Leukocyte Migration Inhibition Responses to MCF-7, Murine Mammary Tumor Virus, and Thomsen-Friedenreich Antigen in a Series of Cancer Patients

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ABSTRACT

Previous reports have shown that breast cancer patients have exhibited leukocyte migration inhibition test (LMIT) responses to antigenic preparations of the MCF-7 breast cancer cell line, murine mammary tumor virus (MTV), and the T-antigen (Thomsen-Friedenreich antigen), a precursor of the M and N blood group system. We have previously demonstrated significant responses by breast cancer patients to the two antigens, MCF-7 and MTV. Springer has shown a high frequency of responses in breast cancer patients to the T-antigen (TAg). We have measured the LMIT to MCF-7, MTV, and TAg in patients with primary breast cancer and in patients with other cancers (colorectal, head and neck, and melanoma). The LMIT to concanavalin A was used to ensure an adequate capacity to generate a response in individual patients. The majority of the cancer patients gave a normal LMIT to concanavalin A.

In this study, we observed a significant response to the TAg in patients with primary breast cancer. This occurred mainly in patients with regionally advanced disease, 38% of whom responded, and less so in patients with Stage I and II disease. None of the other types of cancer patients responded to TAg. We did find a lower frequency of responses in the breast cancer patients to MCF-7 and MTV than that we have reported previously. This may be due to differences in antigen preparations or possibly patient populations. The response rate to MCF-7 was distinctly related to timing of surgery, was lowest during the first and second postoperative months, and returned to the preoperative level at 6 months. There was a high degree of correlation among the paired responses to MCF-7, MTV, and TAg in the breast cancer patients, possibly suggesting the presence of cross-reacting antigens in the preparations tested.

The response to the TAg, although lower than that reported by Springer et al. [Cancer (Phila.), 45: 2349–2954, 1980] suggests promise as a probe in the immunological evaluation of breast cancer.

INTRODUCTION

The LMIT provides useful information regarding states of cellular immunity, including antigen recognition and the ability to generate lymphokines and to respond to them by inhibition of migration (9). The LMIT does provide data regarding preexisting sensitization and cellular responsiveness to defined antigens. By means of this assay, breast cancer patients have been reported by different investigators to have significant responses to 3 apparently independent antigens prepared from the MCF-7 breast cancer line (2, 6, 8, 10), the MTV (1, 5, 15), and the TAg as described by Springer et al. (11–14). We have addressed the question of whether there were similar responses in patients with other types of cancer and of whether there were any correlations of the LMIT responses to these 3 antigens in either breast cancer patients or in patients with selected other cancers including colorectal cancer, head and neck cancer, and melanoma. The LMIT responses to a mitogen, Con A, and a nonspecific antigen, SPL, were also measured to ensure that there was capacity to develop an LMIT response, since that could be a potential limitation of this assay in patients with known depression of cell-mediated immunity.

MATERIALS AND METHODS

Patients and Controls. Control subjects consisted of 66 laboratory and hospital workers from 29 to 56 years old. All were in good health and were not receiving medication at the times of blood donation. Patient studies included 16 with benign breast disease as demonstrated by biopsy and 171 cancer patients who had been admitted to the University of Virginia Hospital. There were 36 patients with early and 33 with regionally advanced breast cancer, 14 with early and 19 with advanced colorectal cancer, 19 with early and 14 with advanced melanomas, and 7 with early and 29 with advanced head and neck cancer. Many of the subjects were followed over time and were therefore tested repeatedly.

Antigen Preparation. The mitogen Con A (Difco Laboratories, Detroit, Mich.) was used as a standardized reference reagent. SPL types I and III (Delmont Laboratories, Inc., Swarthmore, Pa.) were used as commercially available. The MTV preparation was a generous gift from Dr. Arnold Dion, Institute for Medical Research (4) Camden, N. J. The source of the virus was milk from the MTV and RIII mouse. It had been purified through sucrose gradient, had been concentrated by ultracentrifugation, and had been frozen and thawed (methanol:dry ice) 3 times prior to use (16). The MCF-7 had been prepared by freezing and thawing of MCF-7 cells (human adenocarcinoma of breast, our passage 26) 3 times, followed by overnight agitation in 0.9% NaCl solution, removal of particulate materials by centrifugation for 1 hr at 40,000 × g (at 4°), and filtration through 0.22-μm Millipore filters. This material was aliquoted in concentrated form and stored at −85°. Aliquots were defrosted and diluted to working concentrations immediately prior to use. The TAg in lyophilized form was kindly provided by Dr. George Springer (Northwestern University, Evanston, Ill.). These materials were diluted in phosphate-buffered saline (Dulbecco’s IX; Grand Island Biological Co., Grand Island, N. Y.), and the protein concentration was adjusted using the method by Lowry et al. (7).

Migration Inhibition Assay. Leukocyte migration inhibition assays were performed using peripheral blood leukocytes in the capillary tube method as described previously (5, 9). Migration areas were measured with a Talos 7419E digitizer (Talos Systems, Inc., Scottsdale, Ariz.) and an Apple II microcomputer. Con A was used at 3 μg/ml, a concentration which produced consistently positive results without significant agglutination of peripheral blood leukocytes. SPL types I and III were used.

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The abbreviations used are: LMIT, leukocyte migration inhibition test; MTV, murine mammary tumor virus; TAg, T-antigen (Thomsen-Friedenreich antigen, a precursor of the MN blood group system); Con A, concanavalin A; SPL, Staph phage lysate.

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RESULTS

Activity to the various test antigens were examined by calculating a Pearson product moment correlation coefficient. Correlations of reactive or nonreactive relative to the group as a whole. Where analysis of variance produced a significant overall group F (0.05), Duncan’s multiple-range test was used to determine which groups or subgroups were significantly different from that of the control population or from those of other cancer groups, plotting migration index (mean of triplicate migration areas) versus age of individuals within the group.

Definition of Population Groups. Comparison of patients with benign breast disease to the control population showed that patients with benign breast disease were different from controls in reactivity to any of the test substances or migration in control media. These 2 groups were therefore merged.

Each of the 4 groups of cancer patients (breast, colorectal, head and neck, and melanoma) were analyzed in groups and then in subgroups by stage of disease with Stages I and II merged as “early cancer” and Stages III and IV as “advanced cancer.” Comparison of patients with benign breast disease to the control population showed that patients with benign breast disease were not different from controls in reactivity to any of the test substances or migration in control media. These 2 groups were therefore merged. All persons represented in the final analysis were included in the tabulations.

Table 1

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>Mean migration index of cancer patients (by group) and controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean migration area with medium alone</td>
</tr>
<tr>
<td></td>
<td>Con A</td>
</tr>
<tr>
<td>Controls</td>
<td>38 (82)</td>
</tr>
<tr>
<td>Breast</td>
<td>60 (69)</td>
</tr>
<tr>
<td>Colorectal</td>
<td>62 (33)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>47 (33)</td>
</tr>
<tr>
<td>Head and neck</td>
<td>48 (28)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number of patients.
* Numbers in parentheses, number of assays. Certain individuals were tested more than once. All persons represented in the final analysis were included in the tabulations.
* Numbers in parentheses, number of cases. Where analysis of variance produced a significant overall group F (0.05), Duncan’s multiple-range test was used to determine which groups or subgroups were significantly different from that of the control population or from those of other cancer groups, plotting migration index (mean of triplicate migration areas) versus age of individuals within the group.

A Pearson product moment correlation coefficient was used to distinguish among population groups (17). Correlations of reactivity to the various test antigens were examined by calculating a Pearson product moment correlation coefficient. Where analysis of variance produced a significant overall group F (0.05), Duncan’s multiple-range test was used to determine which groups or subgroups were significantly different from others. In addition, nonparametric (Wilcoxon rank sum test) analysis was used to distinguish among population groups (17). Correlations of reactivity to the various test antigens were examined by calculating a Pearson product moment correlation coefficient.

RESULTS

Spontaneous Leukocyte Migration. The spontaneous leukocyte migration (leukocytes in media alone) was nearly identical among controls and breast cancer and melanoma patients but was markedly decreased in patients with colorectal and head and neck cancer (Table 1).

The reactivity to Con A is shown in Chart 1. All groups and subgroups showed good reactivity to Con A, including those which showed decreased migration in control medium. Patients with advanced breast cancer were the most reactive to Con A (p = 0.038), although this was not judged to be significant due to the decision to require a p level of 0.005 for significance when comparing group and subgroup means because of the number of comparisons involved. Because of differences in the spontaneous leukocyte migration in certain patient groups, we examined whether there were significant correlations between spontaneous leukocyte migration and the LMIT with different test substances.

Correlations among Migration Areas in Control Medium and Migration Inhibition in Test Substances. There was a significant correlation between the LMIT to Con A and spontaneous leukocyte migration in the control medium of controls and in patients with cancers of the breast, head and neck, and melanoma but not in colorectal cancer (Table 2). Leukocyte migration inhibition...
Table 2
Correlations among mean migration area in control medium and migration index in each of the test antigens

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Breast cancer</th>
<th>Colorectal cancer</th>
<th>Melanoma</th>
<th>Head and neck cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r$</td>
<td>$p$</td>
<td>$n^*$</td>
<td>$r$</td>
<td>$p$</td>
</tr>
<tr>
<td>Con A</td>
<td>-0.338</td>
<td>0.0001</td>
<td>131</td>
<td>-0.005</td>
<td>0.71</td>
</tr>
<tr>
<td>MCF-7</td>
<td>-0.188</td>
<td>0.08</td>
<td>91</td>
<td>-0.339</td>
<td>0.04</td>
</tr>
<tr>
<td>MTV</td>
<td>-0.041</td>
<td>0.71</td>
<td>81</td>
<td>-0.204</td>
<td>0.22</td>
</tr>
<tr>
<td>TAg</td>
<td>-0.458</td>
<td>0.0005</td>
<td>54</td>
<td>-0.249</td>
<td>0.16</td>
</tr>
</tbody>
</table>

$^*$ $n$, number of assays.

### Reactivity of Putative Breast Cancer-associated Antigens

Previous studies demonstrated a relationship among the LMIT responses to MCF-7 and MTV in breast cancer patients (4, 13). When the current data were analyzed by cancer group or subgroup, only TAg showed a relationship to breast cancer (Chart 2). The responses to MCF-7 and MTV overall were not significantly different from those of the control group. When the LMIT responses to MCF-7 were examined in reference to timing of surgery, there were some apparent differences (Chart 3). The responses to MCF-7 were 20% in the preoperative patients versus 9% in the group 1 to 2 months postoperation. At 6 months, the response rate was again 19%. The migration index corresponding to the 10% cutoff point to TAg was 0.796 in controls; therefore, values less than this were considered positive. Analysis by group, based upon the cutoff point, showed that 26% of the patients with breast cancer were reactive to TAg ($p = 0.025$) (Chart 4). Subgroup analysis revealed that patients with localized breast cancer were not different from controls in their reactivity to TAg, whereas 38% of patients with regionally advanced breast cancer (Stages III and IV) were reactive ($p = 0.005$) (Chart 4). Analysis of these data by subgroup means also showed significance. Patients with advanced breast cancer were more reactive to TAg than were controls ($p = 0.003$). When the assays were analyzed with reference to timing of surgery, the majority of responses were found in patients who were more than 5 months postoperation.

### Correlation Analysis

Reactions to each of the test substances were analyzed for correlations among all possible pairs of test substances (Table 3). Correlations were found among the LMIT responses to the 3-antigen preparation, MCF-7, MTV, and TAg, in the cancer patients as a group, but they were most significant in the breast cancer patients (Chart 5). There were significant correlations among the responses to the antigen pairs: MCF-7 and TAg, $r = 0.69$; MCF-7 and MTV, $r = 0.49$; and MTV and TAg, $r = 0.60$. The $p$ values were all $<0.0001$. No correlations were found in the LMIT responses to any of these 3 antigens and Con A. Among controls, $r$ values were low in correlations between the antigen pairs MCF-7 and TAg ($r = 0.14$; $p$, not significant) and MTV and TAg ($r = 0.31$; $p = 0.037$) but were higher in MCF-7 and MTV pair ($r = 0.58$; $p = 0.037$).

### DISCUSSION

A major objective of this study was to measure the LMIT to 3
were significant only in the breast cancer patients. Positive responses occurred to TAg (p = 0.025). This response was primarily in patients with advanced breast cancer (38% reacted to TAg); patients with localized breast cancer did not respond differently from controls. The response to MCF-7 was overall not significantly different from that of controls. When patients were segregated according to timing of operation, preoperative patients and those who were 6 months or more postoperation had a 20% frequency of response compared to a 9.5% response frequency in patients tested at 1 to 2 months postoperation. Although this was not significant, it suggests that this relationship might be worth pursuing in future studies. We had previously reported LMIT responses to MCF-7 and MTV of 50 and 49%, respectively, in a larger group of breast cancer patients studied at Memorial Sloan-Kettering (New York, N. Y.) (4, 5). Some of these differences may be explained in population and staging differences, as well as differences in the antigen preparations used. The MCF-7 line has been carried in our laboratory for 26 generations, and there may be antigenic differences in the cell line from that originally used, or perhaps there may be differences in antigen expression according to timing of removal from culture. In our previous studies, extracts were made from large batches of cultured MCF-7 and were frozen for testing as a single pooled antigen preparation. Although we attempted to duplicate this, we did work with a different MCF-7 line, and perhaps antigen concentrations under current culture conditions were different. The MTV was obtained from a different laboratory, although from a similar source, milk from the RIII mouse. We would have expected this antigen to have given similar results as obtained previously. Unfortunately, our original pool of antigen is no longer available. Although the method of conducting the LMIT assay has been quite standardized in this laboratory for years, the manner of interpreting positive versus negative responses is open to question. We have customarily used the 10% cutoff of the responses to an antigen in the control population as the indicator level to determine the response in the cancer patients to that antigen. In this regard, we find markedly different cutoff points to different antigens in controls; thus, in controls, the 10% cutoff level was 0.95 with Con A, 0.87 with MCF-7, 0.75 with MTV, and 0.796 with TAg. The end point of a positive test, whether selected on the basis of a 10% cutoff point or using the 90% confidence level, does allow for a great deal of variability when using this assay (2, 4). Our effort to use the Con A response on an individual basis as a method of determining the patient’s baseline level of response was also associated with variability that precluded rigid acceptance of the Con A selected end point as the end point to be used in the test for each antigen. Some patients who showed a positive response to the antigen had only borderline responses (above 0.95) to Con A.

In spite of the problems with lower response rates to MCF-7 and MTV in the breast cancer patients, there were significant correlations in the LMIT responses to MCF-7 and MTV. We have observed this previously (5, 15). There were also apparent correlations between the responses to MCF-7 or MTV and TAg in the breast cancer patients. There was no such correlation with the response to Con A or SPL. Since most of the responses to MCF-7 and MTV fell in the response range of the controls, it is likely that the significant association between MTV and MCF-7 (r = 0.666; p < 0.0001) is due to similarity in incremental variation which occurred within the “nonresponse” range for each test substance. Although this suggests there may be some antigenic cross-reactivity, interpretation is difficult because of the low number of defined positive responses. The apparent correlation of response with either MCF-7, MTV, or TAg is also of interest.

Table 3

<table>
<thead>
<tr>
<th>Test antigens</th>
<th>n</th>
<th>r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7 and MTV</td>
<td>246</td>
<td>0.6660</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MCF-7 and TAg</td>
<td>177</td>
<td>0.5038</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MTV and TAg</td>
<td>174</td>
<td>0.5908</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

n, number of paired observations.

Putative breast cancer-associated antigens in a broad sample of cancer patients and to determine whether the responses were restricted to the breast cancer patients. Measurement of the LMIT to a standardized stimulant, such as Con A, and measuring the spontaneous migration in control media provided data on the ability of individuals to mount a response and to identify patients who might be nonresponsive to Con A and who therefore might be unable to generate a response to the test antigens. Overall, the cancer patients showed a normal range of responses, both in spontaneous migration and in the responses to Con A, except for depressed spontaneous leukocyte migration in patients with colorectal cancer and head and neck cancer. Although the spontaneous migration of leukocytes (in media alone) was depressed in these 2 patient groups, the LMIT to Con A was not significantly different from that in controls. There was correlation of the spontaneous migration and the ability to generate a Con A response in controls and in certain cancer patients (melanoma and head and neck cancer) but not in patients with breast or colorectal cancer. In general, the Con A response did not predict which patients were more likely to respond to individual test substances, and a nonresponse to Con A was not significantly different from that of controls. When patients were segregated according to timing of operation, preoperative patients and those who were 6 months or more postoperation had a 20% frequency of response compared to a 9.5% response frequency in patients tested at 1 to 2 months postoperation. Although this was not significant, it suggests that this relationship might be worth pursuing in future studies. We had previously reported LMIT responses to MCF-7 and MTV of 50 and 49%, respectively, in a larger group of breast cancer patients studied at Memorial Sloan-Kettering (New York, N. Y.) (4, 5). Some of these differences may be explained in population and staging differences, as well as differences in the antigen preparations used. The MCF-7 line has been carried in our laboratory for 26 generations, and there may be antigenic differences in the cell line from that originally used, or perhaps there may be differences in antigen expression according to timing of removal from culture. In our previous studies, extracts were made from large batches of cultured MCF-7 and were frozen for testing as a single pooled antigen preparation. Although we attempted to duplicate this, we did work with a different MCF-7 line, and perhaps antigen concentrations under current culture conditions were different. The MTV was obtained from a different laboratory, although from a similar source, milk from the RIII mouse. We would have expected this antigen to have given similar results as obtained previously. Unfortunately, our original pool of antigen is no longer available. Although the method of conducting the LMIT assay has been quite standardized in this laboratory for years, the manner of interpreting positive versus negative responses is open to question. We have customarily used the 10% cutoff of the responses to an antigen in the control population as the indicator level to determine the response in the cancer patients to that antigen. In this regard, we find markedly different cutoff points to different antigens in controls; thus, in controls, the 10% cutoff level was 0.95 with Con A, 0.87 with MCF-7, 0.75 with MTV, and 0.796 with TAg. The end point of a positive test, whether selected on the basis of a 10% cutoff point or using the 90% confidence level, does allow for a great deal of variability when using this assay (2, 4). Our effort to use the Con A response on an individual basis as a method of determining the patient’s baseline level of response was also associated with variability that precluded rigid acceptance of the Con A selected end point as the end point to be used in the test for each antigen. Some patients who showed a positive response to the antigen had only borderline responses (above 0.95) to Con A.

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Chart 5. Correlations between the responses to various antigen preparations and the corresponding r and p values (Pearson correlation coefficient) for cancer patients (A, B, and C) and controls (D, E, and F).
This could be explained by the possibility that the MCF-7 breast cancer cell line may contain TAg, which is extracted during preparation of the test antigen. The relationship between the response to MTV and TAg is less obvious. Several microorganisms have been shown to possess antigens which cross-react with human blood group substances (3, 11). Also, a T-like antigen has been described in the glycoprotein coat of the murine TA-3 mammary carcinoma (3). However, TAg has not been reported as a MTV component. We are currently attempting to demonstrate whether relationships exist between MCF-7, MTV, and TAg using well-characterized antisera. Whether neuraminidase treatment of MCF-7 and MTV antigen preparations would expose antigenic residues characteristic of TAg also merits exploration.

Springer used the LMIT in agarose plates and measured the sensitization towards T and MN antigens. The antigen was preincubated at a concentration of 10 μg/10 μl of media containing 1.5 x 10⁶ leukocytes, and a migration index less than 0.90 was considered positive. Among 68 patients with breast carcinoma stages II, III, and IV, 51% showed a positive reaction, whereas 31 patients with Stage I disease 32% were positive (14).

In our study, we used TAg in concentrations of 5 and 10 μg/ml in the capillary tube method without preincubation and defined a migration index of 0.78 as the cutoff point indicating a positive response. This was equivalent to the 10% cutoff point among the responses in controls to TAg. We observed a 38% incidence of positive reactions in patients with Stage III and IV disease (most were Stage III). The differences in the 2 studies reside mostly in technical factors such as type of LMIT used, whether preincubation was used, antigen concentrations, and the migration index used as a cutoff point.

We have modified our technique now, and although we still use the capillary tube method because we find it gives sharper end points in our hands, we are now using multiple antigen concentrations and 1-hr preincubation in order to see whether this will enhance the sensitivity of the assay for TAg.

Other aspects of this study which merit comment are the measurements of the mean migration area in medium alone and LMIT response to Con A. The mean migration areas in medium alone were equivalent to controls when the total cancer patient results were examined. Patients with colorectal cancer and head and neck cancer, however, had significant depression of mean values (Table 1). The colon and head and neck cancer patients as groups showed the poorest responses to Con A compared to those of controls. The melanoma patients had intermediate values. Patients with advanced breast cancer had the highest response rates. Where patient groups were subdivided by stage, patients with advanced cancer of the colorectum, melanoma, and head and neck had inferior responses to Con A (these mean responses were not significantly depressed, however). This general trend has been observed in other studies of nonspecific immunity such as dinitrochlorobenzene skin testing and in vitro measurements of lymphocyte blastogenesis (14). It appears that, overall, there is a general trend to impaired leukocyte migration inhibition responses to Con A in colorectal cancer patients and head and neck cancer patients, which one might expect on the basis of other immune function data.

In summary, we have observed a significant response to the TAg in patients with primary breast cancer, most of the responses occurring in the patients with Stage III and IV disease. None of the other cancer patients responded to this antigen. In contrast to previous studies, breast cancer patients had lower response rates to both MCF-7 and MTV. This may be due to differences in antigenic strength in the preparations used or possibly some undefined differences in the test population. The utilization of a standardized stimulant, Con A, ensured the proper functioning of the assay on an individual test basis, although it was not useful for determining cutoff points. These findings do not nullify previous work with MCF-7 and MTV but do point out the limitation of the LMIT assay and the need for standardization of test antigen material. The findings with the TAg, although not strong, are promising and suggest that, with improvements and modifications of the assay and antigen concentration, this test may be useful as an immunological probe in breast cancer patients.

ACKNOWLEDGMENTS

We are grateful to Dr. Arnold Dion for a gift of the MTV and to Dr. George Springer for supplying the TAg.

REFERENCES

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