MAM-6 Antigen, a New Serum Marker for Breast Cancer Monitoring

J. Hilkens, V. Kroezen, J. M. G. Bonfrer, M. De Jong-Bakker, and P. F. Bruning
Departments of Tumor Biology and Clinical Oncology, The Netherlands Cancer Institute (Antoni van Leeuwenhoek Huis), Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

ABSTRACT
Almost all carcinomas contain a cell surface antigen, MAM-6, which has been defined by several monoclonal antibodies, including 115D8 (Hilkens et al., Int. J. Cancer, 34: 197–206, 1984). A quantitative sandwich radioimmunoassay, using 115D8 as catcher and as tracer antibody, has been developed to detect MAM-6 in serum. To quantitate the MAM-6 level, pooled human milk was used as a standard, and arbitrary units were chosen. Less than 5% of the sera of apparently healthy individuals contained more than 5 units/ml. In sera of patients with benign breast lesions, the same low levels were detected. However, concentrations over 5 units/ml were found in 24, 21, 43, and 79% of the sera of patients with pathological Stages I, II, III, and IV breast cancer, respectively. MAM-6 levels were also increased in almost all sera tested from patients with advanced stages of ovarian carcinoma, but in a low percentage of sera from patients with other advanced cancers. A longitudinal study was carried out to test the MAM-6 assay as clinical marker to monitor the therapeutic response of breast cancer. Increasing or decreasing MAM-6 serum levels correlated in 93% of the cases with breast cancer progression or regression, indicating that the assay can be used to monitor the course of the disease during therapy. In some breast cancer patients, elevated MAM-6 levels were observed prior to any clinical indication of tumor recurrence.

INTRODUCTION
Tumor-associated antigens present in the serum of cancer patients can be useful markers for monitoring cancer therapy (1–4). CEA, α-fetoprotein, and chorionic gonadotropin are generally accepted as tumor markers, although none of these is tumor specific. MAbs have been used to improve the specificity and sensitivity of detection of such markers in serum (5) and to define new cancer-associated antigens. The recently introduced markers for ovarian and gastrointestinal carcinoma, Ca 125 and Ca 19.9, have thus been defined by MAbs (1, 2, 6, 7). Both markers have been successfully used for monitoring cancer treatment but proved to be of limited value for the diagnosis of cancer, due to lack of specificity.

No suitable marker has been identified yet to monitor the course of breast cancer routinely. Presently CEA is most commonly used for this purpose. However, CEA has not been very useful for breast cancer, since less than 50% of these patients show elevated CEA serum levels, and there is only a weak correlation with the development of the disease (8–11). Ca 125 and Ca 19.9 were elevated in about 10% of the breast cancer patients (6, 7), which is insufficient for a useful serum marker of this disease. Recently, Papsidero et al. (12), Burchell et al. (13), and Hayes et al. (14) developed radioimmunoassays which detected breast cancer-associated antigens defined by monoclonal antibodies in a high percentage of sera obtained from advanced breast cancer patients. Ceriani et al. (15) observed elevated levels of circulating breast cancer-associated antigens by using a radioimmunoassay with polyclonal antibodies to human milkfat globule membranes. The sensitivity of most of these assays is not very high, since elevated antigen levels were only found frequently in sera from advanced breast cancer patients, while low or normal levels were found in almost all investigated patients with early cancer. A correlation with the course of the disease has been reported in only one case (14).

We have previously reported the production of a panel of monoclonal antibodies to differentiation antigens of the mammary gland (16, 17). One of these antibodies, 115D8, detects the MAM-6 antigen. MAM-6 is an epithelial membrane marker present at the apical side of epithelial cells lining normal ductal and alveolar structures. MAM-6 has also been detected on a variety of carcinomas, and it is often homogeneously present on all cells. In previous studies we have shown that MAM-6 can be used as a carcinoma marker on tissue sections (17, 18). The antigen is heavily glycosylated, and when immunoprecipitated from human skin milk, it appears on sodium dodecyl sulfate-polyacrylamide gels as two bands of an apparent molecular weight of over 400,000 (19). MAb 115D8 is directed to the MAM-6a epitope which is present on the carbohydrate side chains of the glycoprotein. As we have shown in preliminary reports (19, 20), MAM-6 is present in elevated concentrations in the serum from many breast cancer patients. In the present study, we have extended this observation and show that elevated MAM-6 levels are very frequently present in the sera from patients with clinical and pathological Stages III and IV breast cancer, and less frequently in the sera from early breast cancer patients (Stages I and II). Data are presented to show that MAM-6 can be used to monitor the course of the disease during therapy and that elevated MAM-6 levels can be detected before any evidence of recurrent breast cancer.

MATERIALS AND METHODS
Monoclonal Antibodies. 115D8 was raised against human milkfat globule membranes, as described in detail by Hilkens et al. (17).

Clinical Specimens. Serum samples were collected from 132 apparently healthy donors, 9 women in third trimester pregnancy, 12 patients with renal insufficiency, 10 with liver cirrhosis, 8 with hepatitis, 31 patients with benign breast lesions (7 adenofibromas, 14 patients with mastopathia having predominantly epithelial proliferation, and 9 mastopathia with predominantly fibro sclerosis), and cancer patients. A first series of 161 sera was collected from patients with staged breast cancer. Stages I, II, and III of breast cancer were independently determined by the clinician and by the pathologist on surgical specimens taken immediately after the patients’ first admission to the Netherlands Cancer Institute. Staging was based on the UICC classification (21). Metastases in Stages I, II, and III, if present, are restricted to regional lymph nodes; in Stage IV distant metastases are always present. Sera were only considered suitable for this study when the staging determinations of the clinician and the pathologist were in agreement. Stage IV was only determined by the clinician.

A second series of breast cancer sera was prospectively collected from 29 patients with advanced disease (Stage IV) during the time they received therapy according to a trial protocol. At the same time points, the clinical course of the disease was followed by regular intervals of 1 to 3 mo and occasionally 4 mo. Patients described as having progressive disease developed new tumor lesions or showed an increase in size of existing lesions by more than 25%. Regression required a more than 50% reduction of detectable tumor lesions.

A third group of 7 patients with Stage II breast cancer underwent...
surgical removal of the primary cancer and in some cases of the regional lymph nodes. These patients were included in a trial protocol and regularly checked for tumor recurrence, while occasionally blood samples were taken.

Sera from patients with other types of cancer were collected only during advanced stages of the disease when distant metastases were present.

**Human Skim Milk.** Human skim milk was prepared by removing the fat from fresh human milk by centrifugation at 40,000 x g. Protease inhibitors (phenylmethylsulfonyl fluoride and aprotinin, both from Sigma, St. Louis, MO) were added, and the preparation was stored at -20°C. Pooled human skim milk was used as standard for quantitation of the radioimmunoassay.

**Protein Iodination.** In labeling of the MAb 115D8 was carried out using the iodogen method as described by Fraker et al. (22).

**MAM-6 Sandwich Radioimmunoassay.** The sandwich radioimmunoassay was carried out according to the method described before with some minor modifications (20). Briefly, 4.5 µg of Sepharose-Protein A-purified MAB 115D8 (from ascites fluid) in 50 µl of PBS was absorbed to the wells of Falcon PVC microtiter plates at 4°C overnight. The wells were washed 3 times with PBS and preincubated at room temperature for 1 h with 2% rabbit serum in PBS and washed again 3 times in PBS. Fifty-µl samples of at least 4 times diluted human serum in PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate were added in triplicate to the wells and incubated at room temperature for 2 h. Unbound antigen was removed by 3 washes with PBS containing 0.5% Tween 20. Bound antigen was detected with 50 µl of 125I-labeled MAB 115D8 (2-4 ng/ml with a specific activity of 60-90 x 10^3 cpm/ng) in PBS containing Triton X-100, sodium deoxycholate, and sodium dodecyl sulfate containing normal mouse serum and incubated at room temperature for 2 h. Unbound antibody was removed by 3 washes with PBS containing 0.5% Tween 20, and the remaining activity was counted in a gamma counter. The amount of MAM-6 present in sera relative to the amount present in a standard preparation of human skim milk was calculated using a linear regression computer program.

**Statistical Analysis.** The milk standard curve was generated by plotting the bound radioactivity (cpm) and the MAM-6 units after log transformation. Part of the curve could be calculated by linear regression. Dose-response curves of the serum samples were analyzed in the same way, and slopes were compared with the slope of the milk standard. Student’s t test was used to analyze the data statistically. Significant differences of the MAM-6 levels in the control groups and the groups studied were analyzed by the trend test on contingency tables (23).

**RESULTS**

The MAM-6 Assay. A quantitative sandwich radioimmunoassay for MAM-6 was developed by using Mab 115D8 as catcher and as tracer antibody. The assay is similar to the method reported by Bast et al. (1) and assumes that the MAM-6a epitope is repeated on the MAM-6 antigen. Human skim milk which contains large amounts of MAM-6 (19) was used as standard source of antigen. The MAM-6 level present in pooled human skim milk preparations was arbitrarily chosen as 1000 units per ml. The standard curve, which was obtained when various amounts of human skim milk were added in the assay, was reproducible and essentially linear between 0.75 and 40 units/ml in a semilogarithmic plot. The best fit between these levels was obtained by linear regression of the activity bound (cpm) to the log transformed MAM-6 units (r > 0.99; P < 0.01, n = 9). A typical standard curve is shown in Fig. 1. When less than 0.75 or more than 40 units of MAM-6 per ml were added, a nonlinear dose-response curve was observed for which nonlinear regression analysis fit better. When skim milk is used as standard, the slope of the curves obtained with serial dilutions of breast cancer sera should not be significantly different from the one obtained with human skim milk. For this purpose, dose-response curves of 11 positive sera were generated by linear regression. Only the slopes obtained with 2 sera were significantly different from the one obtained with the human milk standard analyzed by Students’ t test (P < 0.05), indicating that skim milk in most cases was suitable as reference.

Table 1 shows the reproducibility of the assay as determined by calculation of the intra- and interassay variation of repeatedly tested, 4 times diluted sera containing different MAM-6 levels. The intra- and interassay coefficients of variation were not uniformly distributed and were lowest in the sera with low MAM-6 concentrations. This difference is probably due to the logarithmic nature of the standard curve, causing small variations in counts to have a relatively large influence on calculations of high MAM-6 values. For that reason we usually diluted the serum samples to less than 30 units/ml.

The possible influence of storage and repeatedly freezing and thawing on the test results was investigated. No difference in MAM-6 levels of 6 positive sera was observed even after 8 freezing and thawing cycles or 3 days of storage at 4°C or room temperature, indicating that the MAM-6a epitope is extremely stable.

**MAM-6 Levels in Healthy Individuals.** Serum samples from 132 apparently healthy adults were assayed. The results are...
presented in Table 2 and Fig. 2. Since the standard curve is linear down till 0.75 units/ml and the assay required at least 4 times dilution of the serum, we could not calculate MAM-6 levels lower than 3 units/ml. Levels less than 3 units/ml were arbitrarily assumed to contain 2 units/ml. Therefore, the actual mean level may be lower. The highest MAM-6 level which was considered normal was set at 5 units/ml, which is about 2 SDs over the mean normal MAM-6 value. Levels over 5 units/ml were considered as positive. Thus, more than 95% of the serum samples from healthy control individuals were negative for MAM-6. Smoking may increase the MAM-6 serum concentration. As is shown in Table 2, 4 of 32 (12%) of the smoking individuals contained levels of over 5 units/ml. However, the MAM-6 serum values of the whole group of smokers were not significantly different from the values obtained from nonsmoking individuals, in the trend test on contingency tables (P = 0.12).

By using similar criteria for significance, sex also had no influence on the MAM-6 serum levels (P = 0.40). In addition, when individuals were separated in age groups of 10 yr, no influence of age could be detected in the trend test (P = 0.22). However, only 8 individuals over the age of 60 were tested. It has to be realized that MAM-6 levels lower than 3 units/ml were arbitrarily considered to contain 2 units/ml; therefore, variation in these lower levels could not be detected.

Considerable variation was observed between individuals, while the intradividual variation measured over more than 1 yr was very small, indicating that the normal MAM-6 level is an individual and stable parameter.

MAM-6 Levels of Noncancer Patients. As demonstrated in Table 3, only 3% of the sera of women with benign breast lesions had serum values above 5 units/ml, which is similar to what was found in the normal population. This was statistically confirmed by the trend test on contingency tables which showed no significant difference between the MAM-6 levels in the normal population and patients with benign breast disease (P = 0.24). MAM-6 levels were elevated in a high percentage of sera from patients with renal insufficiency, in patients with hepatitis and liver cirrhosis, and in third trimester pregnancy (not during earlier stages of pregnancy) (Table 3). These results indicate that health conditions other than cancer may also affect the MAM-6 levels.

MAM-6 Levels in Staged Breast Cancer Patients. Fig. 2 and Table 4 show the MAM-6 levels determined in 161 sera from staged breast cancer patients. Elevated MAM-6 levels were found in 79% of the patients having distant metastases (Stage IV) with a mean value of 142 units/ml. Abnormal MAM-6 levels were less frequently found in earlier stages of breast cancer. More than 40% of the sera from Stage III patients contained over 5 units of MAM-6, per ml but only slightly more than 20% of the sera from Stages I and II patients were positive. Since information about smoking habits or abnormal health conditions other than cancer of these patients was not available, the percentage of positive sera due to breast cancer only may be slightly lower. Elevated MAM-6 levels were found with all histological types of breast cancer. However, we did not investigate whether there was any quantitative relation to the amount of antigen present in sera of patients with different histology.

MAM-6 Levels in Patients with Other Cancers. A limited investigation was carried out with serum from patients with advanced carcinoma, melanoma, or lymphoma. The results are shown in Table 5. Marked elevations were frequently observed in sera of advanced ovarian cancer patients. Approximately the same percentage of positive sera was found with these patients as with advanced breast cancer patients; however, the mean MAM-6 level was lower. A relatively small number of sera from patients with advanced colon, bronchial, and prostate carcinoma, melanoma, and lymphoma showed a small increase of the MAM-6 levels, although all samples were derived from patients with a large tumor burden. These results indicate that increased MAM-6 serum levels are not specific for breast carcinoma, which could be expected since tissue sections from

### Table 2 MAM-6 levels in sera of apparently healthy persons, smokers, and non-smokers

<table>
<thead>
<tr>
<th></th>
<th>Nonsmokers</th>
<th>Smokers</th>
<th>Unknown</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean level (units/ml)</td>
<td>2.8 ± 1.3*</td>
<td>3.3 ± 1.5</td>
<td>2.7 ± 0.9</td>
<td>2.9 ± 1.25</td>
</tr>
<tr>
<td>No. of sera over 5 units/ml</td>
<td>1 (2.2)*</td>
<td>4 (12)</td>
<td>1 (1.9)</td>
<td>6 (4.5)</td>
</tr>
<tr>
<td>No. of sera tested</td>
<td>46</td>
<td>32</td>
<td>54</td>
<td>132</td>
</tr>
</tbody>
</table>

* Mean ± SD.
* Numbers in parentheses, percentage.
other carcinomas were almost always positively staining for MAM-6 (17). However, we also found increased MAM-6 levels in noncancer patients suffering from melanoma and lymphoma, although the mean increase was rather low. The antigen could not have been released by these tumor cells, because tissue sections of melanoma and lymphoma were always MAM-6 negative. Since all sera were derived during a late stage of the disease with a large tumor burden being present, increased MAM-6 levels may be the result of nonspecific tissue damage. This was confirmed by the observation that all 5 melanoma patients with (weakly) positive MAM-6 levels showed large liver metastases, in one case with increased lactate dehydrogenase levels. Most advanced melanoma patients with normal MAM-6 levels showed no large liver metastases. This observation indicates that the antigen levels may be slightly increased by nonspecific liver damage. However, the 3 positive lymphoma patients had no large liver or kidney metastases.

MAM-6 Levels and the Clinical Course of Breast Cancer. In a prospective study, the MAM-6 serum levels were determined in serum samples of 29 breast cancer patients followed over 1- to 6 mo periods. Each patient had previously been treated for breast cancer or received therapy during the observation period. The clinical course of the disease was evaluated by standard criteria and indicated as progression, regression, or stable disease. Only patients with at least one MAM-6-positive serum value were considered for evaluation. In 6 of the 29 patients, it was possible to monitor both regression and progression of the disease, which provided us with 35 evaluable cases. Fig. 3 shows the MAM-6 data, arranged according to the clinical course. Two patients with progressive disease had no change; all others showed increasing MAM-6 serum values. In two patients with clinically stable disease, only small changes of the MAM-6 levels were observed, whereas one patient showed a moderate rise of the MAM-6 level. The only contradictory observation was made in a patient whose skin metastases had regressed but who showed a rising MAM-6 serum level. Computerized tomography revealed that visceral metastases were stable. However, 2 mo after the last MAM-6 assay was performed, the visceral lesions showed progression, while skin metastases were still regressing.

Clinical progression is defined as an increase in size of the tumor lesions of at least 25% and regression as a reduction of at least 50%. If similar criteria were applied to indicate significant changes in MAM-6 levels, MAM-6 increase of 25% and decrease of 50% could be considered as progression and regression, respectively. Using such criteria, a comparison was made with the clinical course of the disease, as is shown in Table 6. MAM-6 levels correlated with the clinical course of the disease in 83% (29 of 35) of the cases (95% confidence interval, 66-94%). If only patients with significantly increasing or decreasing MAM-6 levels were considered, a correlation of 93% (27 of 29) with clinical progression or regression was observed (95% confidence interval, 77-99%).

Lead Time. In a longitudinal study, we determined the MAM-6 levels in 7 Stage II breast cancer patients who eventually developed metastases and from whom sera and clinical data were available prior to the clinical manifestation of metastases. Six patients became MAM-6 positive, and one patient remained negative even when a considerable tumor burden was apparent. Three of the 6 evaluable patients showed rising MAM-6 serum levels before any clinical symptoms of metastases became manifest. The MAM-6 levels of these 3 patients are shown in Fig. 4. If the lead time is defined as the interval between the first elevated MAM-6 value and the last clinical follow up with no clinical evidence of metastases, the lead time varied from a few
cepted for the clinical evaluation of the tumor response, then
or decreased were considered, the correlation was even 93%.
only the cases in which the MAM-6 value significantly increased
significance of serum changes were proportional to those ac
breast cancer on therapy. If the criteria which were applied for
test for its clinical usefulness to monitor the response of
weeks to 4 mo. In 2 other patients, bone metastases were
detected on a bone scan 6 to 12 wk before any rise in MAM-6
level could be observed. The sixth patient showed regional
tumor progression, while the MAM-6 level remained normal.
However, MAM-6 levels became elevated when distant metas-
tases developed later.

**DISCUSSION**

The MAM-6 radioimmunoassay described in this paper was
tested for its clinical usefulness to monitor the response of
breast cancer on therapy. If the criteria which were applied for
significance of serum changes were proportional to those ac-
cepted for the clinical evaluation of the tumor response, then
the correlation between the MAM-6 serum levels and the tumor
response was 83%. When the same criteria were applied and
only the cases in which the MAM-6 value significantly increased
or decreased were considered, the correlation was even 93%.
The latter approach may be more meaningful, since it shows
that patients with significant increasing or decreasing MAM-6
levels have progressive or regressive disease with more than
90% reliability. Our preliminary data also indicate that the
absence of a significant change of the MAM-6 serum level is
not a reliable indicator of the tumor development. The return
of the MAM-6 level to normal does not necessarily indicate
complete tumor regression, since a small tumor load does not
cause an elevated MAM-6 level due to the limited sensitivity of
the assay. This conclusion is based on the observation that
Stage I and II breast tumors cause elevated MAM-6 levels in
only slightly more than 20% of the cases. The latter observation
implicates that the assay at its present sensitivity is unsuitable
for large scale screening of women at risk.

When the threshold level for positivity was established, the
influence which smoking habits, abnormal health conditions,
or very old age may have on the MAM-6 level was not consid-
ered. If the threshold level for positivity would be increased
to 8 units/ml, most of the falsely positive levels would be avoided,
and less than 1% of the tested normal sera would be abnormally
elevated. However, several breast cancer patients showed rising
MAM-6 levels, still lower than 8 units/ml, due to tumor pro-
gression, indicating that a threshold level of 8 units/ml for
positivity is too high. Before the value of the assay as cancer
test can be established more definitely, a more elaborate inves-
tigation is required. We report here mainly on the assay merits
for monitoring breast cancer. Feller et al. described an enzyme-
linked immunosorbant assay using a combination of 2 assays,
one with 115D8 and one with 67D11, the latter being a MAAb
directed to another antigen. They were able to detect elevated
antigen levels at high frequency in all stages of breast cancer.
In our studies, only Stages III and IV were positive at high
frequency. In the sera of Stage III patients, the mean MAM-6
values were still rather low, being only twice above the mean
normal level. Only the mean serum level in Stage IV patients
was more than 50 times over normal with large variations in
the levels of the individual sera, some sera containing even more
than 500 times the mean normal MAM-6 level.

The lead time observed in a small group of patients shows
that the assay can be more sensitive than conventional means
do detection of breast cancer metastases. However, the lead time
observed with the MAM-6 assay greatly depends on the screen-
ing frequency of the patients. Also the localization of the
metastases may have a considerable influence on the detecta-
bility. A large prospective study is needed for more precise
assessment of the lead time.

The MAM-6 assay is not specific for breast cancer. Elevated
antigen levels were also present in the sera of patients with other
epithelial cancers. Particularly, sera from patients with
ovarian carcinoma contained large amounts of antigen. We
have no data available yet to determine the sensitivity of the
assay for this type of cancer. Wargalla et al. (24) compared our
present assay with the Ca 125 assay in longitudinal studies with
ovarian carcinoma patients. They reported that some of the
patients were Ca 125 negative but MAM-6 positive, while the
reverse was also observed, but less frequently. Elevated MAM-
6 serum levels were also found in some advanced colon and
bronchial carcinoma patients, but the mean level was only twice
above the mean normal level. Much larger percentages of these
tumors and their metastases were positive as detected with
histochemical staining of tumor sections (17). This discrepancy
between the histochemical and serum results was also found to
a lower extent for some of the breast cancer cases and may be
explained by diminished expression or release of antigen by
these tumors. It is not completely understood why some of the
sera from advanced melanoma and lymphoma patients were
positive, while tumor sections never stained positive for MAM-
6. From the data obtained with melanoma patients, it seems
likely that nonspecific tissue damage caused by tumor invasion
of the liver is responsible for the observed moderate increase in
MAM-6 levels (maximal increase to 10 units/ml in melanoma

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1 W. F. Feller, J. Kantor, J. Hilken, and J. Hilgers, manuscript submitted for publication.
patients and to 7 units/ml in lymphoma patients). Similarly, the damage of the liver and kidney by other diseases sometimes also raised the serum MAM-6 concentration (Table 3), possibly as a result of the release of antigen, which is present in many tubular organ structures (17), or the degradation rate of MAM-6 glycoproteins may be decreased in patients with impaired liver or kidney function. It can be expected that, in advanced breast cancer, the modest contribution of nonspecific tissue damage to the measured MAM-6 levels is relatively insignificant as compared to the MAM-6 release from the tumor.

Sera from advanced breast cancer patients contained elevated MAM-6 levels more frequently than elevated CEA levels, the latter being elevated in less than 50% of these patients. The sensitivity of the MAM-6 assay in breast cancer is comparable to the Ca 125 assay for ovarian carcinoma (1), the Ca 19.9 assay for pancreatic and colorectal cancer (2), and the CEA assay for colon cancer. Comparable breast cancer assays with similar or less sensitivity have been described by Ceriani et al. (using polyclonal antibodies (15) and by Papsidero et al. (12), Burchell et al. (13), and Hayes et al. (14) using monoclonal antibodies. None of these assays has proven to be suitable for routine monitoring of breast cancer therapy. The MAM-6 assay offers a convenient method to serve this purpose.

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