Mobilisation kinetics of primitive haemopoietic cells following G-CSF with or without chemotherapy for advanced breast cancer

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Summary

Background: The objective of this study was to determine the optimal conditions for blood progenitor cell harvest for transplantation, with main emphasis on the mobilisation kinetics of primitive, marrow repopulating cells.

Patients and methods: Sixteen patients with advanced breast cancer were treated with 4 cycles of dose escalating FAC chemotherapy (5-fluorouracil, adriamycin, cyclophosphamide) each followed by 10 μg/kg/d G-CSF for 13 days. We assessed the number of colony-forming cells (CFC), and estimated the long-term culture initiating cells (LTC-IC) and CD34+ cells during the recovery phase of cycle 1 and 4 of chemotherapy, and during additional periods of G-CSF administration either preceding or following the full course of chemotherapy.

Results: The highest peak numbers of CFC per ml of blood (median 10489, range 860–39282) were mobilised after the first cycle of chemotherapy. The lowest peak numbers of CFC were obtained during the recovery phase from cycle 4 (median 240, range 20–770 x 10⁶). In contrast, the numbers of CD34+ cells per ml of blood were significantly higher in cycle 4 (median 650, range 30–2600 x 10⁶) compared to those of cycle 1 (median 240, range 20–770 x 10⁶). The peak numbers of CFC mobilised by G-CSF before commencement and after the cessation of chemotherapy were equivalent, with a median of 5470 (range 1056–25669) and 5948 (range 2710–38975) per ml of blood, respectively. However, while mononuclear cells (MNC) collected at the days of maximal CFC mobilisation following G-CSF administration before or after cycle 1 were similar to normal bone marrow MNCs in their ability to generate haemopoiesis when seeded onto performed irradiated stroma, those collected after cycle 4 or during G-CSF administration after the cessation of chemotherapy were markedly compromised in this respect.

Conclusions: Our results indicate that repeated cycles of FAC chemotherapy followed by G-CSF result in a far lower number of LTC-IC than of CFC mobilised into the circulation. Furthermore although the combination of chemotherapy and G-CSF mobilised the highest numbers of CFC, G-CSF alone pre-chemotherapy was more effective at mobilising LTC-IC. These data indicate that neither the numbers of CFC mobilised nor the numbers of CD34+ cells are necessarily a reliable indicator for the putative marrow repopulating capability of the blood cells mobilised with chemotherapy plus G-CSF.

Key words: advanced breast cancer, bone marrow stromal cultures, chemotherapy and G-CSF, peripheral blood cell mobilisation

Introduction

Compared to conventional treatment, high-dose chemotherapy followed by transplantation of autologous peripheral blood cells (PBPC) may prolong the duration of remission in selected cases of advanced breast cancer [1–4]. Several studies have shown that sufficient numbers of PBPC for transplantation can be obtained in the recovery phase after myelosuppressive chemotherapy with or without the support of growth factors [5–10]. Moreover, PBPC mobilised into the circulation by chemotherapy and/or growth factors are more effective than bone marrow cells for early engraftment following autologous transplantation [1, 11–15]. However, although the mobilisation kinetics of developmentally restricted cells (presumed to be responsible for early marrow reconstitution) has been well documented, relatively little is known about the mobilisation into the blood stream of the more primitive cells which are presumably responsible for intermediate or long-term engraftment. Usually, the numbers of circulating colony forming cells (CFC) or CD34+ cells harvested for transplantation are used to predict the ‘quality’ of the graft and to assess the optimal timing for PBPC collection [16, 17]. However, the former assay mainly measure committed progenitor cells, while each about 10% of CD34+ are clonogenic progenitor cells, and such about 1 in 200 of CD34+ cells has the capacity to generate lasting haemopoiesis in vitro [19, 20]. Thus, these parameters may not reflect the proportion of primitive cells in the PBPC collection. To assess these more primitive cells, their ability to generate and main-
tain haemopoiesis in LTC has been used by us and others as a model system [9, 18, 19].

We have now studied the effect of single or multiple cycles of FAC chemotherapy with G-CSF support for advanced breast cancer on the release kinetics of putative stem cells, assessed by their capacity to generate long-term haemopoiesis in vitro [18], of progenitor cells in clonal assays, and of CD34+ cells. We have also compared the cell release resulting from administration of G-CSF alone either preceding or following the cessation of chemotherapy with the aim of determining the optimal conditions for PBPC harvest for transplantation.

**Patients and methods**

**Patients**

Sixteen patients with inoperable locally advanced or metastatic breast cancer were entered into a phase II study of dose escalating FAC chemotherapy with G-CSF support. The median age was 44 years (range 32–53). The patients had not received prior treatment for advanced breast cancer, or treatment with haemopoietic growth factors. The study protocol was approved by the South Manchester Ethics Committee and the patients were entered into the study after giving written informed consent.

**Treatment plan**

The design of the study is shown diagramatically in Figure 1. One aim of the study was to identify a regimen with a high-dose intensity but acceptable toxicity. The detailed clinical results have been reported elsewhere [21].

Four cycles of FAC chemotherapy (5-fluorouracil (5-FU), adriamycin (A), cyclophosphamide (C)) at four levels of escalating dose intensity were administered. At level 1, 500 mg/m^2 5-FU, 50 mg/m^2 A and 500 mg/m^2 C were administered at day 1 with an interval of three weeks between each cycle. An equal dose was administered at level 2 but with an interval of two weeks between each cycle to achieve dose intensification. Increase of chemotherapy was given at level 2 but with an interval of two weeks between each cycle. An equal dose was administered at level 3 with an interval of two weeks: 500 mg/m^2 5-FU, 75 mg/m^2 A, 1000 mg/m^2 C at day 1. Lenograstim, (metHuG-CSF) provided by Chugai-Rhone-Poulenc was given subcutaneously daily from days 2–11 of each chemotherapy cycle at a dose of 10 µg/kg. Four patients were entered into the study at each dose level. Two patients at each dose level received 10 µg/kg/day G-CSF for ten days preceding the chemotherapy (period A) while the other two received 10 µg/kg/day G-CSF for 10 days after chemotherapy (period B).

**Blood and bone marrow samples**

Blood was obtained every day during periods A and B and at days 1, 4, 7, 9, 10, 11, 12, 13 of cycles 1 and 4 of chemotherapy. Normal bone marrow was obtained, with informed consent, from allogeneic transplant donors and normal volunteers.

**Preparation of blood and bone marrow samples for in vitro assays**

Each sample was collected in 200 U/l preservative-free heparin. Blood MNC were separated using a Ficoll Hypaque gradient (density 1.077 g/ml, Pharmacia, Germany). MNC were washed twice in phosphate buffered saline supplemented with 2% fetal calf serum (FCS) and counted in a Neubauer haemocytometer.

**Clonogenic assays**

Isco's modified Dulbecco's medium (IMDM, Gibco) was supplemented with 4 x 10^{-3} M glutamine, 10^{-2} M sodium selenite, 2.5 x 10^{-4} M alpha thioglycerol, 30% pretested FCS, 1% medium conditioned by the cell line 5637 as a source of growth factors, 1% deionised bovine serum albumin (BSA) (Sigma Chemical Co.) and 2 units of recombinant erythropoietin (Epo) (Boehringer Mannheim) per ml of culture. Cells were cultured in 1.35% methylcellulose with supplemented IMDM in 24 well standard tissue culture plates (Falcon) in triplicate to a concentration of 1 x 10^5 mononuclear cells/ml. The cells were incubated at 37°C in a humified atmosphere of 5% CO_2 and 5% in O_2 in nitrogen. Colonies were counted after 14 days of incubation and assigned as previously described to granulocyte-macrophage (GM-CFC), burst-forming unit-erythroid (BFU-E), or multipotent (Mix-CFC) progenitor cells [22].

**Bone marrow stromal cultures**

Depletion of erythrocytes in the bone marrow samples was achieved by gravity sedimentation through 0.1% methylcellulose for 30–40

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**Figure 1.** The chemotherapy protocol. Chemotherapy (5-fluorouracil, adriamycin, cyclophosphamide) was administered at 4 dose levels. Level I: 500/50 500 mg/m^2/3 wk; level II: 500/50 500 mg/m^2/2 wk; level III: 500/75/500 mg/m^2/2 wk; level IV: 500/75/1000 mg/m^2/2 wk. Patients received 4 cycles at each level. Lenograstim (G-CSF) was administered from day 2–11 at a dose of 10 µg/kg/day. G-CSF before or after chemotherapy (hatched frame) — period A or B.
minutes at room temperature. Erythrocyte depleted bone marrow cells were washed once in IMDM and inoculated in 25 cm² tissue culture flasks (Falcon) to a concentration of 2 x 10⁶ cells/ml with 10 ml of medium consisting of 10% FCS, 10% horse serum (HS), 5 x 10⁻⁷ M hydrocortisone sodium succinate (Sigma), 0.5% streptomycin sulfate (Sigma), 500 U/ml benzylpenicillin (Glaxo) and single-strength IMDM (340 mOsm/kg). Flasks were gassed with 5% CO₂ in air and incubated at 33°C. The cultures were maintained by replacing weekly 1/2 of the supernatant with fresh medium. Confluent stromal layers were used after 3 to 8 weeks. They were irradiated with 15 Gy from a ¹³⁷Cs source at a dose rate of 4 Gy/min to suppress endogenous hematopoeisis. The supernatant was removed and replaced with 10 ml fresh LTBMC medium containing the test cells.

Two stage long term cultures

Blood mononuclear cells at a concentration of 1 x 10⁹ cells/ml in a volume of 10 ml LTC medium were seeded onto preformed irradiated stromal layers. Half of the supernatant and non-adherent cells were removed weekly during feeding. The cells were counted and assayed for colony forming cells as described above. The cultures were usually maintained for 8 weeks. Control cultures were seeded with equal numbers of normal bone marrow MNC.

Determination of CD34+ cells

The percentage of cells reactive with the HPCA-2 antibody was determined by immunofluorescence staining followed by flow cytometry analysis using a direct labelling procedure. Briefly, MNC were washed twice in PBS supplemented with 3% BSA and incubated with a mouse CD34 antibody conjugated to fluorescein isothiocyanate (HPCA-2-FiTC, Becton Dickenson). Non-specific isotype matched mouse antibodies conjugated to FITC were used as controls. The cells were incubated for 30 minutes at 4°C and then washed twice in PBS containing 3% BSA. CD34+ analysis was performed either immediately or after overnight storage in a 1% formaldehyde solution, on a Coulter Epics V flow cytometer (Coulter Electronics, Luton, UK) and the data saved in a list mode. Live cells were gated and 30,000 events analysed.

Statistical methods

As there were no significant differences in the results either between the patients who received the different dose intensification regimens within each cycle, or between the patients who received G-CSF before or after chemotherapy, data from all patients within a given cycle were pooled before further analysis.

Data from all cycles were compared by non-parametric (ranking) methods. Statistical significances were analysed by the Wilcoxon matched-pairs signed rank test. The results are shown as median ± range. The magnitude of GM-CFC generation in LTC was expressed by the area under the curve (AUC) over 8 weeks. The Mann-Whitney test was used for comparison of the AUC.

Results

Administration of G-CSF alone, prior and post chemotherapy

The eight patients who received 10 µg/kg G-CSF before commencing chemotherapy (period A) showed a 9.2-fold increase of the WBC count over pretreatment level. The median peak of the WBC count was 49.5 x 10⁹/l (range 35.1–70.0 x 10⁹/l). The total number of colony forming cells (CFC, comprising GM-CFC, BFU-E and Mix-CFC) released per ml of blood amounted to a 176-fold increase over pretreatment levels with a median of 5470 (Table 2). The increases for the different colony forming cell populations were 436-fold for GM-CFC and 91-fold for BFU-E. Mix-CFC, which were not detectable in the blood before treatment with G-CSF showed a median number of 56 per millilitre of blood (Tables 1 and 2). The eight patients who received G-CSF after the cessation of cycle 4 of chemotherapy

Table 1. Baseline numbers of cells per ml of blood assessed before G-CSF administration. The data are shown as median (range).

<table>
<thead>
<tr>
<th></th>
<th>G-CSF alone</th>
<th>Chemotherapy + G-CSF</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Period A</td>
<td>Period B</td>
</tr>
<tr>
<td></td>
<td>Cycle 1</td>
<td>Cycle 4</td>
</tr>
<tr>
<td>No. of patients</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>GM-CFC</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>BFU-E</td>
<td>31</td>
<td>116</td>
</tr>
<tr>
<td>MIX-CFC</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total CFC</td>
<td>31</td>
<td>52</td>
</tr>
<tr>
<td>CD34 x 10²</td>
<td>18</td>
<td>24</td>
</tr>
<tr>
<td>MNC x 10³</td>
<td>223</td>
<td>250</td>
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Table 2. Peak numbers of cells mobilised during the administration of G-CSF (lenograstim) preceding chemotherapy (period A), following the cessation of chemotherapy (period B), and during cycle 1 and 4 of FAC chemotherapy plus G-CSF. Numbers per ml of blood are shown. The data are presented as median (range).

<table>
<thead>
<tr>
<th></th>
<th>G-CSF alone</th>
<th>Chemotherapy + G-CSF</th>
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<tbody>
<tr>
<td></td>
<td>Period A</td>
<td>Period B</td>
</tr>
<tr>
<td></td>
<td>Cycle 1</td>
<td>Cycle 4</td>
</tr>
<tr>
<td>No. of patients</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>GM-CFC</td>
<td>2615 (274–12787)</td>
<td>4094 (2255–17709)</td>
</tr>
<tr>
<td>BFU-E</td>
<td>2830 (159–12787)</td>
<td>2152 (90–19866)</td>
</tr>
<tr>
<td>MIX-CFC</td>
<td>56 (8–400)</td>
<td>35 (0–1400)</td>
</tr>
<tr>
<td>Total CFC</td>
<td>5470 (1056–25669)</td>
<td>5948 (2710–38975)</td>
</tr>
<tr>
<td>CD34 x 10²</td>
<td>180 (22–1700)</td>
<td>265 (93–2800)</td>
</tr>
<tr>
<td>MNC x 10³</td>
<td>1950 (1200–4000)</td>
<td>4400 (1500–14000)</td>
</tr>
</tbody>
</table>

|                | 15          | 2462 (40–16965)  |
| Chemotherapy + G-CSF | 12          | 1199 (0–13270)   |
| Cycle 1         | 145 (0–2910) | 55 (0–572)       |
|                | 10480 (860–39282) | 4739 (40–26789) |
|                | 240 (2–770)  | 650 (3.1–2600)   |
|                | 2900 (168–5500) | 1890 (300–12000) |
chemotherapy (period B) showed a 12.7-fold increase of WBC count from pretreatment level with a median peak count of $59.8 \times 10^9/l$ (range $51.0-73.4 \times 10^9/l$). The increase of CFC was 25-fold. This relatively low-fold increase was attributable to an elevated baseline after 4 cycles of chemotherapy (Table 1), with the absolute peak numbers being comparable in periods A and B (Table 2).

The increase of mononuclear cells (MNC) during G-CSF treatment was 8.7-fold before and 23-fold after chemotherapy, respectively (Table 2).

The peak numbers of CD34+ cells were higher after cycle 4 (Tables 1 and 2).

The median day of the maximal WBC count was day 8 (range 7–10) for period A and day 9 (range 9–10) for period B (data not shown). The peak release of CFC per ml of blood during period A occurred at day 6 (median) and in period B at day 8 (Table 3). MNC peaked at day 6 and CD34+ cells at day 7 in both cycles.

**Administration of FAC chemotherapy plus G-CSF**

For the four FAC dose levels, the WBC nadirs in cycle 1 were 2.8, 1.7, 1.1 and 0.5 $\times 10^9/l$ and in cycle 4 were 3.6, 1.5, 0.85 and 0.35 $\times 10^9/l$. The WBC nadir occurred for all dose levels in cycle 1 at median day 8 (range 8–9) and in cycle 4 at day 9 (range 8–9). The WBC recovery showed a median peak of 29.9 (range 1.5–49.9 $\times 10^9/l$) in cycle 1 and 11.9 (range 2.6–42 $\times 10^9/l$) in cycle 4.

The CFC release during the recovery periods from cycles 1 and 4 of chemotherapy was first analysed separately for the eight patients who received G-CSF before cycle 1. Administration of FAC chemotherapy plus G-CSF alone and period B (G-CSF pre- and post-chemotherapy) with chemotherapy + G-CSF showed a higher CD34+ cell release in cycle 4 compared to cycle 1. This difference between cycle 1 and 4 was significant (Wilcoxon test $P = 0.045$).

The peak of WBC count occurred at median day 12 for both cycle 1 (range 10–13) and 4 (range 12–13).

The day of maximal CFC release was delayed by one day in cycle 4 compared to cycle 1 (Table 3). In cycle 4, however, 8 patients demonstrated a peak at day 13, compared to only 1 patient in cycle 1, 3 patients at day 12 and 1 patient at day 11. Since no further assessment of progenitor cells was performed after day 13 of cycle 4, it is possible that the number of CFC could have increased further at later times for 8 of 12 patients, and that the true median peak day is >13 although G-CSF was only given until day 11 (Table 3).

**Comparison of total numbers of CFC during period A and period B (G-CSF pre- and post-chemotherapy) with cycle 1 and cycle 4**

The numbers of CFC released per ml of blood over time are shown in Figure 2. These differences in the numbers of CFC between period A, period B, cycle 1 and cycle 4 were not statistically significant. (Wilcoxon test $P = 0.18$).

**Haemopoiesis in two stage long term cultures (LTC)**

The generation of GM-CFC in LTCs initiated with MNC from the period of peak (± 2 days) CFC release during period A and cycle 1 were not significantly different from that of normal bone marrow MNC but those for cycle 4 and period B were (Figure 3). These
results calculated as the area under the curve (AUC) are shown in Figure 4.

Cultures initiated with MNC collected 3–5 days after the median peak of CFC release in periods A or B generated low CFC numbers as well as MNC obtained 3–4 days before the median peak of CFC release in cycle 1 and 4 of chemotherapy (data not shown).

Figure 2. CFC (GM-CFC, BFU-E, Mix-CFC) released after cycle 1 or cycle 4 of FAC chemotherapy and during the administration of G-CSF alone either before commencing FAC chemotherapy (period A) or after its cessation (period B). The curves of individual patients are shown. The median is shown in bold, respectively.

Figure 3. Haemopoiesis over 8 weeks (GM-CFC and nucleated cells) in two stage long-term culture (LTC). Blood MNC were obtained within 2 days of the peak release of CFC into the circulation and were seeded at 10^6 cells per ml onto irradiated bone marrow stroma. G-CSF before chemotherapy (period A, n = 8): open squares, cycle 1 (n = 12): closed squares, cycle 4 (n = 11): open triangles, G-CSF after the cessation of chemotherapy (period B, n = 7): open diamond, normal bone marrow (n = 8): closed triangle. The data are shown as median. The interquartile range is shown for normal bone marrow.

Figure 4. Median values for area under the curve (AUC) for the data shown in Figure 3. The values represent GM-CFC measured over 8 weeks of LTC shown as median AUC.

Discussion

We have compared the effect of G-CSF with or without FAC chemotherapy for advanced breast cancer on the release both of progenitor cells (CFC) and of the more immature cells that generate them and maintain haemopoiesis in LTC.

At days of maximal CFC release during treatment with G-CSF alone, before commencing FAC chemotherapy (period A), the capacity of PBPC to generate haemopoiesis in vitro, was broadly similar to that of normal bone marrow [23]. This in vitro observation has recently been confirmed by successful autologous and allogeneic transplantation of PBPC mobilised by G-CSF [24–26].

In agreement with others [10, 11, 27] and with our previous work [9, 28], we have found that myelosuppressive chemotherapy in combination with G-CSF is a potent stimulus for the release of progenitor cells from the bone marrow to the blood. In cycle 1 of chemotherapy, the total peak number of CFC was almost twice as high as that obtained after G-CSF alone. In both cases, the increase of GM-CFC over pretreatment level was more than 400-fold, suggesting that a single apheresis would collect sufficient PBPC for autologous transplantation. Indeed, following high-dose consolidation chemotherapy, 3 patients in the series reported here have been transplanted successfully with MNC collected during a single apheresis [29].

Although there is general agreement in the literature with respect to the numbers of CFC considered save to achieve haemopoietic regeneration following PBPC transplantation, few data exist on more primitive cell types [9, 18, 19]. Long-term culture initiating cells (LTC-IC), a primitive cell type which supports haemopoiesis in LTC after 5 weeks [19], are found in normal blood in small, but detectable numbers [30]. After treatment with chemotherapy and/or growth factors, an increase over normal levels has been reported [19, 20]. Different degrees of mobilisation between LTC-IC and CFC numbers have been found: cyclophospha-
mid, with or without growth factors, increased the mobilisation of CFC 26-fold, but that of LTC-IC only 6-fold over normal levels [19].

However, other regimes can achieve a similar degree of mobilisation of CFC and LTC-IC [31]. Apart from the capacity to mobilise cells into peripheral blood the cytotoxic effects of chemotherapy need to be considered when cumulative doses of chemotherapy in HDCT are administered. Cyclophosphamide, for example, does not appreciably damage the stem cell compartment in mice after a single administration [32] but does cause significant damage after repeated cycles [33, 34].

An effect of previous chemotherapy on the mobilisation of progenitor cells has been reported [35]. Similarly, we found that after the first cycle of FAC chemotherapy, LTC-IC collected at the peak of CFC release were only slightly reduced compared to period A, but after 4 cycles of chemotherapy the reduction was 6-fold (Figure 4). However, the peak numbers of CFC per ml of blood were only decreased about 2-fold. Moreover, while a similar peak in the numbers of CFC mobilised was seen either after the administration of G-CSF following 4 cycles of FAC chemotherapy (period B) than following G-CSF administered pre-chemotherapy (period A), there was a 5-fold lower AUC in LTC, reflecting the poorer mobilisation of the cells with the capacity to repopulate LTC compared to CFC. It should be noted that we have not performed limiting dilution assays. However, the data in Figure 3 indicate that the maximum difference found in the generation of GM-CFC in the LTC is found between weeks 5 and 8, the times classically used for determination of LTC-IC.

The enumeration of CD34+ cells has been proposed to be a favourable indicator for estimating the 'quality' of a PBPC collection [16, 17]. Recently, Sutherland et al. showed a significant correlation between CD34+ cells and CFC but not between CD34+ cells and LTC-IC in PBPC harvest [19]. In our study, the release of CD34+ cells was significantly higher in cycle 4 than in cycle 1. This did not follow the pattern of either CFC or of LTC-IC. The CD34+ cells are a heterogeneous population, with the CD34 antigen present on both progenitor cells and more immature, putative stem cells [36], as well as on accessory cells [37–39]. It is not known if different treatment protocols (chemotherapy plus growth factors) change the composition of the CD34+ cell subpopulations released into the circulation. To address this question, the assessment of additional surface markers, and functional assessment need to be performed [19, 35].

In conclusion, we have shown differences in the kinetics of mobilisation of CFC and LTC-IC during single or multiple cycles of FAC chemotherapy supported by G-CSF. After repeated cycles a significant but small decrease in CFC was accompanied by a much more marked decline in LTC-IC. In contrast, the number of CD34+ cells increased significantly over this period. This suggests that caution should be applied in assuming that the numbers of CFC are a true reflection of the quality of the graft in any treatment regimen and also questions the reliability of CD34 estimation alone in predicting the optimal time for PBPC collection (particularly if only a single apheresis is collected and the graft is used after myeloablation). Furthermore, the lack of synchronisation in the release of colony forming cells and of LTC-IC after repeated intensive FAC chemotherapy, and the discrepancy with the release of CD34+ cells, underline the need to extend the kinetic studies for different chemotherapeutic regimens in order to determine the timing for optimal harvests for the cell populations required for different clinical needs, when aiming for example for fast regeneration after cytotoxic treatment, or for reestablishment of haemopoiesis from the graft after myeloablation. We found the preferential time for PBPC harvest with respect to CFC and in vitro (vivo?) marrow repopulating cells to be either during G-CSF administration before FAC chemotherapy, or in the recovery period after the first cycle of FAC chemotherapy. However, consideration of the possible tumor cell contamination is relevant in this context.

Acknowledgements

We thank Chugai-Rhone-Poulenc for the supply of lenograstim and the support of the study and the research nurses A. Griffiths, N. Whelan, J. Kiernan for excellent support.

Supported by the Cancer Research Campaign. I. Baumann is a recipient of a Dr. Mildred Scheel Stiftung grant. T. M. Dexter is a Gibb Research Fellow of the Cancer Research Campaign.

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