Implications of Humoral Antibody in Mice and Humans to Breast Tumor and Mouse Mammary Tumor Virus-associated Antigens

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Summary

As a part of a program directed toward the elucidation of the role of viruses in mouse and human breast cancer, a variety of immunological techniques were applied to a study of the humoral immune response of mice and of humans to their breast tumors. Tumor-bearing mice were found to produce antibodies against a complex array of tumor cell-associated antigens, including mouse mammary tumor virus (MMTV), components, heterophile and Forssman-like antigens, embryonic antigens, and possibly other tumor-associated antigens. Mice bearing MMTV-positive tumors had high titer antibodies against both viral and heterophile antigens. Tumor-free mice, whether of high or low mammary cancer strains, were remarkably free of antibodies that could label MMTV particles, although some sera contained antibodies to viral components.

Patients with breast cancer also had antibodies against a variety of antigens associated with their own and homologous breast cancer cells. These antibodies reacted with heterophile, embryonic, and other tumor-associated antigens, some of which appeared to be viral. Sera of some patients with breast cancer gave positive immunofluorescence reactions with mouse mammary tumor cells grown in tissue culture and producing MMTV. Most of these reactions were due to heterophile antibodies in the sera, but a small number of sera contained antibodies apparently directed specifically toward MMTV particles, as determined by immunoperoxidase electron microscopy. Although human-mouse cross-reactions must be interpreted with caution, these data suggest that a virus putatively associated with human breast cancer is antigenically related to MMTV.

Introduction

The role of the MMTV3 in the induction of mouse mammary cancer, and the importance of genetic, hormonal, and other factors in mammary tumorigenesis, have been the subject of extensive investigation by many laboratories. The literature on this subject is very extensive and will not be considered here.

Although mice were once considered immunologically tolerant to MMTV, more recent studies have shown that this is not always the case. Precipitating or neutralizing antibodies to MMTV virions or to virion components have been induced in mice of several inbred strains including C3H and RIII (2, 17, 21, 22, 28, 37). Mice developing spontaneous mammary tumors have, in some instances, been found to possess precipitating antibodies to MMTV (7, 27).

Tumor-specific transplantation resistance has been induced against some spontaneous mammary tumors in mice congenitally infected with MMTV (18–20, 23, 25). These studies have indicated the existence of a weak tumor-specific antigenicity restricted, as in the case of chemically induced tumors, to the immunizing tumor. Sera of mice with spontaneous mammary tumors were found to contain a factor abrogating in vitro colony inhibition induced by autologous lymphocytes (18).

It appears, therefore, that mammary tumors induced in mice by MMTV type B particles possess at least 2 antigenic systems. Of these, the major system is directly related to the presence of MMTV in the tissues. The 2nd system, more difficult to demonstrate, is tumor associated and may be relatively independent from MMTV.

The demonstration of the viral origin of mouse mammary cancer has led to the assumption that human breast cancer may also be induced by virus (or viruses). There are morphological findings (8–10, 12, 13, 16, 18, 33, 34) and biochemical data (1, 6, 31, 32, 36) suggesting an association between a virus with properties similar to those of MMTV and the origin of human breast cancer. These data raise the question whether antigens of a putative human breast cancer virus may be detectable in breast cancer cells.

It has been shown previously that sera of breast cancer patients contain antibodies against cytoplasmic antigens of their own and homologous breast cancer cells (3, 4, 12, 14, 15, 29, 30, 35). Furthermore, we have reported detection by immunofluorescence tests of antibodies to antigens in MMTV-producing mouse mammary tumor cells in the sera of patients with breast cancer and of some of their normal relatives (29). Some of these sera directly labeled MMTV particles as detected in indirect immunoperoxidase tests (3, 9, 11, 12, 24).

The present communication describes a continuation of...
these studies and extends the findings on the humoral response of tumor-bearing mice to MMTV and other antigens associated with mammary tumor cells and on comparable reactions between sera of breast cancer patients and antigens of human and mouse breast tumor cells.

Materials and Methods

Mice. All mice used in these studies were either obtained from the Texas Inbred Mice Company, Houston, Texas, or were produced in our own breeding colony. Strains used include C3H/HeJ/Tex; C3Hf/He/Tex; RIII/Dk; A/Dk; C3Hfz/Dk; and C57BL/6/Tex.

Tissue Cultures. Tissue cultures were established from spontaneous or induced mammary tumors of various strains of mice (including RIII/Dk; C3H/Dk; C3H/Tex; C3Hf/HeJ; A/Dk; A/Tex; C57 × Af F1; and BALB/c) by standard methods. Cells were monitored for virus production, freedom from pleuropneumonia-like organisms, and morphological characteristics by electron microscopy.

Tissue cultures were established from human breast tumor biopsy specimens or pleural effusions by a variety of techniques. Biopsy specimens were either minced and cultured directly or after mild trypsin treatment. Pleural effusions were centrifuged, resuspended in medium, and directly cultured. Culture medium for most cultures was Eagle’s minimum essential medium supplemented with 10 or 20% fetal calf serum and buffered with N-tris(hydroxymethyl)methylglycine-bicarbonate. Human tissue cultures were monitored by electron microscopy, as with mouse tumor tissue cultures.

FIF Tests. FIF tests were performed by the indirect method on acetone- or methanol-fixed cells, by the method described by Priori et al. (30). Fluorescein-conjugated goat anti-human IgG (Hyland Div., Corta Mesa, Calif., or Microbiological Assoc., Inc., Bethesda, Md.) was used to develop the reaction.

Immunoelectron Microscopy. Immunoelectron microscopy was performed either by indirect immunoferritin test or by indirect immunoperoxidase test. For the immunoferritin test, ferritin-conjugated rabbit antimouse IgG was obtained from Cappel Laboratories, Downingtown, Pa. Each batch was tested and titered with a known positive system. Aliquots of sera from mice of all strains were tested by the indirect immunoferritin method against MMTV particles produced by cells of an established cell line derived from a spontaneous mammary tumor of a C3H/HeJ mouse. Cells were prefixed in situ with either phosphate-buffered 2% formalin or 1% acrolein. Cells were then washed with PBS, scraped from the tissue culture flasks, incubated with serum for 3 hr at room temperature, washed with PBS, and further incubated in ferritin conjugate for 3 hr. After incubation in conjugate, the cells were washed with PBS and fixed in 3% glutaraldehyde, followed by further fixation in 2% osmium tetroxide, then processed for electron microscopy. Immunoferritin tests with human sera followed the same protocol, using ferritin-conjugated rabbit antimouse IgG also obtained from Cappel Laboratories. The methodology for the immunoperoxidase test has been described in detail by Hoshino and Dmochowski (24).

Results

Study of Naturally Occurring Antibodies to Mouse Mammary Tumor Cell-associated Antigens in Sera of Tumor-bearing and Tumor-free Mice. Initially, emphasis was placed on analysis of the occurrence, distribution, and nature of antigen-antibody reactions in normal and mammary tumor-bearing mice of different high and low mammary cancer strains, especially various sublines of C3H, C3H/Dk; C3Hf/Tex, C3H/HeJ; RIII/Dk; A/Dk; A/Tex, and C57BL/6/Tex.

The results of immunoferritin and FIF tests of sera of mice with spontaneous mammary tumors are summarized in Table 1. A similar study of the sera of tumor-free mice is summarized in Table 2. A total of 14 specimens of sera comprising 34 sera of mice bearing spontaneous mammary tumors and 16 specimens of sera comprising 68 sera of tumor-free mice were tested. The studies were carried out with MMTV-producing cells derived from a C3H/HeJ mammary tumor cell line as substrate. Sera of nearly all tumor-bearing mice were tested against MMTV produced by a C3H/HeJ culture, and the results are summarized in Table 1.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Sera of mammary tumor-bearing mice tested against MMTV produced by a C3H/HeJ culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sera</td>
<td>Immunoferritin</td>
</tr>
<tr>
<td>---------</td>
<td>----------------</td>
</tr>
<tr>
<td>A/Dk (single)</td>
<td>+(1:16)</td>
</tr>
<tr>
<td>A/Dk (pool of 5)</td>
<td>+</td>
</tr>
<tr>
<td>A/Dk (pool of 3)</td>
<td>+</td>
</tr>
<tr>
<td>A/Dk (pool of 4)</td>
<td>+</td>
</tr>
<tr>
<td>A/Tex (single)</td>
<td>+(1:32)</td>
</tr>
<tr>
<td>A/Tex (single)</td>
<td>+(1:32)</td>
</tr>
<tr>
<td>A/Tex (single)</td>
<td>Neg(1:8)</td>
</tr>
<tr>
<td>C3H/Z/Dk (single)</td>
<td>Neg(1:8)</td>
</tr>
<tr>
<td>C3H/Z/Dk (pool of 4)</td>
<td>+</td>
</tr>
<tr>
<td>C3H/HeTex (single)</td>
<td>+</td>
</tr>
<tr>
<td>RIII/Dk (single)</td>
<td>Neg(1:8)</td>
</tr>
<tr>
<td>RIII/Dk (pool of 3)</td>
<td>+</td>
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<tr>
<td>RIII/Dk (pool of 3)</td>
<td>+</td>
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<tr>
<td>RIII/Dk (pool of 5)</td>
<td>+</td>
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</tbody>
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* Neg, negative.
Cancer Tissues.

Antigens of Cells Derived from Human and Mouse Breast designed to determine whether any of the sera from breast specific antigens. These observations led to experiments antigens associated with mouse mammary cancer cells.

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The number of sera of patients and of their relatives that were positive with cells of any of these cell lines was not influenced by the time at which the disease occurred in the patient.

Sera of 40% (8 of 20) of apparently normal women reacted with cells of a line producing only type C particles, while 15% (3 of 20) reacted with cells of a line producing type B virus particles. Twenty % (4 of 20) of sera of these normal women gave a positive FIF test with cells of a line apparently negative for virus particles. Sera of 3 male relatives gave a positive FIF test with cells of mouse mammary tumor lines producing type B and C virus particles, or producing only type B virus particles.

The results of absorption studies of sera of patients and of their relatives with suitable material demonstrated in these sera the presence of Forssman, tumor tissue, and MMTV antibodies.

One of us (E. S. Priori) has extended a part of this investigation to include a study of sera of breast cancer patients at the time of surgery and of sera of breast cancer patients receiving chemotherapy and Bacillus Calmette-Guérin immunotherapy. This part of the study is being done in collaboration with Dr. Jordan U. Gutterman of the Department of Developmental Therapeutics of our institution. These sera were tested by FIF tests against cells of the same C3H/HeJ mouse mammary tumor cell line previously described.

Among patients undergoing 4 to 6 months of chemotherapy and immunotherapy, 70% (25 of 35) gave positive cytoplasmic FIF reactions with cells of the MMTV-producing mouse mammary tumor culture. Absorptions thus far carried out indicate considerable complexity of these positive reactions, and suggest that the sera of treated patients contain antibodies to heterophile antigens and, in some cases, to MMTV antigens. This study is still in the preliminary stages, however, and no definite conclusions can be drawn until additional experiments are performed.

The indirect immunoferritin or immunoperoxidase test was used to determine whether sera from breast cancer patients contained antibodies directed toward antigens of MMTV particles produced by C3H/HeJ mouse mammary tumor cell line. Difficulty was encountered with the immunoferritin technique, in the case of human sera, apparently due to poor or low-titer conjugates for human IgG. Immunoperoxidase conjugates proved satisfactory, however, so the immunoperoxidase test was applied, by methods described by Hoshino and Dmochowski (24). In experiments thus far carried out, 4 of 22 sera of breast cancer patients have given direct labeling of budding and mature MMTV type B particles. The indirect immunoferritin test was also observed.

Absorption of positive human sera with MMTV particles purified from mouse milk completely removed the labeling of type B particles, further indicating the antiviral specificity of the reaction.

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Examples of the positive immunoperoxidase reaction between sera of 2 breast cancer patients and MMTV particles in the C3H/HeJ mouse mammary tumor cell line are shown in Figs. 3 and 4. Fig. 5 shows, for comparison, a preparation identically treated but without a positive human serum.

In studies initiated in collaboration with Dr. M. Hoshino, now at the Aichi Cancer Center, all of 13 selected sera from Japanese women with breast cancer have given positive immunofluorescence reactions with the MMTV-producing mouse mammary tumor cell line. In addition, 1 serum tested gave peroxidase labeling of type B particles. The nature of these reactions is currently under study.

In a further collaborative study with Dr. M. Muller and his colleagues of the Medical Academy “Carl Gustav Carus” Institute of Pathology in Dresden, Germany, we have examined a serum specimen from a woman with breast cancer previously found by them to react in FIT tests with intracytoplasmic type A particles of murine mammary carcinomas. This serum, in our hands, gave a similar reaction with intracytoplasmic type A particles of other mouse mammary carcinomas examined. This reaction was not removed by absorption of the serum in vivo (in C57BL/6/Dk mouse) or by absorption of the serum with sheep erythrocytes or guinea pig kidney tissue. The FIT reaction of the serum was completely removed by absorption with RIII/Dm mouse milk virus preparations. Furthermore, the serum gave positive immunoperoxidase reactions with cells of the same mouse tumor, with similar cytoplasmic localization. The peroxidase reaction was also removed by absorption of the serum with MMTV preparations.

In reciprocal antibody tests, sera of tumor-bearing mice found positive in immunofluorescence tests with MMTV-producing mouse mammary cells have been tested by the FIT test with cells of cultures derived from human breast tumors. Eleven serum pools, including sera from a C57BL/Tex mouse with spontaneous leukemia, were tested. Positive cytoplasmic and perinuclear fluorescence was observed with 3 of 8 sera from tumor-bearing RIII/Dm, A/Dm, or C3H/Dm mice. The remaining 5 pools of sera from tumor-bearing mice, the serum from the leukemic C57BL/Tex strain mouse, and 2 pools from tumor-free C57BL/Tex strain mice were negative. However, the results of absorption of the 3 positive serum pools with heterophile or MMTV antigens demonstrated that the reactions were due to heterophile antibodies and were apparently not virus specific.

Discussion

Mice bearing mammary tumors produce antibodies against a variety of antigens associated with autologous, isologous, and homologous mammary tumor cells. These consist of heterophile and Forssman-type antigens, antigens associated with MMTV components, and possibly other nonviral tumor antigens as well. These antigens and their specific antibodies can be demonstrated by several methods and are shown to be intracellular (cytoplasmic and perinuclear) at the cell surface and associated with both intracytoplasmic type A particles and budding, immature, and mature type B MMTV particles.

With respect to antibodies against MMTV antigens, the sera of mammary tumor-bearing mice appear to fall into at least 2 categories. One category contains sera that react with a broad spectrum of antigens, including viral envelope antigens. When tested against mouse mammary tumor cells producing type B virus particles, these sera give strong cytoplasmic fluorescence with fixed cells, give strong membrane immunofluorescence reactions with viable cells, and specifically label budding and mature type B virus particles with either ferritin- or peroxidase-labeled antibody. Furthermore, the results of preliminary radioimmunoprecipitation tests indicate that these sera of mice of this category also precipitate lactoperoxidase-radioiodinated type B particles.

In similar tests against the same cells, sera of the 2nd category characteristically give strong cytoplasmic fluorescence with fixed cells but give little or no reaction with viable cells, and do not label type B virions. This very interesting observation is currently under intense investigation.

Current data do not permit reliable conclusions about the relationship of mouse antibodies to the appearance and progression of mammary tumors. The role of the humoral immune response to mammary tumor cells and the part played by MMTV antigens in the immune response must be elucidated by further study. It is certainly clear, however, that mice are not tolerant to MMTV antigens, since even high mammary cancer strains produce antibodies against viral antigens, often to high titers.

The results presented here confirm and extend our previous observations (3, 12, 24, 29), and those of others, that sera of some breast cancer patients, of some of their relatives, and of some normal individuals contain antibodies against mouse mammary tumor cells producing MMTV type B particles. In human sera positive by immunofluorescence tests with MMTV-producing mouse mammary tumor cells, both heterophile and MMTV antibodies are responsible for the reactions. A small number of the sera of breast cancer patients can be shown by immunoelectron microscopy to directly label MMTV particles. The labeling of MMTV particles by positive sera can be removed by absorption of the sera with purified MMTV preparations, but not with heterophile antigens, mouse tissue, or preparations of MMTV-free mouse milk. Our findings are in general agreement with the findings of Charney and Moore (5), who observed anti-MMTV-neutralizing antibodies in the sera of some breast cancer patients, and of Muller and his associates, who have demonstrated MMTV antibodies in sera of breast cancer patients (26, 37, 38).

The reason for the small percentage of human sera positive by immunoelectron microscopy is not clear and requires much further study. One possible explanation would be quantitative differences in the reactions, but this appears unlikely, since there was no correlation between high and low titers by FIT tests and immunoperoxidase labeling of MMTV particles. Another possible explanation may be that the human sera, like mouse sera, contain different kinds of anti-MMTV reactions, some labeling the viral envelope and others directed toward other, possibly internal, virion components. The latter explanation is speculative at the pre-
sent, but is the subject of extensive investigation now in progress.

The observation of cross-reactions between sera of human breast cancer patients and MMTV antigens has a number of potentially important implications. The implication for establishing the etiological role of a virus related to MMTV to human breast cancer is obvious. In addition, these findings have implications for the design of both chemotherapeutic and immunotherapeutic protocols based on exploitation of metabolic and/or antigenic differences between malignant cells and normal tissues. Further study is required to determine the nature and extent of the human-mouse cross-reactivity. It is emphasized that these results should be interpreted with great caution, just as with any other cross-species reaction. On the basis of the present evidence, however, there is a strong case for the association of a human breast cancer virus, similar to and related to MMTV.

This hypothesis serves to point the direction for future research on the etiology and management of this neoplasm.

References

Fig. 1. Ferritin labeling of budding MMTV particles by the pooled sera of 5 tumor-bearing A/Dm mice. MMTV-producing cells of a tissue culture derived from a spontaneous tumor of a C3H/HeJ mouse were tested against the serum pools, as described in the text. × 60,000.

Fig. 2. Peroxidase labeling of budding and immature MMTV particles by serum of a tumor-bearing C3H2/Dm mouse. Virus-producing cells were the same as in Fig. 1, and were prepared as described in the text. × 60,000.

Fig. 3. Peroxidase labeling of MMTV particles by a serum from a patient with breast cancer. Virus-producing cells were the same as in Fig. 1, and were prepared as described in the text. × 60,000.

Fig. 4. Peroxidase labeling of MMTV particles by serum from another patient with breast cancer, prepared as described in legend to Fig. 3. × 60,000.

Fig. 5. A portion of an ultrathin section of the MMTV-producing culture shown in Figs. 1 to 4, carried completely through the indirect peroxidase-labeling procedure, but without use of a positive mouse or human serum. × 60,000.
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