

# Use of a Murine Monoclonal Antibody for Detection of Circulating Plasma DF3 Antigen Levels in Breast Cancer Patients

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## Abstract

The murine monoclonal antibody (MAb), designated DF3, reacts with a 300,000-mol wt mammary epithelial antigen. A sequential double-determinant radioimmunoassay (RIA) has been developed to monitor circulating DF3 antigen. Using this assay, we have demonstrated that 33 of 36 normal women had plasma RIA antigen levels < 150 U/ml. In contrast, 33 of 43 patients (76%) with metastatic breast cancer had RIA DF3 antigen levels  $\geq$  150 U/ml. The difference between these two groups was statistically significant ( $P < 0.001$ ). Similar results have been obtained with a double-determinant enzyme-linked immunoassay (EIA). Only 6 of 111 age-matched normal subjects had EIA DF3 antigens levels  $\geq$  30 U/ml, while 42 of 58 patients (72%) with breast cancer had levels equal to or above this value. Thus, similar patterns of specificity are obtained with the EIA or RIA. The elevation of circulating DF3 antigen levels in breast cancer patients has been confirmed by transfer blot assays. MAb DF3 reactivity occurred predominantly with circulating antigens of three different molecular weights ranging from 300,000 to  $\sim$ 400,000 mol wt. We also demonstrate that patients with both primary and metastatic breast cancer who were free of detectable disease at the time of sampling have DF3 antigen levels that are similar to those obtained from normal subjects. While patients with hepatoma (27%) and ovarian carcinoma (47%) also had elevated circulating DF3 antigen levels, the results suggest that DF3 antigen levels may be useful in distinguishing breast cancer patients from those with esophageal, gastric, colorectal, pancreatic, and lung carcinomas. Furthermore, the results of the RIA, EIA, and transblot analyses demonstrate that the measurement of circulating DF3 antigen levels provides a new and potentially useful marker to follow the clinical course of patients with metastatic breast cancer.

## Introduction

We have previously defined a human mammary epithelial antigen using a murine monoclonal antibody, designated DF3 (MAb DF3),<sup>1</sup> prepared against a membrane-enriched fraction

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1. *Abbreviations used in this paper:* EIA, enzyme-linked immunoassay; MAb DF3, murine monoclonal antibody, designated DF3; NVD, nonvisceral disease; VD, visceral disease.

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of a human breast carcinoma (1). This antigen has a molecular weight of 300,000 and is detectable on the surface of human breast carcinoma cells. Immunoperoxidase staining patterns with MAb DF3 clearly distinguish malignant and benign breast lesions. DF3 antigen is present on apical borders of secretory mammary epithelial cells and in the cytosol of less differentiated malignant cells. This apical and cytoplasmic staining pattern of human mammary epithelium has also been described using antibodies produced against milk fat globule membrane (2, 3) and breast carcinoma cells (4). MAb DF3, however, can be distinguished from other MAbs generated against milk fat globule membrane and breast tumors on the basis of the molecular weight of the cross-reactive antigen (1-15).

We have developed two double-determinant immunoassays with MAb DF3 to monitor circulating DF3 antigen levels in patients with breast cancer. DF3 antigen has been assayed in apparently healthy controls, in patients with breast cancer, and in individuals with a prior history of breast cancer but who were free of detectable disease at the time of sampling. The results indicate that DF3 antigen levels are significantly elevated in the plasma and/or serum of patients with breast cancer. These findings have been confirmed by transfer blot analysis. Furthermore, the results indicate that circulating DF3 antigen provides a new and potentially useful marker for following the clinical course of breast cancer.

## Methods

*Preparation of MAb DF3.* MAb DF3 was produced by the immunization of BALB/c mice with a partially purified membrane-enriched fraction of a human breast carcinoma metastatic to liver (1). The antibody was purified from ascites by protein A-sepharose-CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) with elution by a pH gradient at pH 5.5. The purity of the IgG<sub>1</sub> was confirmed by SDS polyacrylamide gel electrophoresis.

*Sequential DF3 double-determinant radioimmunoassay (RIA).* The purified antibody was radiolabeled with Na<sup>125</sup>I (New England Nuclear, Cambridge, MA) by the Iodogen method (Pierce Chemical Co., Rockford, IL) (16) to achieve specific activities of 0.5-0.8 [<sup>125</sup>I]/DF3 IgG<sub>1</sub> (mole per mole).

Vinyl plates (96 wells/Costar, Cambridge, MA) were coated with purified antibody (1  $\mu$ g/well) and dried overnight at room temperature. Plates were incubated with 1% bovine serum albumin/phosphate buffered saline (BSA/PBS) for 30 min at 37°C before use and washed with 0.1% polyoxyethylene sorbitan monolaureate (Tween 20; Sigma Chemical Co., St. Louis, MO) in PBS, pH 8.0 (Tween buffer).

Plasma samples were obtained from breast cancer patients and normal females after separation of blood drawn into EDTA-treated collection tubes. These samples were diluted in 0.5% BSA/Tween buffer/PBS at 1:5, 1:25, 1:625, 1:1,250, 1:6,250, and 1:12,500. All assays were performed in duplicate. Aliquots (150  $\mu$ l) were added to the MAb DF3-coated wells and incubated overnight at 37°C. The wells were then washed twice with Tween buffer. The [<sup>125</sup>I]MAb DF3 ( $\sim$ 250 ng/well diluted in 0.5% BSA/Tween buffer/PBS) was added to each well, incubated for 3 h at 37°C, and then washed five times with

Tween buffer. The bound  $^{125}\text{I}$  radioactivity was determined as a relative measure of DF3 antigen content and was compared on each run to a frozen primary reference standard (membrane-enriched fraction of a breast carcinoma metastatic to liver).

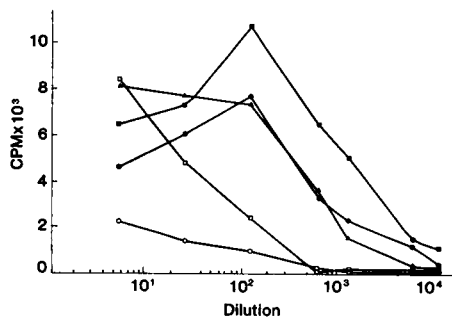
**Sequential DF3 double-determinant enzyme-linked immunoassay (EIA).** Vinyl plates (96 wells, Costar) were coated with 4  $\mu\text{g}$  MAb DF3/well. The plates were then incubated with 1% BSA/PBS for 60 min at 37°C and washed with Tween buffer. Plasma or serum samples at appropriate dilutions were added to the MAb DF3-coated wells and incubated overnight at 37°C. The wells were then washed three times with Tween buffer. Purified MAb DF3 was conjugated (17) to horseradish peroxidase type VI (Sigma Chemical Co.) and added to each well (0.7  $\mu\text{g}$  in 100  $\mu\text{l}$  0.5% BSA/Tween buffer). After a 1-h incubation at 37°C and then 2 h at room temperature the wells were washed with Tween buffer and developed by adding 100  $\mu\text{l}$  of 0.012% hydrogen peroxide, 5.5 mM *o*-phenylenediamine, and 0.1 M citrate buffer (pH 4.5). Absorbance at 490 nm was monitored after 30 min of incubation at room temperature using a Mini Reader II (Dynatech Laboratories, Alexandria, VA). Relative DF3 antigen values were determined by comparison with a frozen primary reference standard.

**Determination of molecular weights of MAb DF3 reactive antigens by transblot analysis.** 1  $\mu\text{l}$  of plasma or serum from selected patients and controls was analyzed on 3–15% SDS polyacrylamide gels. After electrophoresis, transfer from the polyacrylamide gel to nitrocellulose paper (Schleicher & Schuell, Keene, NH) was performed using a Bio-Rad transblot apparatus (Bio-Rad Laboratories, Richmond, CA) with a 25 mM Tris, 192 mM glycine, and 20% methanol buffer (pH 8.3). The nitrocellulose paper was treated with 5% BSA, washed, and incubated with MAb DF3 IgG (0.25  $\mu\text{g}/\text{ml}$ ) for 2 h (18). The paper was again washed, incubated with sheep anti-mouse Ig [ $^{125}\text{I}$ ]F(ab')<sub>2</sub> (16  $\mu\text{Ci}/\mu\text{g}$ , Amersham Corp., Arlington Heights, IL) for 2 h and then exposed to X-AR film (Eastman Kodak Co., Rochester, NY) for 12 h. Quantification of the relative DF3 antigen level was determined by densitometric calculation of area under the curve using a Quickscan densitometer (Helena Laboratories, Beaumont, TX) and comparison with a frozen primary reference standard.

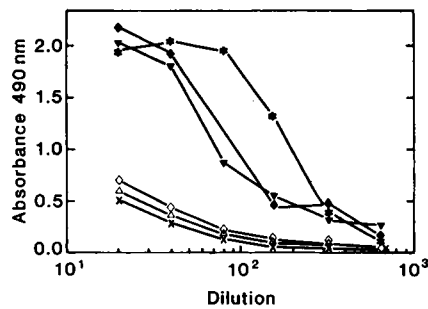
**Statistical determinations.** The significance of differences in DF3 antigen levels between groups of subjects was determined by single factor analysis of variance by ranks (Kruskal-Wallis test) (19). Pearson product moment correlation coefficients were calculated to compare data between assay techniques and their significance was analyzed by the *t* test (20).

## Results

Plasma samples obtained from patients with metastatic breast cancer and from normal females were diluted and initially



**Figure 1.** Effect of plasma dilution on binding of  $^{125}\text{I}$ -MAb DF3. Plasma samples from patients with metastatic breast cancer (■, ●, ▲) and from normal females (□, △) were diluted serially and assayed for DF3 antigen in the sequential double-determinant RIA.

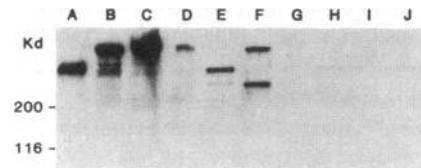


**Figure 2.** Effect of plasma dilution on binding of peroxidated MAb DF3. Plasma samples from patients with metastatic breast cancer (◆, ▼, \*) and from normal females (◇, △, ×) were diluted serially and assayed for DF3 antigen in the sequential double-determinant EIA.

assayed for DF3 antigen in the sequential double-determinant RIA. Fig. 1 illustrates representative profiles obtained from the plasma of three patients with breast cancer and two normal females. DF3 antigen was detectable in each plasma. However, the level of DF3 antigen was higher in the patients as compared to controls. For example, DF3 antigen levels were undetectable at a 1:625 dilution of plasma from both normal females, while over 3,000 cpm  $^{125}\text{I}$ -MAb DF3 bound at similar dilutions of plasmas from the three patients.

Similar findings have been obtained using the sequential double-determinant EIA. Fig. 2 illustrates representative profiles using this assay. DF3 antigen was detectable in the plasmas from three patients with breast cancer and from three control subjects. However, DF3 antigen levels were undetectable at a 1:125 dilution of plasmas from all three normal females, while significant amounts of peroxidated MAb DF3 bound at similar dilutions of plasmas from the three patients. The EIA profiles thus appeared similar to those obtained by RIA, but this assay was not inhibited at lower plasma dilutions and undetectable plasma DF3 antigen levels were reached at approximately fivefold lower dilutions than in the RIA. Plasma or serum samples yielded identical results.

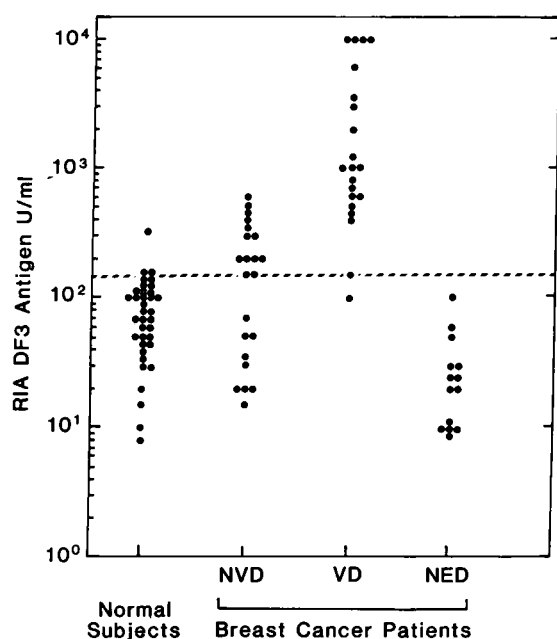
The detection of circulating DF3 antigen by RIA and EIA prompted further analysis using transblots to determine the molecular weight of the reactive species. Fig. 3 shows the results obtained with plasmas from breast cancer patients and normal subjects. Antigenic heterogeneity was observed in both patients and controls. MAb DF3 reactivity was found predom-



**Figure 3.** Reactivity of MAb DF3 with a metastatic breast carcinoma and plasma samples. (A) Membrane-enriched fraction of a breast carcinoma metastatic to liver (0.5  $\mu\text{g}$ ); (B–F) Plasma samples from patients with metastatic breast cancer (1  $\mu\text{l}$ ); (G–J) Plasma samples from normal subjects (1  $\mu\text{l}$ ). The antigen preparations were subjected to electrophoresis on a 3–15% SDS-polyacrylamide gel, transferred to nitrocellulose paper, and analyzed for reactivity with MAb DF3. Kd, molecular weight.

inantly with antigens of three different molecular weights ranging from 300,000 to ~400,000 mol wt. Although similar patterns were observed in breast cancer patients and controls, the extent of reactivity was clearly greater with plasma obtained from breast cancer patients. These findings by transblot analysis are thus in concert with the RIA and EIA results. Further, the transblot approach yields additional information regarding the molecular weight of the cross-reactive antigens.

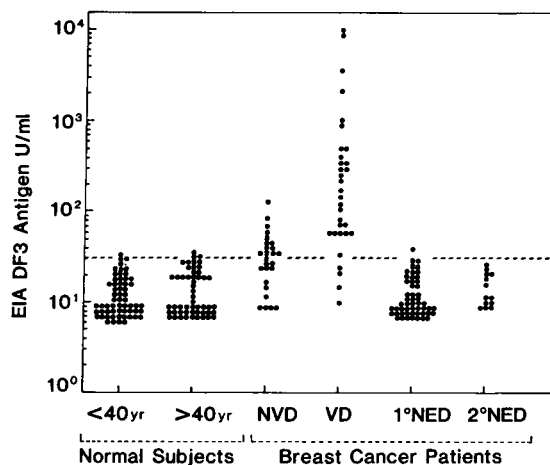
The RIA, EIA, and transblot assays have been used to monitor circulating DF3 antigen levels in a larger series of breast cancer patients and normal subjects. The results obtained by RIA are illustrated in Fig. 4. The absence of purified DF3 antigen for construction of a standard curve has precluded a determination of actual plasma concentration. However, we have determined a relative DF3 RIA value based upon a plasma dilution necessary to bind 2,000 cpm  $^{125}$ I-MAb DF3 above background (<200 cpm). Further, each assay was performed with a frozen primary reference standard to insure comparative results between different experiments. Using this approach, we have assayed plasmas from 43 patients with metastatic breast cancer and from 36 apparently normal females. No significant difference was observed between values obtained from normal women above and below the age of 40. Further, the menstrual cycle and pregnancy had no effect on plasma DF3 antigen levels (data not shown). 33 of 36 control subjects had plasma RIA DF3 antigen levels < 150 U/ml (mean±SD; 82±54). In contrast, 33 of 43 patients (76%) with



**Figure 4.** RIA DF3 antigen levels in plasma from normal subjects, patients with metastatic breast cancer, and breast cancer patients free of detectable disease. Samples from 36 normal subjects, 43 patients with metastatic breast cancer, and 14 patients with a history of breast cancer but who were free of detectable disease at the time of sampling were assayed for RIA DF3 antigen levels. Relative RIA DF3 antigen units per milliliter were determined at 2,000 cpm bound and by reference to the frozen breast tumor standard. The dotted line represents an arbitrary level to distinguish elevated values. NVD (local recurrence, skin recurrence, bone metastases); VD (liver or lung metastases); NED, no evidence of detectable disease.

breast cancer had RIA DF3 antigen levels  $\geq 150$  U/ml (mean±SD; 1,497±2,894). The highest plasma RIA DF3 antigen levels detected in patients with breast cancer were 10,000 U/ml. The difference between RIA DF3 antigen levels obtained for normal subjects and for breast cancer patients was statistically significant ( $P < 0.001$ ). The breast cancer patients were also separated into two categories: (1) nonvisceral disease (NVD), encompassing patients with local recurrence, skin recurrence, or bone metastases, and (2) visceral disease (VD), encompassing patients with liver and lung metastases. As illustrated in Fig. 4, >90% of VD patients had RIA DF3 antigen levels  $\geq 150$  U/ml, while only 50% of NVD patients had elevated levels. The difference between patients with VD (mean±SD: 3,000±3,736) and those with NVD (mean±SD: 195±172) was significant ( $P < 0.001$ ). Finally, 14 of 14 patients with a previous history of breast cancer who were free of detectable disease at the time of sampling were found to have RIA DF3 antigen levels < 150 U/ml (mean±SD; 34±27).

A more extensive series of breast cancer patients and normal subjects was monitored for circulating DF3 antigen levels by EIA. Fig. 5 summarizes the EIA assay results of samples from 58 patients with metastatic breast cancer and 111 apparently normal females. Relative EIA DF3 antigen values were based upon the plasma dilution resulting in an absorbance of 0.75 at 490 nm (background < 0.05). Each assay was performed with a frozen primary reference standard to insure comparative results between different experiments. Using this approach, only 6 of 111 normal subjects had EIA DF3 antigen levels  $\geq 30$  U/ml (mean±SD: 13±8). Furthermore, similar results were obtained with normal subjects younger or older than 40 yr. In contrast, 42 of 58 patients (72%) with breast cancer had EIA DF3 antigen levels  $\geq 30$  U/ml (mean±SD: 545±1,781). The difference between all breast

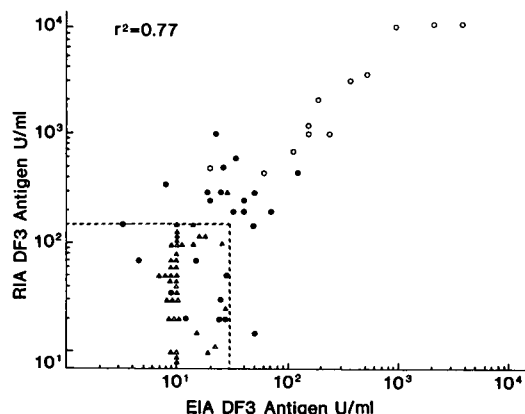


**Figure 5.** EIA circulating DF3 antigen levels in normal subjects, patients with metastatic breast cancer, and patients with breast cancer free of detectable disease. Samples from 111 normal subjects, 58 patients with metastatic breast cancer (NVD: 26 patients; VD: 32 patients), and 65 patients with a prior history of breast cancer (1° or primary disease; 2° or metastatic disease) but who were free of detectable disease (NED) at the time of sampling were assayed for EIA DF3 antigen level. Relative EIA DF3 antigen units per milliliter were determined at an absorbance of 0.75 (490 nm) and by reference to the frozen breast tumor standard. The dotted line represents an arbitrary level to distinguish elevated values.

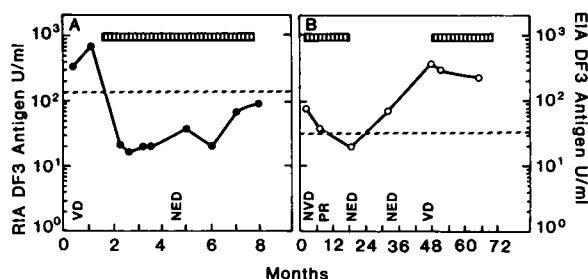
cancer patients and all normal subjects was significant ( $P < 0.001$ ). 28 of 32 patients (88%) with visceral disease had EIA DF3 antigen levels  $\geq 30$  U/ml, while 14 of 26 patients (54%) with nonvisceral disease had EIA DF3 antigen values above this level. EIA DF3 antigen levels in patients with visceral disease (mean $\pm$ SD: 973 $\pm$ 2,350) were significantly different from those patients with nonvisceral disease (mean $\pm$ SD: 37 $\pm$ 27) ( $P < 0.001$ ). The EIA antigen levels were elevated in NVD patients with disease restricted to bone (mean $\pm$ SD: 49.5 $\pm$ 32.3), but not in patients with disease restricted to skin or lymph nodes (mean $\pm$ SD: 24.4 $\pm$ 14.4). The difference between EIA DF3 antigen levels obtained with NVD patients and all normal subjects was significant ( $P < 0.001$ ). Although the EIA DF3 antigen levels for patients with disease restricted to bone was significantly different ( $P < 0.001$ ) from that obtained for all normal subjects, the difference between values obtained for patients with skin or nodal recurrence and normal subjects was not as significant ( $P < 0.05$ ). Finally, 50 of 52 patients with primary and 13 of 13 patients with metastatic breast cancer who were free of detectable disease at the time of sampling had EIA DF3 antigen levels that were  $< 30$  U/ml.

The results obtained by RIA and EIA thus demonstrate the presence of elevated plasma DF3 antigen levels in patients with metastatic breast cancer as compared to normal subjects and breast cancer patients free of detectable disease. A comparison of results obtained with these two assays is illustrated in Fig. 6. The correlation coefficient for the RIA and EIA data was  $r^2 = 0.77$  ( $P < 0.001$ ). The relationships among assays were extended further by comparing the RIA DF3 antigen levels and those obtained by transblot analysis (58 samples). The correlation between these two determinations was significant ( $r^2 = 0.68$ ,  $P < 0.001$ ). Similarly, the correlation between the EIA and transblot data (120 samples) was also significant ( $r^2 = 0.49$ ,  $P < 0.001$ ). The use of the RIA, EIA, or transblot analysis thus provides concordant measurements of DF3 antigen levels.

It was of further interest to determine whether serial measurements of circulating DF3 antigen levels would vary with clinical course. In this regard, we monitored DF3 antigen levels in patients with metastatic breast cancer who were

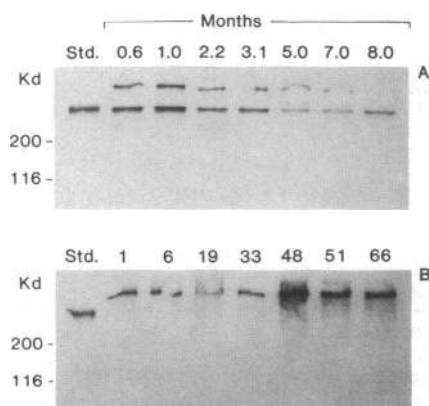


**Figure 6.** Relationship between RIA and EIA DF3 antigen units per milliliter for normal subjects ( $\blacktriangle$ ), patients with metastatic breast cancer (NVD:  $\bullet$ ; VD:  $\circ$ ), and breast cancer patients free of detectable disease (NED:  $\blacktriangle$ ). Dotted lines represent arbitrary levels distinguishing elevated values for the respective assays.



**Figure 7.** Circulating DF3 antigen levels and clinical course. (A) Serial samples from a patient with metastatic breast cancer assayed for RIA DF3 antigen levels. (B) Serial samples from another patient with metastatic breast cancer assayed for EIA DF3 antigen levels. The dotted line represents arbitrary levels to distinguish elevated values in the RIA (150 U/ml) and EIA (30 U/ml) assays. Hatched areas represent treatment with chemotherapy.

subsequently treated with chemotherapy. A representative profile obtained by RIA is illustrated in Fig. 7 A. This patient had an RIA DF3 antigen level of 700 U/ml just before beginning chemotherapy. Clinical response was obtained after 1 mo of therapy and at that time the RIA DF3 antigen level had declined to 20 U/ml. The RIA DF3 antigen levels ranged between 15 and 70 U/ml while the patient received chemotherapy and remained in clinical remission. A similar example of a patient monitored for EIA DF3 antigen levels is illustrated in Fig. 7 B. This patient initially presented with an EIA DF3 antigen level of 80 U/ml. After achieving a complete response with chemotherapy, the EIA DF3 antigen level decreased to below 30 U/ml. The EIA DF3 antigen level, however, increased while this patient remained off chemotherapy, predating clinical detection of recurrent disease (32 mo). Hepatic and pulmonary metastasis were eventually detected at 48 mo and an unsuccessful attempt at retreatment with chemotherapy was associated with persistently elevated levels of EIA DF3 antigen. Serial findings have been confirmed by transblot analysis (Fig. 8). Finally, serial EIA DF3 antigen levels were monitored in five



**Figure 8.** Reactivity of MAb DF3 by transblot analysis and clinical course. (A) Serial samples (1  $\mu$ l) from patients shown in Fig. 7 A. (B) Serial samples (1  $\mu$ l) from patients shown in Fig. 7 B. The initial lanes in A and B represent the membrane-enriched extract of a human breast carcinoma metastatic to liver (0.5  $\mu$ g) used as a standard (Std.). The numbers above each lane represent duration of the clinical course (months) and correspond to the respective data points in Fig. 7. Kd, molecular weight.

patients with progressive breast cancer and in each case there was an increase in DF3 antigen level (Fig. 9). Three of these patients were subsequently treated with hormonal or chemotherapy and declines in EIA DF3 antigen level paralleled partial and complete clinical responses.

It was also of interest to monitor for circulating DF3 antigen levels in patients with other benign and malignant diseases. In this regard, serum EIA DF3 antigen levels were determined for 17 patients with benign breast diseases (Table I). None of these patients had EIA DF3 antigen levels  $\geq 30$  U/ml (mean  $\pm$  SD:  $14.8 \pm 17.3$ ). Table I also lists the results obtained from patients with other malignancies and benign liver disease. There was no detectable elevation of circulating DF3 antigen in 71 patients with esophageal, gastric, or colorectal carcinomas. 25 of the 48 patients with colorectal tumors had liver involvement, which thus suggests that hepatic metastases per se did not cause elevations in circulating DF3 antigen. Furthermore, only 2 of 58 patients with pancreatic malignancies and 1 of 11 patients with lung cancer had DF3 antigen levels  $\geq 30$  U/ml. Elevated DF3 antigen levels were, however, detected in 27% of patients with hepatoma and 47% of patients with ovarian cancer. Finally, 10 of 66 patients with benign liver disease had DF3 antigen levels  $\geq 30$  U/ml (Table I).

## Discussion

Although circulating levels of carcinoembryonic antigen and gross cystic disease protein are presently used as serologic markers of human breast cancer (21, 22), there is clearly a need for more specific and sensitive assays. In contrast, tissue markers such as casein (23),  $\alpha$ -lactalbumin (24), glycosyltransferases (25), glycolipids (26), and phospholipids (27) have been detected in plasma by a variety of techniques, but none has gained acceptance as a breast cancer marker. Human mammary epithelial antigens detected by MAbs prepared against human milk fat globules have been shown to be elevated in sera of patients with disseminated breast cancer (28). This 46,000-mol wt mammary epithelial antigen is distinct from the 300,000-mol wt antigen reactive with MAb DF3. Similarly, breast epithelial antigens defined by MAb 24-17.2 ( $M_r = 100,000$ ) and by MAb F36/22 ( $M_r > 400,000$ ) have been detected in increased amounts in the sera of patients with metastatic breast cancer (9, 29). These assays appear promising and may prove useful in monitoring clinical course.

The present study demonstrates by RIA, EIA, and transblot analysis that DF3 antigen is significantly elevated in plasma and serum from patients with metastatic breast cancer. The

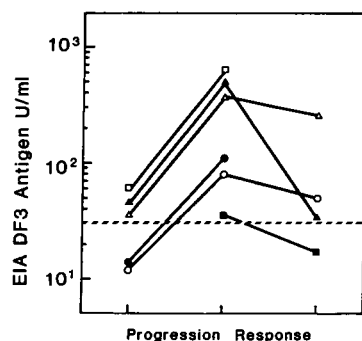


Figure 9. Serial changes in circulating DF3 antigen levels in patients with breast cancer after progression of disease or response to chemotherapy. Complete responder: ■, ▲, ○.

Table I. EIA DF3 Values from Patients with Benign Diseases and Nonmammary Malignancies

Diagnosis	Number of patients	Number of patients with elevated EIA DF3 value (%)	EIA DF3 value*
Benign breast disease‡	20	0 (0)	$14.9 \pm 17.3$
Cancer			
Esophageal	7	0 (0)	$10.2 \pm 4.8$
Gastric	16	0 (0)	$9.9 \pm 6.4$
Colo-rectal	48	0 (0)	$13.7 \pm 6.7$
Pancreatic	38	2 (5)	$14.2 \pm 7.9$
Hepatoma§	56	15 (27)	$29.9 \pm 18.7$
Lung	11	1 (9)	$17.4 \pm 27.2$
Ovarian	43	20 (47)	$52.4 \pm 69.7$
Benign liver disease			
Cirrhosis¶	32	6 (19)	$20.8 \pm 11.8$
Chronic active hepatitis	4	2 (50)	$27.0 \pm 25.0$
Acute hepatitis			
Type A	14	0 (0)	$10.3 \pm 2.4$
Type B	16	2 (13)	$22.0 \pm 21.8$

\* Mean units per milliliter  $\pm$  standard deviation.

‡ Includes gross cysts (4), fibrocystic disease (10), fibroadenoma (1), mastalgia (1), and physiologic nodularity (4).

§ Includes idiopathic (29), associated alcoholic cirrhosis (8), and associated post-necrotic cirrhosis (19).

|| Includes oat cell (3), adeno (4), and squamous (4).

¶ Includes alcoholic (4), idiopathic/postnecrotic (25), and hepatitis B associated (3).

results obtained by RIA correlated with those obtained by EIA. Furthermore, quantitation of circulating DF3 antigen by transblot analysis and densitometric calculation of area under the curve correlated with results obtained by both RIA and EIA. These results demonstrate the presence of elevated DF3 antigen levels in up to 70% of patients with metastatic breast cancer by three different methods of analysis. In contrast, >94% of age-matched normal subjects had nonelevated DF3 antigen levels. The results obtained by RIA and EIA were quite similar with regard to sensitivity and specificity. The arbitrarily chosen cut off values of  $\geq 150$  RIA DF3 antigen U/ml and  $\geq 30$  EIA DF3 antigen U/ml resulted in a specificity of <6% false positives and a sensitivity of >70% true positives. Furthermore, the transblot analysis provided another approach to monitor both DF3 antigen level and molecular weight of the reactive moiety. Although heterogeneity was seen in terms of DF3 antigen molecular weight, there did not appear to be a particular pattern associated with breast cancer or site of metastatic disease.

The results also demonstrate that DF3 antigen levels are not significantly elevated in patients with benign breast disease. Similarly, patients with esophageal, gastric, and colorectal carcinomas had no detectable elevation of circulating DF3 antigen. The absence of elevated circulating DF3 antigen levels in patients with colorectal carcinomas distinguishes our assay from the results obtained with carcinoembryonic antigen (21, 22) and other breast carcinoma-associated antigens (29). Furthermore, >90% of patients with pancreatic and lung carcinomas had EIA DF3 antigen levels < 30 U/ml. In contrast, the detection of an elevated DF3 antigen level would be less helpful in distinguishing patients with breast cancer from those with either hepatoma or ovarian cancer. Transblot analyses of

plasma from patients with hepatoma and ovarian carcinoma have revealed cross-reactive DF3 antigens with molecular weights similar to that found in breast cancer (data not shown). Thus, although elevated circulating DF3 antigen has been detected in certain other malignancies, the pattern of specificity suggests that measuring DF3 antigen levels may be useful diagnostically and in terms of monitoring clinical course.

MAB DF3 has previously been shown to react with 87% of 52 primary breast carcinomas and with 100% of breast tumors metastatic to axillary lymph nodes or distal sites (1). The DF3 antigen is present on apical borders of differentiated secretory mammary epithelial cells and in the cytosol of less differentiated malignant cells. Other studies (18) have demonstrated a selective enhancement of DF3 antigen expression after exposure of human MCF-7 breast carcinoma cells to sodium butyrate, an inducer of differentiation in leukemias (30) and carcinomas (31). Further, butyrate treatment enhanced synthesis of a higher molecular weight MAB DF3 reactive antigen which was detectable in human milk (18). The presence of DF3 antigen in human milk and on the apical borders of more differentiated cells therefore suggests that the synthesis of this antigen represents a differentiated secretory function of mammary epithelium.

The detection of elevated circulating levels of DF3 antigen may represent tumor degradation with release of antigen or secretion by a mechanism such as reverse pinocytosis. The detection of other milk fat globule membrane antigens in the sera of breast cancer patients (28) suggests that several antigens may be released and that this process could be accelerated in malignant cells. It is of interest that the circulating antigen levels in patients with visceral disease were significantly higher than those obtained in patients with bone, skin, or locally recurrent disease. However, only patients with visceral or bone lesions had significantly elevated DF3 antigen levels when compared to controls. These findings may be related to tumor burden, degree of tumor differentiation, and/or site of disease. Patients with only skin or nodal disease might be considered to have less tumor burden than those with bone or visceral involvement who are generally considered to have more

extensive disease (32). Furthermore, the degree of breast tumor differentiation has recently been shown to be a determinant of intracellular DF3 antigen content (33) and therefore may also influence circulating levels. Another possibility is that the presence of breast cancer metastases in nonvisceral organs may not be conducive to secretion of the DF3 antigen. The DF3 antigen may be degraded by the liver. Thus, the presence of intrahepatic metastases could allow shedding of the antigen directly into the systemic circulation. The DF3 antigen is a glycoprotein (unpublished data) and may be susceptible to hepatic degradation. Our studies in metastatic colon cancer suggest that liver metastases are not sufficient to elevate circulating DF3 antigen levels, although cirrhosis or hepatitis can result in elevated values. The findings in cirrhosis and hepatitis could be related to decreased hepatic clearance of the DF3 antigen. Finally, separation of tumor site and burden as independent factors contributing to the elevation of DF3 antigen levels will require a prospective study with objective clinical staging criteria (34, 35).

DF3 antigen levels are thus elevated in 70% of all patients with metastatic breast cancer and in nearly 90% of patients with visceral disease. In contrast, 65 patients with primary and secondary breast cancer who at the time of sampling were considered to be clinically free of disease were monitored for DF3 antigen, and only two of these patients had an elevated level. Further, eight patients with metastatic breast cancer have been monitored during progression of disease and/or response to therapy. In each case, DF3 antigen levels reflected progression or response. These findings suggest that DF3 antigen levels are useful in monitoring the clinical course of breast cancer patients. In contrast, our results do not demonstrate that elevated DF3 antigen levels are specific for the diagnosis of breast cancer. The diagnostic value of monitoring DF3 antigen levels will require extensive investigations that examine plasmas from large numbers of patients with a wide variety of malignancies. Additional studies will also be needed to determine the predictive value of DF3 antigen levels and whether these levels will be useful in monitoring breast cancer recurrence before detection of disease by current diagnostic approaches.

*Appendix. Circulating DF3 Antigen Values and Sites of Disease in Patients with Metastatic Breast Cancer*

Patient	Sites of disease										DF3 value (U/ml)	
	Pr	Bo	BM	Sk	LN	Li	Ab	Lu	PE	Br	EIA U	RIA U
1	+	+	+			+					180	2,000
2	+	+	+			+					45	
3	+	+									20	1,500
4		+	+				+				75	
5		+				+		+	+		500	
6		+	+				+				1,000	
7		+			+	+					180	
8				+		+		+			150	1,200
9				+				+			60	
10				+				±	+		300	1,000
11		+	+	+		+			+		22	
12		+	+	+	+	±	+	+		+	500	3,500
13	+			+							350	
14		+				+		+			350	3,000
15					+	±		+			10	
16							+	+			2,100	10,000
17					+	±		±			300	

Appendix (Continued). Circulating DF3 Antigen Values and Sites of Disease in Patients with Metastatic Breast Cancer

Patient	Sites of disease										DF3 value (U/ml)	
	Pr	Bo	BM	Sk	LN	Li	Ab	Lu	PE	Br	EIA U	RIA U
18	+			+		+			+		11,000	
19		+				±			+		80	
20		+		+				+			60	
21	+	+				+		+			900	10,000
22				+	+	±	+				120	
23					+	+		+	+		3,600	10,000
24		+				+					150	1,000
25		+		+	+		+		+		35	
26				+				+	+		60	450
27		+			+	±					400	
28		+			+			+			24	
29		+				+					250	
30		+		+		+					110	700
31		+		+		+		+		+	230	1,000
32		+				+		+			9,000	
33		+						+	+			6,000
34	+							+				800
35					+			+	+			600
36		±				+						400
37					+	+	+	+	+			600
38				+				+				100
39	+	+				+		+		+		10,000
40	+	+		+							40	300
41		+		+	+						28	350
42	+			+							16	500
43		+									33	600
44		+									50	450
45	+	+		+		±				+	13	150
46				+	+						25	300
47		+	+								70	200
48		+									70	150
49		+		+							85	
50				+							29	50
51				+							58	
52		+		+							20	20
53		+									32	200
54				+	+						30	20
55				+							40	
56				+	±						25	150
57		+									35	
58				Muscle							<5	
59				+							<5	
60				+							7	
61		±		+	+						25	
62					+						17	35
63		+									42	
64	+	+									130	
65		+		+							35	
66				+								50
67	+	+		+							70	
68				+								200
69		+										200
70		+										30
71		+										20
72	+	+		+								15
73	+			+								200

Pr, primary; Bo, bone; BM, bone marrow; Sk, skin; LN, lymph node; Li, liver; Ab, abdominal; Lu, lung; PE, pleural effusion; Br, brain; +, known involvement; ±, suspected involvement; and blank space, no involvement.

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