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DNA Flow Cytometry and Response to Preoperative Chemotherapy for Primary Breast Cancer

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Between October 1988 and June 1990, 22 patients with locally advanced, inoperable breast cancer entered a pilot study of four cycles of anthracycline based cytotoxic chemotherapy followed by surgery and tamoxifen. Fine needle aspirate samples of tumour were obtained for DNA flow cytometry before treatment and during the first cycle of chemotherapy. 21 patients are eligible for assessment of response and toxicity. Chemotherapy was well tolerated with > WHO grade 2 vomiting or stomatitis in 4 patients. Granulocytopenia < 10^9/l was noted in 16/21 patients but there were no episodes of neutropenic sepsis. There were 7 complete responses (CR) and 11 partial responses (PR), giving an overall response rate to chemotherapy (CR+PR) of 18/21 (86%). Responses were observed more commonly in patients who had aneuploid tumours ($P = 0.06$) and in patients whose tumours had a high S-phase fraction ($P = 0.1$). Tumours which responded to chemotherapy (CR or PR) had a significantly higher median SPF compared with tumours which did not regress ($P < 0.05$). There was no consistent pattern of change in SPF values during the first cycle of chemotherapy, either for patients who responded to treatment or for those whose tumours did not regress. This combination therapy is well tolerated with a high response rate. The results of this pilot study support the recent suggestion that tumours with rapidly proliferating, aneuploid populations of cells exhibit the best short-term response to chemotherapy.
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INTRODUCTION

THERE IS increasing interest in the use of preoperative chemotherapy to downstage breast cancer and allow breast conservation in patients who would otherwise need a mastectomy for local control of disease [1]. One problem with this approach, however, is that any delay in definitive locoregional treatment consequent on the tumour failing to respond to chemotherapy could be detrimental to outcome. At present, there is no reliable method of predicting the response of the primary tumour to chemotherapy, but recent reports have suggested that pretreatment DNA flow cytometric measurement of tumour ploidy and of the fraction of cells in the S-phase of the cell cycle (SPF) may be indicators of tumour cell chemosensitivity [2, 3]. This prospective study investigates the relationship between flow cytometry measurement of both DNA ploidy and SPF and response to chemotherapy. In addition, changes in DNA ploidy and SPF during treatment were studied using serial fine needle aspirates.

PATIENTS AND METHODS

Patients

Between October 1988 and June 1990, 22 patients with locally advanced, inoperable breast cancer entered a therapeutic trial of combined modality treatment. All patients had the diagnosis of breast cancer confirmed by incision or trucut biopsy. Fine needle aspirate specimens were taken for flow cytometric analysis at diagnosis and, in some cases, serially during chemotherapy. Aspirates were obtained using the method described by Lever *et al.* [4]. Briefly, a 23 gauge needle attached to a 10 ml syringe was inserted into the tumour and the plunger of the syringe then pulled back to exert suction. As negative pressure was maintained, the needle was moved through the tumour three or four times, thus aspirating material into the syringe. Part of the extracted material was expelled onto slides for cytological examination and the remainder suspended in 0.5 ml of RPMI and kept on ice until flow cytometric analysis.

Treatment and response

Primary treatment consisted of four cycles of anthracycline containing combination chemotherapy. Patients were then offered mastectomy if this was felt to be technically possible. Those whose tumours were still judged to be inoperable, or who declined surgery, received radiotherapy to breast and axilla. All patients then received tamoxifen 20 mg daily for 2 years.

The chemotherapy regimen evolved during this study as part of a dose ranging exercise. All patients received cyclophosphamide and 5-fluorouracil, combined with an anthracycline (doxorubicin or epirubicin). The first 12 patients received doxorubicin 30 mg/m^2 intravenously and 5-fluorouracil 600 mg/m^2 intravenously on days 1 and 8 with cyclosphosphamide 100 mg/m^2/day orally days 1 to 8, repeated monthly. A further 4 patients were treated according to the same schedule, but with doxorubicin replaced by epirubicin 40 mg/m^2. 2 patients received epirubicin 50 mg/m^2 intravenously, 5-fluorouracil 500 mg/m^2 intravenously and cyclophosphamide 500 mg/m^2 intravenously repeated every 21 days. The doses were then

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increased for the final 4 patients to epirubicin 70 mg/m^2 intravenously, 5-fluorouracil 700 mg/m^2 intravenously and cyclophosphamide 700 mg/m^2 intravenously every 21 days.

Response to chemotherapy was assessed by clinical evaluation after completion of four cycles of treatment using UICC criteria. Complete response (CR) was defined as the disappearance of all palpable disease, partial response (PR) as a regression of 50% or more and no change (NC) as a regression of less than 50%.

Flow cytometry

Fine needle aspirate samples were centrifuged, resuspended in 1 ml 5% serum in minimal essential medium and then passed sequentially twice through 19, 21, 23 and finally 25 gauge needles and stained with DAPI (final concentration 1 μg/ml). Chicken red blood cells (CRBC) were added as a DNA control to check ploidy (ration CRBC: human diploid cells = 3.04). A minimum of 10 000 cells were scanned on a Becton Dickinson FACS analyser to construct each histogram. Samples were considered aneuploid only when two clear G_1 peaks were present; the identity of the diploid peak was checked by reference to the CRBC peak (ratio 3.04:1). Flow cytometry on paraffin-embedded specimens was performed according to the method we have described previously [5], calculating the DNA index by measuring the position of any aneuploid G_1 peak relative to the normal G_0/G_1 peak, with a DNA index of 1.0 indicating the presence of only diploid cells. A histogram was considered interpretable if the coefficient of variation (CV) was less than 8%.

For DNA diploid samples, the SPF was calculated using the method of Baisch *et al.* [6]. For aneuploid tumours with a DNA index > 1.2, a modification of this method was used to calculate the SPF for the aneuploid cells alone. If the aneuploid peak accounted for ≤ 10% of the total number of cells, the SPF was not calculated.

RESULTS

21 patients completed four cycles of chemotherapy and thus were considered evaluable for response. The remaining patient developed a severe chest infection and was withdrawn from the study. Chemotherapy was well tolerated with > WHO grade 2 vomiting or stomatitis in 4 patients. Granulocytopenia < 10^9/l was noted in 16/21 patients but there were no episodes of neutropenic sepsis. An objective response (CR or PR) was observed in 18/21 (86%) evaluable patients, with a clinical CR noted in 7 patients (33%).

Samples for flow cytometry analysis were obtained prior to chemotherapy for 20/22 patients (fine needle aspirate samples for 13 patients and fixed tissue biopsies for 7 patients). Histograms from which DNA ploidy could be estimated were obtained from 16/20 samples. The remaining four samples either contained insufficient material for analysis or had a CV of > 8%. 4 patients (25%) had diploid tumours and 12 (75%) had aneuploid tumours. S-phase fraction could be calculated for 14/22 patients. The median SPF was 10.2% (range 2.7–17.8%). Table 1 shows the relationship between pretreatment tumour ploidy, SPF and response to chemotherapy. Objective response was observed in 2/4 patients with diploid tumours and in all 12 patients with aneuploid tumours (Fisher's exact test, $P = 0.06$). When the median SPF was used as a cut-off, response to treatment was noted in 3/5 patients with a low SPF and in all 9 patients with a high SPF (Fisher's exact test, $P = 0.1$). The relationship between SPF and response is illustrated in Fig. 1. The median SPF was 14.1% for patients who had a CR, 10.4% for patients who had a PR, and 4.7% for patients with NC (Mann-Whitney non-parametric test, CR vs. NC, $P < 0.01$; PR vs. NC, $P < 0.01$; CR vs. PR, $P > 0.05$).

Table 1. The relationship between DNA ploidy, SPF and response to chemotherapy

	CR	PR	NC
Diploid	0	2	2
Aneuploid	5	7	0
SPF < 10.2%	0	3	2
> 10.2%	5	4	0

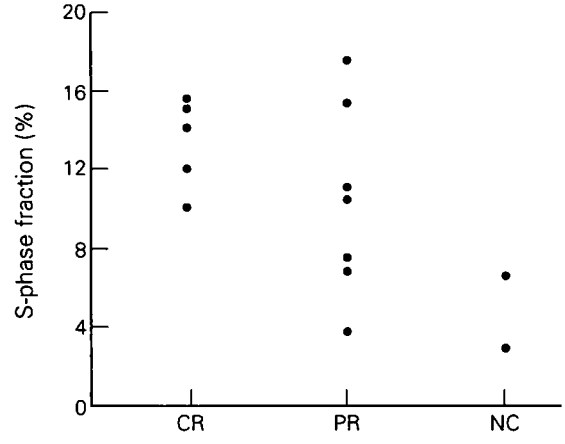

Fig. 1. Pretreatment SPF values and response to chemotherapy.

11 patients had a repeat fine needle aspirate for flow cytometry performed on day 8 of the first cycle of chemotherapy and 7 patients had a further fine needle aspirate on day 21 or 28. There was no variation in DNA ploidy in 8 cases. In 1 patient, an aneuploid cell line was no longer detected on day 8 while, in another patient an aneuploid population appeared after treatment. The serial SPF measurements are shown in Table 2. There was no consistent pattern of change in SPF values during

Table 2. Serial SPF values from fine needle aspirate specimens taken during the first course of chemotherapy

	S-phase fraction (%)		
Response	Pretreatment	Day 8	Day 21/28
CR	15.6	10.4	NA
CR	14.1	15.6	NA
PR	11.0	8.6	7.5
PR	7.5	7.8	5.7
PR	15.3	17.0	NA
PR	17.8	15.0	NA
PR	3.7	5.1	4.7
PR	10.4	6.8	7.2
PR	6.8	NA	7.2
NC	2.8	3.7	3.2
NC	6.5	5.6	6.2

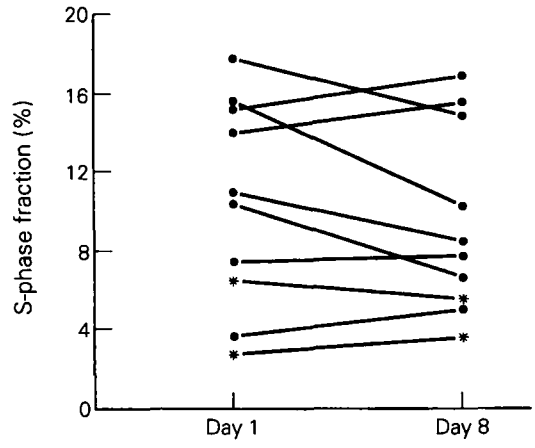

Fig. 2. Pretreatment and day 8 SPF values related to response to chemotherapy. ● Response (CR or PR); ★ no change.

the first cycle of treatment, either for patients who responded to treatment or for those whose tumours did not regress (Fig. 2).

14 patients had a modified radical mastectomy after four cycles of chemotherapy. 1 patient had no pathological evidence of carcinoma in either the breast or axillary nodes and 2 further patients had no disease in the breast but positive lymph nodes. All these patients had a clinical CR after chemotherapy. The remaining 10 patients all had breast carcinoma visible in both the breast and axillary lymph nodes.

DISCUSSION

The results of this pilot study, with response to chemotherapy being more commonly observed in aneuploid tumours and tumours with a high SPF, are consistent with the recent reports from two French centres. Briffod *et al.* noted a significantly higher objective response rate to combination chemotherapy in aneuploid tumours (15/25) than in diploid tumours (1/10, $P = 0.08$) [2]. Remvikos *et al.* [3] also reported that responses were more common in the aneuploid group although, as in our study, this difference did not achieve statistical significance (overall response 82% vs. 63%, $P = 0.15$). They did, however, report that tumour chemoresponsiveness was significantly related to SPF, with all 12 patients who had SPF of 10% or more showing demonstrable tumour regression.

While it would be useful, for reasons of loco-regional control, to be able to predict the chemosensitivity of the primary tumour, the target cells of most interest in early breast cancer are those of occult micrometastases. Micrometastases may exhibit different cell cycle kinetics, especially after removal of the primary tumour [7], and thus flow cytometry measurement of ploidy and SPF from the primary tumour may not predict chemosensitivity at metastatic sites. There is, however, some evidence of an association between proliferative activity of the primary tumour and response to adjuvant chemotherapy. In a report on adjuvant chemotherapy for node negative breast cancer, the Milan group commented that chemotherapy appeared to act maximally in those patients whose tumours had a high thymidine labelling index, with little or no effect in the subgroup whose tumours had a low thymidine labelling index [8]. In addition, a recent report on adjuvant chemotherapy for patients with node positive disease noted that, while treatment does significantly improve prognosis for premenopausal patients whose tumours have a low SPF, the magnitude of the effect is greater for patients with more rapidly proliferating tumours [9].

We did not find serial flow cytometry measurements during chemotherapy helpful in predicting response in this study. There are inherent problems, however, in sequential flow cytometry analysis on fine needle aspirate samples. One study has reported marked differences in DNA profile in 12/17 patients when aspirates taken for diagnosis were compared with samples obtained 1–3 weeks later, without any intervening systemic therapy [10]. This variability in DNA profile, which is presumably related to tumour heterogeneity, suggests that monitoring changes in flow cytometry parameters by serial fine needle aspirates may not be feasible. Our results would tend to support this conclusion.

The results of this pilot study support the recent suggestion that tumours with rapidly proliferating, aneuploid populations of cells exhibit the best short-term response to chemotherapy. While these results are interesting, larger prospective studies are needed to decide whether DNA flow cytometry gives any clinically useful information.

1. Ragaz J. Emerging modalities for adjuvant therapy of breast cancer: neoadjuvant chemotherapy. *NCI Monogr* 1986, **1**, 145–152.
2. Briffod M, Spyratos P, Tubiana-Hulin M, *et al.* Sequential cytopunctures during preoperative chemotherapy for primary breast cancer. *Cancer* 1989, **63**, 631–637.
3. Remvikos Y, Beuzeboc P, Zajdela A, Voillemot N, Magdelenat H, Pouillart P. Correlation of pretreatment proliferative activity of breast cancer with the response to cytotoxic chemotherapy. *J Natl Cancer Inst* 1989, **1**, 1383–1387.
4. Lever JV, Trott PA, Webb AJ. Fine needle aspiration cytology. *J Clin Pathol* 1985, **38**, 1–11.
5. O'Reilly SM, Camplejohn RS, Barnes DM, *et al.* DNA index, S-phase fraction, histological and prognosis in breast cancer. *Br J Cancer* 1990, **61**, 671–674.
6. Baisch H, Gohde W, Linden WA. Analysis of PCP-data to determine the fraction of cells in the various phases of the cell cycle. *Radiat Environ Biophys* 1975, **12**, 31.
7. Fisher B, Gunduz N, Saffer EA. Influence of the interval between primary tumour removal and chemotherapy on kinetics and growth of metastases. *Cancer Res* 1983, **43**, 1488–1492.
8. Bonadonna G, Valagussa P, Tancini G, *et al.* Current status of Milan adjuvant chemotherapy trials for node-positive and node-negative breast cancer. *NCI Monogr* 1986, **1**, 45–49.
9. O'Reilly SM, Camplejohn RS, Millis RR, *et al.* Proliferative activity, histological grade and benefit from adjuvant chemotherapy in node positive breast cancer. *Eur J Cancer* 1990, **26**, 1035–1038.
10. Mullen P, Miller WR. Variations associated with the DNA analysis of multiple fine needle aspirates obtained from breast cancer patients. *Br J Cancer* 1989, **60**, 688–691.