*Featured Article*

# Circulating Tumor Cells in Patients with Breast Cancer Dormancy

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## ABSTRACT

***Purpose:*** **The purpose of this study was to test the hypothesis that circulating tumor cells (CTCs) are present in patients many years after mastectomy without evidence of disease and that these CTCs are shed from persisting tumor in patients with breast cancer dormancy.**

***Experimental Design:*** **We searched for CTCs in 36 dormancy candidate patients and 26 age-matched controls using stringent criteria for cytomorphology, immunophenotype, and aneusomy.**

***Results:*** **Thirteen of 36 dormancy candidates, 7 to 22 years after mastectomy and without evidence of clinical disease, had CTCs, usually on more than one occasion. Only 1 of 26 controls had a possible CTC (no aneusomy). The statistical difference of these two distributions was significant (exact $P = 0.0043$). The CTCs in patients whose primary breast cancer was just removed had a half-life measured in 1 to 2.4 hours.**

***Conclusions:*** **The CTCs that are dying must be replenished every few hours by replicating tumor cells somewhere in the tissues. Hence, there appears to be a balance between tumor replication and cell death for as long as 22 years in dormancy candidates. We conclude that this is one mechanism underlying tumor dormancy.**

## INTRODUCTION

Recurrence of tumor can occur a prolonged time after removal of the primary tumor. These very long intervals that some patients have before recurrence are not consistent with constant kinetic growth of tumor cells, so there must be a dormant state in the tumor cell population. Short-term presence of circulating tumor cells (CTCs) or marrow tumor cells may represent residual disease and is known to be associated with a higher risk of recurrence (1). However, CTCs in patients in long remissions who are most likely cured of disease (dormancy candidates) could represent an altogether different process and may provide important insights into mechanisms of tumor control. Our objective was to determine whether very sensitive techniques could detect these tumor cells in such a population of patients whose risk of recurrence at this point in time is minimal and to further characterize these tumor cells. Experimental tumor dormancy has been created by immunization (2–7), angiogenic inhibitors (8–17), hormonal deprivation (18–20), and altering signaling pathways by a variety of methods (21–27). However, it is unclear which, if any, of these models applies to human cancer dormancy.

We have studied a murine lymphoma ($BCL_1$) model in which mice immunized against the idiotype of $BCL_1$ were then challenged with $BCL_1$ and developed a state of dormancy (7, 22, 23, 28, 29). Despite the continued replication of tumor cells, each mouse that remained dormant had approximately $10^6$ $BCL_1$ cells in its spleen for the 430 days of observation (22, 30). Relapses occurred randomly, as in breast cancer 10 to 20 years after mastectomy (31, 32). We concluded that in the mice with a dormant tumor population, replication was balanced by cell death. We therefore hypothesized that in human dormancy candidates, the tumor cell population dynamics might be similar.

We have developed a sensitive and specific assay to identify and characterize CTCs on slides. CTCs are detectable in many patients at the time of a primary breast cancer and in almost all patients who have a recurrence and are not yet undergoing treatment (33–35). To test this hypothesis, we used this assay to detect CTCs in patients with prolonged freedom from recurrence after early-stage breast cancer who are at low risk of recurrence. These patients are thereby candidates for breast cancer dormancy.

## MATERIALS AND METHODS

### Patient Selection and Data Recording

Breast cancer patients with no clinical evidence of disease for 7 or more years after mastectomy and normal women age-

Received 6/7/04; revised 8/27/04; accepted 9/15/04.
**Grant support:** Nasher Cancer Research Program.
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**Note:** J. Uhr holds stock in Immunicon Corporation.
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matched by decades were studied. For half-life studies, we recruited five patients who were having a primary breast tumor removed that was at least 2 cm in diameter, without gross metastases. All specimens were obtained after informed consent and collected using protocols approved by the Institutional Review Board at the University of Texas Southwestern Medical Center (Dallas, TX).

### Collection of Samples

Thirty milliliters of blood were drawn in EDTA vacutainer tubes (BD Biosciences, San Diego, CA) from patients and controls. For the half-life studies, 40 to 60 mL were drawn at each time point. The samples were processed within 1 to 2 hours of collection.

### Cell Lines

Carcinoma cell lines SKBr3 (breast), Colo 205 (colorectal) and PC3 (prostate), maintained in RPMI 1640 plus 10% fetal calf serum, were used to evaluate new batches of antibody and antibody-fluorochrome conjugates for potency and specificity and as positive or negative control cells.

### Antibodies

**Flow Cytometry.** Antibodies used for flow cytometry were as follows: anti-pancytokeratin C11-phycoerythrin from Immunicon Corp. (Huntingdon Valley, PA), which recognizes cyokeratin (CK) 4, 5, 6, 8, 10, 13, and 18; and anti-CD45-PerCP (clone 2D1; BDIS, San Jose, CA), which recognizes white bloods cells.

**Slides.** Monoclonal mouse anti-pancytokeratin C11-fluorescein isothiocyanate (Sigma, St. Louis, MO); a $F(ab')_2$ fragment prepared from a rabbit anti-mammaglobin (36, 37) and conjugated to AlexaFluor 594 (Molecular Probes, Eugene OR); and monoclonal mouse anti-CD45 (clone 9.4; American Type Culture Collection, Manassas, VA) grown, purified, and conjugated to AlexaFluor 546 (Molecular Probes) in our laboratory recognized white bloods cells. Control antibodies were mouse monoclonal isotype-matched antibodies of irrelevant specificities and a $F(ab')_2$ fragment of normal rabbit IgG.

### Ferrofluids for Circulating Tumor Cell Enrichment

The method used to isolate CTCs is similar to that used by others (38–47). CTCs were immunomagnetically enriched with ferrofluid (44, 48) conjugated to antibody against EpCAM [specific for epithelial cells (49, 50)]. The anti-EpCAM antibody GA73.3 (provided by Dr. Dorothee Herlyn, Wistar Institute, Philadelphia, PA) was used to make slide preparations. For flow cytometry, anti-EpCAM VU1D9 attached to Immunicon Corp. ferrofluid was used.

### Isolation of Circulating Tumor Cells

**Flow Cytometry for Half-Life Studies.** The collected blood was pooled, and 7.5-mL aliquots were distributed into 15-mL conical centrifuge tubes and processed as described previously (44), except that the residue was resuspended in 500 μL of CellFix (Immunicon Corp.) and 10 μL of nucleic acid dye (thioflavin from Sigma), and 10 μL containing 10,000 fluorospheres (Flow Set Fluorospheres; Coulter, Miami, FL) were added. Samples were collected and analyzed as described previously (33).

**Slide Preparation.** Blood was processed as described previously (34), except that the wash buffer consisted of PBS containing 0.5% bovine serum albumin and 2 mmol/L EDTA, and the cells were not permeabilized. If more than one aliquot of blood was being processed, up to four tubes were combined. Samples were transferred to a 15-mL conical tube, 10 mL of washing buffer were added, and the tubes were centrifuged at $300 \times g$ for 10 minutes. The supernatant was aspirated and resuspended in 100 μL of PBS per 5 mL of blood; 100 μL were placed on each slide and air dried at 37°C. After fixing for 10′ in acetone, slides were stored at −80°C until needed.

### Immunofluorescent Staining

Immunofluorescent staining was carried out as described previously (34). Blood slides from healthy individuals of similar ages served as negative controls, and SKBr3 breast carcinoma cells served as positive controls in the staining experiments.

### Screening for Circulating Tumor Cells

Screening for CTCs was performed as described previously (34). In this study, the immunophenotypic definition of a CTC as $CD45^-$, $CK^+$ was used. The expression of the breast tissue-specific antibody mammaglobin was also evaluated at this time. The location of each candidate cell was recorded and stored. Slides from normal donors and patients were coded so that investigators were "blinded."

### Fluorescence *In situ* Hybridization Procedure

Pretreatment and denaturation of slides have been described in detail elsewhere (34). Chromosome enumerator probes (CEPs) for repetitive sequence regions of chromosome 1 (satellite II/III), α-satellites 3, 8, 11 and 17 were kindly provided by Vysis, Inc. (Downers Grove, IL) and used for tricolor combination (CEP 1, 17, and 8, SpectrumOrange, SpectrumGreen and SpectrumAqua, respectively). Dual-color combination (CEP 3 and 11, SpectrumGreen and SpectrumAqua or SpectrumOrange and SpectrumGreen) was used for reprobing. Hybridization and posthybridization washes were performed according to the manufacturer's instructions. Slides were counterstained and prepared with mounting media containing 4′,6-diamidino-2-phenylindole. Leukocytes from patients served as controls. Reprobing was performed as described previously (34).

### Analysis of Fluorescence *In situ* Hybridization Results

Hybridized cells were relocated with the same fluorescence microscope used for scanning. Hybridization signals in recorded cells were enumerated separately for each CEP through the appropriate single-pass filter.

### Criteria for Classification of a Blood Cell as a Circulating Tumor Cell

The criteria for classification of a blood cell as a circulating tumor cell are outlined in detail in Table 1. Fig. 1*A* shows the cytomorphology, immunophenotype, and fluorescence *in situ* hybridization (FISH) results of representative CTCs.

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*Table 1* Criteria for diagnosis of CTCs

| General Principles | Cytomorphology | Immunophenotype | FISH |
|---|---|---|---|
| 1. "Blinded" studies. All individuals involved in processing and interpreting blood samples have no information about the patient. | 1. High nuclear to cytoplasmic ratio | 1. $CK^+$ and/or mammaglobin$^+$, nucleic acid$^+$, $CD45^-$ | 1. 50 WBCs on same slide have easily readable signals. |
| 2. For all monoclonal antibodies used, there are isotype-matched controls (for polyclonal antibodies, a species control) to evaluate nonspecific staining. | 2. Larger than WBC | 2. CK stains periphery of cells. | 2. There is no more than 1 WBC in 50 with amplification of a single chromosome. |
| | 3. Nucleus is usually granular or stippled. | 3. CK may stain web over nucleus (cytoskeleton). | 3. There are no more than 2 WBCs in 50 with loss of a single chromosome. |
| | | 4. CK and mammaglobin staining pattern are *not* identical. | 4. Aneusomy of the CTCs is a requirement: 2 CEPs amplified in 1 CTC; 1 CEP amplified to ≥4 in 1 CTC; 1 CEP amplified in 2 CTCs; loss of same CEP in 2 CTCs in 1 or repeat blood samples; using CEP 1, 8, and 17 and, in some patients, also CEP 3 and 11 |

### Statistical Methods

To determine that the CTCs observed in these studies were restricted to dormancy candidates, we used the two-sample binomial proportion test [StatXact-5 version 5.0.3, statistical software for exact nonparametric inference (Cambridge, MA)] to compare 1 CTC in the 26 controls to 13 patients with CTCs in the 36 dormancy candidates.

## RESULTS

**Criteria for Classification of a Cell as a Circulating Tumor Cell.** Because the patients in the present study were clinically cancer-free and at low risk for recurrence, classification of a cell in the blood as a malignant tumor cell had to be unambiguous. A cell had to meet *all* the criteria listed in Table 1 for it to be classified as a CTC. Fig. 1*A* shows CTCs from dormancy candidates that fulfilled all of the criteria, including typical cytomorphology of a large cell with a very high nuclear to cytoplasmic ratio and virtually indistinguishable from CTCs of metastatic patients and tumor cells from breast cancer cell line SKBr3. There were three immunophenotypic patterns in the tumor cells: $CK^+$ mammaglobin$^+$ was the dominant one; $CK^+$ mammaglobin$^-$ was infrequent; and $CK^-$ mammaglobin$^+$ was rare. The CTCs from disease-free patients 7 or more years after mastectomy (dormancy candidates) appeared smaller than the other tumor cells. Therefore, the CTCs from 13 dormancy candidates (23 cells), 9 patients with metastatic breast cancer (50 cells), 5 patients with primary breast cancer (50 cells), SKBr3 breast tumor cell line (50 cells), and white bloods cells (50 cells) were measured for cell (cytoplasmic) and nuclear "size." Diameters (average of two diameters at 90-degree angles; in micrometers) were determined for cytoplasm and nucleus. The results can be summarized, as follows: (*a*) The nuclear to cytoplasmic ratio (nuclear size/cytoplasmic size) of CTCs from all of the tumor cells had a mean value of 0.8 with a SD of 0.1, which was significantly different ($P < 0.0001$) from that of white bloods cells (mean, 0.55; SD, 0.05). This result is consistent with the importance of this criterion for interpreting the cytomorphology of circulating cells. (*b*) The mean cell size of CTCs in dormancy candidates was 29.8 (SD, 6.5) compared with a mean of 33.9 (SD, 8.3) in patients with metastatic disease and mean of 32.0 (SD, 5.8) in patients with primary tumors. The smaller size of the CTCs in the dormancy candidates compared with the other two groups was significantly different for both patients with metastatic disease and patients with primary tumors ($P = 0.0047$ for metastatic CTCs and $P = 0.047$ for primary tumors, based on one-sided Student's *t* test). This, together with the DNA disruption as observed by the microscopist, suggests but does *not* prove that the CTCs in dormancy candidates may represent advanced apoptosis.

CD45 is uniquely associated with white bloods cells. There was modest nonspecific binding of anti-CK and anti-mammaglobin to some $CD45^+$ white bloods cells; therefore, *any* cell that was $CD45^+$ was not counted as a CTC.

The control group consisted of randomly recruited women age-matched by decade who came to our Komen Breast Cancer Center for their annual mammogram. We drew the blood samples after the mammogram was read as normal. This served the additional purpose of controlling for any breast cells that might enter the circulation due to the trauma of the procedure. In fact, no such cells were found in the control group.

Of 26 normal age-matched controls, there was one cell that had the cytomorphology and immunophenotype of a CTC, but was *not* aneusomic for CEP 1, 8, or 17. Nevertheless, we counted such a cell as a false positive.

Initially, small blood volume equivalents were studied, and tricolor FISH was performed with CEP 1, 8, and 17. Later in the study, larger blood volumes were analyzed to increase the number of CTCs per patient. In some patients, reprobing was

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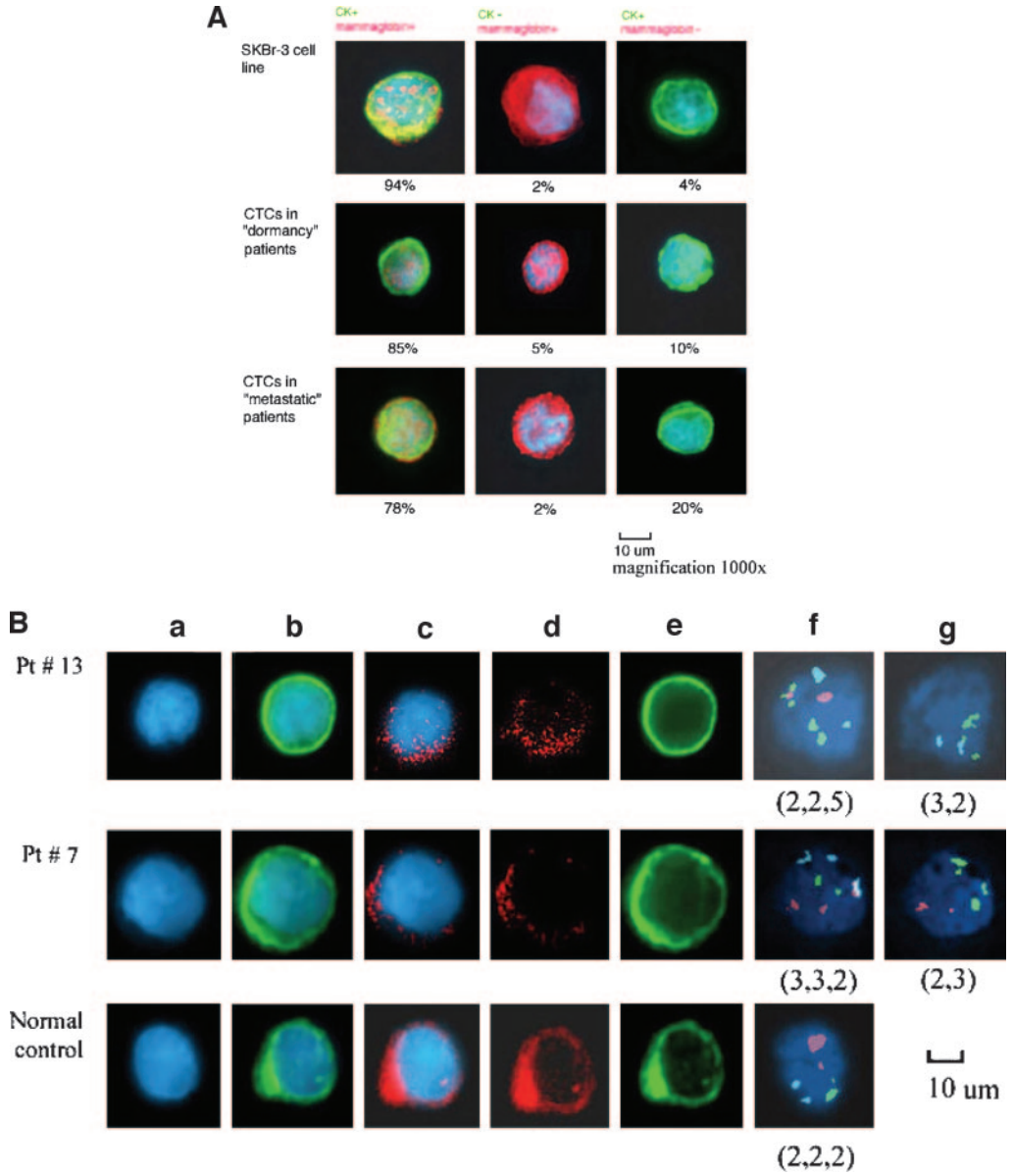


*Fig. 1* *A*, three different immunophenotypes of CTCs from patients with breast cancer dormancy, metastatic disease, and a breast cancer cell line. Tumor cells were stained with anti–CK-fluorescein isothiocyanate (*green*) and anti–mammaglobin-AlexaFluor 594 (*red*). A *yellow color* is produced when the *red* and *green staining* overlap. Nuclei are stained *blue* with 4′,6-diamidino-2-phenylindole. The percentage of each pattern is shown *below* the immunofluorescence photo (SKBr3, $n = 100$; dormancy candidate CTCs, $n = 40$; metastatic CTCs, $n = 100$). Two epidermal cells are also shown. Mammaglobin does not stain all breast cancer cells, even in cell lines such as SKBr3, shown above. CK staining is less variable. There was a statistical difference by *t* test in cell size between dormancy candidate CTCs from 22 patients (CTCs = 42) and metastatic CTCs from 9 patients (CTCs, $n = 50$; $P = 0.0095$) and SKBr3 cells ($n = 50$ cells; $P = 0.00015$). There was no difference in cell size between metastatic CTCs and SKBr3 cells ($P = 0.396$). *B*, decomposing the immunophenotype and displaying aneuploidy of CTCs from each of two dormancy patients and one non-CTC from a normal control. Single band filters were used to block out the fluorescence of one or more fluorochromes used to stain for CTCs. Columns (*a*–*g*) show cells staining for nucleic acid only (*a*), nucleic acid and CK (*b*), nucleic acid and mammaglobin (*c*), mammaglobin only (*d*), CK only (*e*), FISH analysis for CEP (CEP 1, 8, and 17; *f*) and (CEP 3 and 11; *g*). The *top two rows* show classical CTCs. Note that mammaglobin and CK stain different portions of the CTC. The *third row* shows identical staining between mammaglobin and CK on the non-CTC cell. This is nonspecific staining and is seen in an occasional cell in both dormancy patients and normal controls. Such a cell can display aneusomy. For hybridization for FISH, CEP 1 (SpectrumOrange), CEP 8 (SpectrumAqua), and CEP 17 (SpectrumGreen) were used (*f*), and reprobing was performed with CEP 3 (SpectrumOrange or SpectrumGreen) and CEP 11 (SpectrumGreen or SpectrumAqua; *g*). Under each FISH panel, the chromosome copy numbers are shown. Note that the photos are taken in only one Z-plane, whereas the microscopist can focus on the entire Z-plane. Hence, spots more than 1 signal diameter apart can be distinguished more accurately by microscopy; otherwise, the spots are counted as 1 copy number. Spots that are not seen in the Z-plane of the photo can also be detected by microscopy.

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performed with CEP 3 and 11 (a total of five probes) to increase the probability of demonstrating aneuploidy.

Fig. 1*B* shows a "decomposition" of the immunophenotype for two CTCs and a mammaglobin$^+$ CK$^+$ cell that is *not* a CTC in the normal control. Two patients, each with at least one CTC that met all of the criteria in Table 1, are shown to indicate that each CTC has classical cytomorphology, immunophenotype, and sufficient aneusomy.

Statistical analysis indicates that the cells designated as CTCs are restricted to the dormancy candidates. We found the two distributions of CTCs in controls and dormancy candidates to be significantly different (exact $P = 0.0043$). In contrast, adherence to the criteria may fail to detect a proportion of CTCs, as will be discussed later. Our conclusion from all of the above studies is that CTCs in candidates for cancer dormancy are malignant breast cancer cells derived from metastases. This is consistent with earlier observations that CTCs obtained at the same time as removal of the primary malignant breast tumor are derived from the primary tumor. This was deduced from the similarity of the aneusomic pattern between clones in the primary tumor and the CTCs (34).

**Detection of Circulating Tumor Cells in Dormancy Candidates.** The results of the examination of CTCs in dormancy candidates are shown in Tables 2 and 3. As can be seen in Table 2, 36 patients who were at ≥7 years post-mastectomy were examined one or more times for CTCs, and 13 had CTCs in their blood on at least one occasion. Of seven patients who had CTCs in the first blood sample, five had one or more CTCs in the second sample.[11] Of the six patients who had no CTCs in the first blood sample, two had CTCs in the second sample. None of the 13 CTC-positive dormancy candidates had any clinical evidence of recurrence. The incidence of recurrence in dormancy candidates 8 to 20 years after mastectomy is about 1% per year (31, 32). Our finding that 36% (95% confidence interval, 21–54%) of 36 dormancy candidates have CTCs without clinical evidence of disease indicates that virtually all of these patients were in a dormant state ($P < 0.0001$). A significant proportion of the patients with CTCs would not be expected to experience a recurrence during their lifetime.

The clinicopathological features of the primary tumor might be different between the dormancy candidate with detectable CTCs and those without detectable CTCs. However, we were unable to obtain all of the pertinent information on many of the patients because of the long time interval that had elapsed after removal of the primary tumor and the fact that in many cases the primary tumor was removed at other hospitals. We compared the age at removal, years post-mastectomy, stage, and pathology of the 13 dormancy candidates who had CTCs and the 23 candidates who did not show evidence of CTCs using the multiple logistic regression approach. None of these variables was found to be significant in distinguishing these two groups. The staging criterion has changed many times during the last two decades, and the particular criteria used are not available for most of these patients. We found that the age at primary surgery was slightly higher in dormancy candidates without CTCs compared with the dormancy candidates with CTCs (57.4 *versus* 52.2 years); however, this difference is not statistically significant.

Table 3 shows the pattern of aneusomy in every dormancy candidate who had a CTC. All of the patients had 1 to 4 CTCs that met all of the criteria. The concentration of CTCs appears very low, but in fact, it is similar to the range of what is found in very small primary breast tumors (≤1 cm). Measuring CTCs by examination of slides involves far more loss of CTCs than quantifying events representing CTCs by flow cytometry. Seven initially CTC-positive patients had a second blood sample examined from 3 to 9.5 months later. Six patients continued to have CTCs. Some CTCs in both samples had the same pattern of aneusomy, but there were many new patterns in the CTCs of the second sample. This was not unexpected because of the enormous heterogeneity of aneusomy in breast cancer (53) and the constant replication of the tumor cells with a high rate of mutation that give rise to the CTCs (four of these six patients gave a third blood sample 7.8–20.1 months after their first one). In patient 8, we obtained a third blood sample of 50 mL to determine whether we could increase the number of CTCs for future studies. The sample contained 4 CTCs (see the footnotes in Table 3).

**Half-Life of Circulating Tumor Cells.** Because of the apparent balance between proliferation and cell death, an evaluation of the half-life of CTCs was performed. Five patients (age, 40–85 years) were recruited with a primary breast carcinoma of 2.5 to 10 cm in diameter without gross metastases. All were ductal or lobular carcinomas, $T_2$ to $T_4$. We examined the number of events corresponding to CTCs in blood samples taken from these patients either just before or immediately after removal of the primary tumor and at intervals thereafter. Only a rough estimate can be obtained because the CTCs in dormancy candidates may have a different half-life than those shed from a primary tumor. Also, in contrast to examining CTCs on slides, flow cytometry has a fluctuating background level of events in normal samples. As shown in Fig. 2, we used one-compartment and two-compartment models for describing the decay of CTCs. Both models gave a good fit for the number of CTCs per 10 mL of sample ($P < 0.0001$). Based on the two-compartment model, we estimate that the half-life of CTCs is 1 hour, whereas based on the one-compartment model, the estimate is 2.4 hours. By 24 hours, the number of events that could be CTCs had reached background levels in *every* patient. This is consistent with the statistical analyses. Because of the aforementioned caveat, it can only be concluded that the half-life is very short, probably measured in hours.

## DISCUSSION

The objective of the present studies was to determine whether patients who are candidates for breast cancer dormancy have tumor cells in their blood. Cancer cells have not previously been described in patients with remote primary cancer who are at very low risk of recurrence.

The major findings to emerge from this study are, as follows: (*a*) Circulating breast carcinoma cells have been identified in patients who are candidates for tumor dormancy at a

[11] Because these patients believe they are cured of cancer, it is difficult to obtain repeated blood samples without causing unacceptable anxiety. Bone marrow aspirations are unacceptable to our institutional review board, and magnetic resonance imaging can only be justified if the CTC count is rising or if there is other evidence that a recurrence is imminent.

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*Table 2* Dormancy candidates: CTCs and tumor characteristics

| Patient no./Age at removal (y) | Years post-mastectomy* | No. of CTCs | Blood volume (mL) | Tumor-Node-Metastasis | Stage† | Pathology | Biology of tumor |
|---|---|---|---|---|---|---|---|
| CTC-positive patients | | | | | | | |
| 1/40 | 13 | 2 | 12.5 | $T_{is}N_0M_0$ | 0 | High-grade DCIS‡ | ER+, PR+, S phase 3.9% |
| 2/59 | 18 | 1 | 12.5 | $T_{1c}N_0M_0$ | I | IDC | ER+, PR− |
| 3/61 | 14 | 1 | 25 | NA | I | NA | NA |
| 4/51 | 8 | 2 | 25 | $T_{1a}N_0M_0$ | I | ILC, IDC | ER+, PR+, aneuploid |
| 5/63 | 8 | 1 | 1 | $T_{1b}N_0M_0$ | I | IDC | ER−, PR−, S phase 3.5%, diploid |
| 6/40 | 14 | 2 | 12.5 | $T_1N_1M_0$ | IIA | IDC | Probably ER+, PR+§ |
| 7/48 | 22 | 1 | 12.5 | $T_2N_0M_0$ | IIA | IDC | ER−, PR− |
| 8/46 | 13 | 2 | 13 | $T_{1c}N_1M_0$ | IIA | ILC | ER+, PR+ |
| 9/46.5 | 15.5 | 1 | 15 | $T_2N_0M_0$ | IIA | IDC | ER−, PR−, S phase 7.7%, aneuploid |
| 10/57 | 18 | 2 | 25 | $T_{1c}N_0M_0$ | IIA | ILC | 1/17 nodes+, no ER/PR done |
| 11/55.5 | 13.5 | 2 | 25 | $T_{1c}N_0M_0$ | IIA | IDC | ER+, PR+, S phase 0.5%, diploid, 1/33 nodes+ |
| 12/62 | 9 | 1 | 12.5 | $T_2N_1M_0$ | IIB | ILC | ER+, PR+, Her2/neu−, S phase 4% |
| 13/50 | 15 | 2 | 12.5 | $T_3N_1M_0$ | IIIA | IDC | ER+, PR+, aneuploid, 2/28 nodes+ |
| CTC-negative patients | | | | | | | |
| 14/36 | 7 | 0 | 15 | $T_{1c}N_0M_0$ | I | MC | ER+, PR+, Her2/neu− |
| 15/60 | 10 | 0 | 25 | NA | IIA | IDC | NA |
| 16/42 | 18.5 | 0 | 15 | $T_2N_0M_0$ | IIB | ILC | ER+, PR+ |
| 17/65 | 16 | 0 | 15 | $T_{1c}N_0M_0$ | IIB | IDC | ER+, PR+, S phase 4.3%, diploid, 2/16 nodes+ |
| 18/79 | 13 | 0 | 15 | NA | NA | IDC | Not available |
| 19/76 | 8 | 0 | 12.5 | $T_{1b}N_0M_0$(R) | I | IDC | ER+, PR+ |
| 20/62 | 12 | 0 | 12.5 | $T_{1b}N_0M_0$ | I | IDC | ER+, PR+, S phase 2.4%, diploid |
| 21/67 | 8 | 0 | 12.5 | $T_1N_0M_0$ | I | IDC | ER +, PR + |
| 22/45 | 18 | 0 | 12.5 | $T_{1c}N_0M_0$ | I | IDC | ER+, PR+ |
| 23/46 | 8.5 | 0 | 10 | $T_1N_0M_0$ | I | IDC | ER−, PR− |
| 24/45 | 20 | 0 | 10 | $T_{1b}N_0M_0$ | I | IDC | ER, PR not done |
| 25/53 | 9 | 0 | 10 | $T_1N_0M_0$ | I | IDC | Probably ER+, PR+§ |
| 26/54 | 8 | 0 | 10 | $T_{1b}N_0M_0$ | I | IDC | ER−, PR−, tetraploid |
| 27/50 | 10 | 0 | 15 | $T_{1b}N_0M_0$ | I | IDC | ER+, PR+ |
| 28/52 | 19 | 0 | 15 | $T_{1b}N_0M_0$ | I | IDC | ER+, PR+ |
| 29/47 | 12 | 0 | 12.5 | $T_{1b}N_1M_0$ | IIA | IDC | ER−, PR−, S phase 11.3%, aneuploid, 1/19 nodes+ |
| 30/76 | 7 | 0 | 12.5 | $T_1N_1M_0$ | IIA | IDC | ER+, PR+, Her2/neu−, S phase 6%, diploid, 1/24 nodes+ |
| 31/67 | 16.5 | 0 | 15 | $T_{1c}N_0M_0$ | IIA | IDC | ER+, PR+ |
| 32/29 | 19 | 0 | 10 | $T_2N_0M_0$ | IIA | IDC | ER−, PR+ |
| 33/53 | 20 | 0 | 15 | $T_1N_0M_0$ (R) | IIA | IDC | ER, PR not done |
| 34/71 | 14 | 0 | 12.5 | $T_2N_1M_0$ | IIB | IDC | ER+, PR+ |
| 35/68 | 18 | 0 | 12.5 | NA | NA | NA | NA |
| 36/72 | 7 | 0 | 10 | NA | NA | IDC | NA |

Abbreviations: IDC, infiltrating ductal carcinoma; DCIS, intraductal carcinoma *in situ*; ILC, infiltrating lobular carcinoma; ER, estrogen receptor; PR, progesterone receptor; NA, not available; MC, mucinous carcinoma.

* We have based our studies on years after mastectomy because all studies in the past have used mastectomy as the beginning time point for defining dormancy. However, patients in this study, like others reported in the literature, have received appropriate adjuvant therapy.

† The difference in recurrence risk based on stage and grade diminishes over time. In other words, the year-to-year risk at 10 years out is very low in patients who initially were at either high or low risk. Patients at higher risk tend to have recurrence earlier, and the annual hazard of high-risk and low-risk groups tends to merge at low yearly levels as time goes on (51).

‡ Those oncologists on our staff who have had patients diagnosed with ductal carcinoma *in situ* who later developed recurrent breast cancer uniformly believe that areas of invasiveness were missed by the pathologist. Many of these patients had their tumors removed at small hospitals in rural areas many years ago, which may contribute to the discrepancies. Furthermore, involvement of axillary lymph nodes in up to 13% of patients with ductal carcinoma *in situ* underscores the fact that invasive cells can exist in small foci that evade detection even with contemporary pathological analysis (52). Finally, the one case with ductal carcinoma *in situ* found to have CTCs in our series was of high nuclear grade and therefore more likely to be associated with microinvasive disease.

§ Based on tamoxifen therapy received. For simplicity, the patients have been grouped according to CTC status and numbered sequentially.

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*Table 3* Patterns of aneusomy in each dormancy candidate who had a CTC

| | First blood sample | | | Time between first and second sample (mo) | Second blood sample† | | | Time between first and third sample (mo) | Third blood sample† | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient no. | No. of CTCs | Blood volume (mL) | Aneusomic pattern* | | No. of CTCs | Blood volume (mL) | Aneusomic pattern* | | No. of CTCs | Blood volume (mL) | Aneusomic pattern* |
| Initially positive | | | | | | | | | | | |
| 6 | 2 | 12.5 | 1x(4,3,2)<br>1x(3,2,2) | 8.5 | 2 | 25 | 1x(3,3,2) (2,3)<br>1x(4,1,3) (2,3) | 20.1 | 1 | 25 | 1x(2,3,3) |
| 13 | 2 | 12.5 | 2x(3,4,3,) | 3 | 1 | 25 | 1x(5,1,2) | 7.8 | 1 | 25 | 1x(2,3,3) (3,2) |
| 7 | 1 | 12.5 | 1x(4,3,5) | 9 | 0 | 15 | | | | | |
| 12 | 1 | 12.5 | 1x(5,3,4) | 9.5 | 1 | 15 | 1x(5.3.4) | | | | |
| 3 | 1 | 25 | 1x(2,2,4) (3,3) | 4 | 1 | 15 | 1x(2,2,4) | | | | |
| 4 | 2 | 25 | 1x(3,2,3) (4,2)<br>1x(2,3,3) (2,2) | | | | | | | | |
| 5 | 1 | 10 | 1x(2,3,2) (3,3) | 6 | 1 | 25 | 1x(2,2,3) (3,2) | 14.2 | 0 | 25 | |
| 8 | 2 | 15 | 1x(1,1,2) (2,2)<br>1x(2,2,2) (2,4) | 3 | 1 | 15 | 1x(1,1,2) | 8.8 | 4 | 50 | 1x(4,3,5); 1x(2,2,4)<br>1x(5,2,5); 1x(3,2,3) |
| 9 | 1 | 15 | 1x(2,3,2) (3,3) | | | | | | | | |
| 10 | 2 | 25 | 1x(1,2,5)<br>1x(3,3,2) (3,2) | | | | | | | | |
| 11 | 2 | 25 | 1x(2,2,5)<br>1x(2,2,4) (4,2) | | | | | | | | |
| Initially negative | | | | | | | | | | | |
| 1 | 0 | 12.5 | | 1.5 | 2 | 12.5 | 1x(2,2,3)<br>1x(2,3,3) | | | | |
| 2 | 0 | 12.5 | | 2.5 | 1 | 12.5 | 1x(3,2,3) | 9.5 | 2 | 15 | 1x(2,3,1);<br>1x(2,2,3) |
| 14 | 0 | 12.5 | | 13 | 0 | 15 | | | | | |
| 15 | 0 | 25 | | 3 | 0 | 15 | | | | | |
| 36 | 0 | 12.5 | | 12 | 0 | 15 | | | | | |
| 20 | 0 | 12.5 | | 2.5 | 0 | 12.5 | | | | | |

* All cells were probed for chromosomes 1, 8, and 17. Some cells were reprobed for chromosomes 3 and 11. x = number of CTCs. The parentheses containing three numerals represent copy number of CEP 1, 8, and 17, respectively. The parentheses containing two numerals represent copy number of CEP 3 and 11, respectively.

† Only a portion of patients has been drawn more than once to date.

significantly higher frequency than their risk of recurrence. Of 36 breast cancer patients with no evidence of clinical disease, at least 36% had CTCs 8 to 22 years after mastectomy.[12] This finding is consistent with the previously published studies (54–56), which demonstrated that the persistent idiotype in serum and cells of patients with non-Hodgkin's lymphoma (NHL) in long-term remission was the same as that of the corresponding primary tumor. (*b*) CTCs from primary breast cancer have a short half-life measured in hours. This is consistent with prior reports that epithelial cells (normal or malignant) separated from the stroma and neighboring epithelial cells enter an apoptotic program (57–64) and have a short half-life. They die by a combination of apoptosis (64, 65) and uptake by the liver (66, 67) and lung (66, 68, 69). Apoptosis has been demonstrated in CTCs shed from both primary and recurrent breast cancer,[13] but it has not been proven for CTCs in dormancy candidates. Regardless, a source of replicating cells, presumably from micrometastases, is necessary to maintain their presence in the blood of dormancy candidates. (*c*) Patients who are candidates for breast cancer dormancy can have replicating tumor cells for as long as 22 years after removal of their tumor without evidence of progressive growth of the tumor cell population; hence, replication appears to be balanced by cell death in these patients. To explain this balance, there must be unknown innate mechanisms that do not allow the size of the tumor population to increase. This finding is conceptually different from patients with recent breast cancer who have CTCs. These patients have an increased risk for recurrence compared with those who do not have CTCs after removal of the primary tumor (1). In contrast, the statistical risk of recurrence in dormancy candidates (~1% per year) is lower than the percentage of dormancy candidates who have CTCs (36). However, the implications of the presence of CTCs on their risk of recurrence can only be established by long-term follow-ups.

[12] Although we have stringent criteria for designating a cell as a CTC, it is not a completely objective test. A small number of cells are borderline in one or more criteria, making a definitive conclusion difficult. However, with experience, our stringent criteria, and erring on the conservative side, three "blinded" observers (S. Meng, J. Uhr, and T. Tucker) rarely disagreed on the classification of a CTC. The strongest evidence to support our conclusion is that only one cell that could possibly be mistaken for a CTC was found in the 26 controls.

[13] T. Fehm, S. Meng, J. Wang, T. Tucker, N. Lane, J. Uhr, unpublished data.

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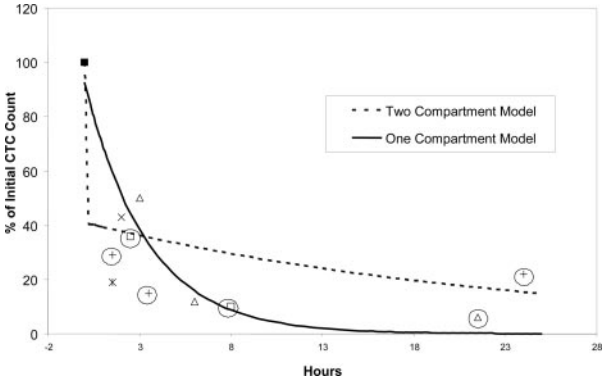


*Fig. 2* Estimation of half-life of CTCs. The *curve* represents the accumulation of data from five patients. Each patient has a different symbol for her CTC counts. For each patient, the first blood sample was obtained before surgery or just after removal of the primary tumor and was plotted as 100%. The CTCs were measured by flow cytometry in which the background level of events can differ among normal patients. Therefore, those events that are within the background are circled.

The definition of dormant cancer in the past has necessarily been a clinical one, namely, recurrence of tumor a long disease-free period of time after removal of a primary tumor. This is a purely descriptive definition, but until recently, there has been no realistic opportunity to detect tumor cells in such patients. Now that there are sensitive techniques available to detect and characterize persisting tumor cells, it seems appropriate to consider expanding the definition. We suggest that the patients that we have called dormancy candidates with CTCs are patients with tumor dormancy for the following reasons: (*a*) They have replicating tumor cells for as long as two decades after mastectomy without expansion of the tumor population. This is deduced from the finding that the CTCs have a short half-life probably measured in hours. Hence, they must constantly be replenished by replicating tumor cells in metastatic foci. Clearly, from the biological viewpoint, this is tumor dormancy. (*b*) None of the small group of patients followed for 1 to 2 years has had any clinical evidence of recurrent disease, consistent with past data that about 1% of breast cancer patients disease-free for ≥7 years after mastectomy will have a recurrence in a given year. Also, of the 13 dormancy candidates who have had two or more blood tests with 1.5 to 20.1 months between the first and last sample, all have shown a relatively steady, low concentration of CTCs. There were several patients with no detectable CTCs in one blood sample and 1 or 2 CTCs in another sample, possibly due to technical variations in the assay or oscillations in the balance (70). (*c*) There are striking similarities, clinically and biologically, to both NHL and the $BCL_1$ murine model of dormant lymphoma. In both cases, because there is a unique clonal marker on the tumor cells (the idiotype of the tumor IgM), it can be shown that the original clone persists for a very long time without clinical manifestations and that clinical recurrences taking place at intervals are composed of tumor cells bearing the same clonal marker. Taken together, the above findings represent strong evidence that dormancy candidate patients with CTCs have tumor dormancy. To make this definition also fit the clinical classification will take a long time. Because there is no clonal marker for breast cancer at this time, usually only 1 to 2 CTCs per patient are obtained, and the rate of recurrence is so low in this group of patients a follow-up of a very large number of patients for an extended period of time will be necessary to obtain a sufficient number of recurrences and material to perform microarray and/or proteomic assays on both the CTCs and recurrent tumor cells to prove identity.

The finding that breast cancer patients with tumor dormancy can have an apparent balance between replication and cell death was not entirely unexpected. As discussed previously, similar observations were made in the $BCL_1$ murine lymphoma model of dormancy. Also, Holmgren *et al.* (8) suggested that angiogenesis suppression could produce such a balance based on experiments in mice. It is not known whether the dormancy candidate patients who did not have detectable CTCs were free of tumor or had undetectable levels at the time of the blood sampling. Because a portion of dormancy candidates who had no CTCs in the first blood sample had CTCs in a second blood sample, it is possible that a higher percentage of the patients in our study have CTCs and thus have cancer dormancy.

The present observations cannot be extrapolated to embrace the entire life history of the population of tumor cells responsible for the state of dormancy. For example, after tumor removal, disseminated tumor cells could be in $G_0$-$G_1$. Therefore, the balance in replication and cell death may occur at sometime after this putative phase (71). If there are two phases, it is not known when the "switch" from $G_0$-$G_1$ to "balanced" replication takes place. However, leaving aside the early history of micrometastases, the simplest explanation of our data is that the balance described above has been in place for many years, if not decades.

The mechanisms underlying the control of the size of the tumor cell population are unknown. It is important to characterize them because they may reveal novel homeostatic mechanisms that will lead to development of new drugs. Should this be explained by known antitumor mechanisms such as antitumor immune responses (24), angiogenic suppression (8–17), and so forth, the results could help to determine which of the current therapeutic approaches for controlling metastases should be emphasized experimentally and accelerated in clinical trials.

As mentioned above, the relapse rate in breast cancer 7 or more years after tumor removal is stochastic at about 1% per year for 20 years (31, 32). A new cancer in the other breast or any remaining tissue in the breast that was the site of the original primary tumor is conventionally excluded by clinical, laboratory, and imaging studies. Because the total percentage of relapses over two decades is about 20%, relapse is a significant problem. At present, as long as the CTC count is stable or absent and the dormancy patients remain clinically disease-free, it is impossible to predict who, if anyone, should receive treatment.

The present data add to the increasing evidence that cancer is probably a chronic disease in many patients. There is abundant evidence that tumor cells disseminate before an apparently organ-confined cancer is detected (1, 33, 34, 38, 40–43, 45, 46, 72–82) and that the persistence of neoplastic hematopoietic cells is not incompatible with clinical cure (54, 83). The prevalence of dormancy in breast cancer (84–86), melanoma (87–89), renal carcinoma (90, 91), and NHL (54–56); the less

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frequent late recurrences in other types of cancer (83, 88, 92–97); and the lack of information about tumor cell persistence in cancers that are not associated with late recurrences or even in patients with breast cancer who do not display persisting tumor argue that chronicity of cancer may be a widespread phenomenon.

## ACKNOWLEDGMENTS

We thank Drs. John Minna and Ellen Vitetta for their comments on the manuscript; Mahdieh Iran-Nejad Parizi and Lauren Loftis, our research coordinators; Marcia Conner for help with recruitment of patients; and Linda Owens for administrative assistance.

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*Clin Cancer Res* 2004;10:8152-8162.

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