analysis for cell surface or enzyme markers. Furthermore, aneuploid cells are often assumed to be terminal cells resulting from the general disorganization of tumour cell cycle control. Our data suggest that, despite their abnormal chromosome load, they are more than capable of either themselves metastasising or of constituting the bulk of metastatic deposits. In countries where lymph node metastases are still removed prior to chemotherapy, it might be useful in clinical studies of prognostic indicators to focus on the metastatic subpopulation in addition to primary tumours for monitoring of predictive factors such as lectin binding [30] or oestrogen receptor content. Moreover, the use of dual-parameter flow cytometry employing a CK-antibody to detect epithelial tumour subpopulations has the potential for allowing the sorting of selectively metastatic subpopulations of aneuploid nuclei. These can then be used for comparative analysis of genetic factors such as proto-oncogene amplification or loss of heterozygosity, which predispose different cell populations within an individual towards or against metastasis. We conclude that subpopulations of the primary tumour predominate in metastatic deposits. This observation has considerable biological relevance in the understanding of tumour development and metastatic capacity in breast cancer patients.

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Cytotoxicity of 5-Aza-2′-deoxycytidine in a Mammalian Cell System

Sean Davidson, Penelope Crowther, John Radley and David Woodcock

After the addition of 5-aza-2′-deoxycytidine, a potent inhibitor of DNA methylation and S-phase-specific cytotoxic agent, metaphase chromosomes of Chinese hamster ovary (CHO) cells exhibited a highly decondensed and extended morphology (numerous "fragile sites") at the first mitotic division. However, when a lethal dose of this drug was added in early G_1 phase to cells synchronised by mitotic selection, the majority subsequently divided at the same time as an untreated control cell population with few division abnormalities and with few of the more usual types of chromosome aberrations such as gaps, breaks and exchanges. The drug-treated cells also entered and completed the second S-phase without significant delay and it was only at the second mitosis after addition of 5-azadeoxycytidine that cells showed delays in entering mitosis and significant increases in abnormal divisions concomitant with a modest increase in chromosome aberrations. If cells in a tumour behave similarly, the tumour mass would be expected to double before any reduction in tumour burden could be expected to occur.
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INTRODUCTION

IT IS IMPORTANT to elucidate the detailed biological effects of 5-aza-2′-deoxycytidine (5-azadC) on three grounds. Firstly, 5-azadC has been shown to be an active antileukaemic agent and has undergone phase I and II trials with different types of human tumours [1–3]. 5-azadC is highly cytotoxic to mammalian cells with maximal sensitivity in S-phase [4]. However, the actual mechanism of cytotoxicity has not been definitively demonstrated. Secondly, 5-azadC is a potent inhibitor of DNA methylation in mammalian cells with approximately 10 times the activity on a molar basis than the corresponding ribonucleotide, 5-azacytidine (5-azaC) [5, 6]. Both these agents have been used extensively in studies to elucidate the role of DNA methylation in mammalian cells. Both agents have been shown to reactivate inactive X-chromosomes [7] and induce the conversion of undifferentiated cell lines into myocytes, chondrocytes and adipocytes [9, 8]. Thirdly, 5-azadC (and 5-azaC) induces unusual morphologies in metaphase chromosomes which have variously been

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