Immunohistochemical Evidence for RNA Virus Related Components in Human Breast Cancer*

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ABSTRACT

The localization of antigenic components with cross-reactivity to a 52,000 dalton group specific glycoprotein (gp52) of the mouse mammary tumor virus (MMTV) in paraffin sections of human breast carcinomas is described using an indirect immunoperoxidase method. This method was first optimized on paraffin sections of mouse mammary tumors. The specificity of the reaction observed in the human tissues was established by absorption of the specific IgG with: a) purified gp52; b) several relevant and irrelevant viral preparations; c) normal human plasma, leukocytes, breast tissue, milk, actin, collagen, and hyaluronic acid; d) sheep erythrocytes, bovine mucin and fetal calf serum. Only MMTV and purified gp52 eliminated the immunohistochemical reaction in human breast tumors.

Positive reactions were seen in 171 of 376 (45.5 percent) randomly selected breast carcinomas of various histopathologic types, while negative reactions were obtained in all 137 normal and benign cases tested. In those invasive tumors with an intraductal component, a higher percentage (63.9 percent) of positive cases was seen. A positive reaction of different specificity was observed in foci of apocrine metaplasia. With one exception, 99 carcinomas from 13 organs other than breast and eight cystosarcomas were negative.

Introduction

During the four decades since Bittner described a "factor" in certain high cancer-incidence strains of mice capable of initiating mammary tumors, a considerable experimental effort has been directed towards establishing useful correlations between the mouse mammary tumor model and human breast cancer.

The subsequent identification of the Bittner factor as a B-type ribonucleic acid (RNA) virus stimulated a search leading to the ultrastructural finding of particles resembling murine B-type and C-type viruses in human breast cancer tissues as well as in human milk. The eventual isolation and biochemical characterization of these particles revealed further similarities to the oncornaviruses, principally the presence of RNA-dependent desoxyribonucleic acid (DNA) polymerase (reverse transcriptase) complexed...
to a 70S RNA molecule. In addition, molecular hybridization studies demonstrated a partial homology between RNA molecules found in human breast tumors and the RNA genome of the mouse mammary tumor virus (MMTV).\(^2,3,29,33\)

In recent years, much experimentation has been focused on exploring a possible antigenic relationship between MMTV proteins and components in human breast cancer. Studies with sera of breast cancer patients have demonstrated the presence in such sera of antibodies capable of neutralizing MMTV\(^7\) and of localizing antigens in mouse mammary tumors by immunofluorescence,\(^19,20\) and ultrastructurally, by immunoferritin\(^9\) and immunoperoxidase\(^6,10\) techniques. The migration inhibition studies of Black et al\(^5\) using leukocytes from breast cancer patients suggest a crossreactivity between the major glycoprotein of MMTV and a protein component extracted from human breast cancer tissues. In addition, the immunofluorescent identification of MMTV crossreactive antigens in the MCF-7 human breast carcinoma cell line has been reported.\(^35\)

This apparent immunologic crossreactivity of MMTV proteins and antigens in human breast cancer is of considerable interest in view of the fact that in the mouse mammary tumor model a 52,000 dalton viral glycoprotein (gp52) is an excellent indicator of disease status.\(^24,25,26\) Thus, earlier studies in this laboratory have established that plasma levels of gp52, measured by radioimmunoassay, could be accurately correlated with the existence,\(^24\) size,\(^25\) and recurrence after surgical excision\(^26\) of mouse mammary tumors, frequently in the absence of gross physical signs of the disease. Attempts to use radioimmunoassays to detect crossreacting proteins in the plasma of breast cancer patients,\(^6,36\) however, have unfortunately not been very successful, probably owing to the thousand-fold blood volume differences between mice and humans which would conceivably dilute the signal in human plasma beyond the sensitivity of present radioimmunoassays.

Our efforts, therefore, have been concentrated on the immunohistologic localization of crossreactive antigens in the tumors themselves using the very sensitive and convenient immunoperoxidase method of antigen localization.\(^17\) After optimizing our immunohistochemical method in the localization of MMTV antigens in frozen and paraffin sections of mouse mammary tumors,\(^13\) we were able to apply successfully the same methodology and reagents on routinely processed, paraffin sections of human breast carcinomas.\(^15,16\) The present paper updates our experience and observations with respect to the localization in human breast cancer tissues of an antigen which crossreacts with gp52, the group-specific glycoprotein of the mouse mammary tumor virus.

**Materials and Methods**

**Tissue**

Paraffin blocks of tissues used for diagnostic purposes were selected from the files of the divisions of Surgical, Gynecologic and Anatomic Pathology at the College of Physicians and Surgeons of Columbia University, as previously described.\(^15\) These tissues had initially been fixed in Bouin’s solution or, in the case of larger specimens, in 10 percent buffered formalin followed by Bouin’s fixation. Additional tumor samples were received fresh from Memorial Sloan-Kettering Cancer Center. One small block from each of these was fixed in Bouin’s and routinely processed. From all of the aforementioned, 5 μm serial sections were cut for immunohistochemical staining; occasional sections were stained with hematoxylin and eosin for comparison.
TABLE I
Immunoperoxidase Staining of Carcinoma of the Breast
Total Cases Classified as to Source

<table>
<thead>
<tr>
<th>Source</th>
<th>Cases</th>
<th>Positive</th>
<th>Percent Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Columbia-Presbyterian</td>
<td>238</td>
<td>110</td>
<td>46.2</td>
</tr>
<tr>
<td>Michigan Cancer Foundation</td>
<td>104</td>
<td>48</td>
<td>46.2</td>
</tr>
<tr>
<td>Memorial Sloan-Kettering</td>
<td>34</td>
<td>13</td>
<td>38.2</td>
</tr>
</tbody>
</table>

376 171 45.5

One hundred and four additional breast cancer cases were received as unstained paraffin sections from Dr. Marvin Rich, of the Michigan Cancer Foundation.

ANTISERA AND IgG PREPARATIONS

The two principal antisera used in the present study were raised in rabbits by using whole (disrupted) MMTV isolated from the milk of Paris RIII mice, and contained antibodies to the MMTV proteins in proportional amounts as reflected by their radioimmunoprecipitation profiles. Other antisera used for comparison included rabbit anti-MMTV (C₈H), rabbit anti-gp52 and goat anti-gp52. The IgG fractions from these sera were prepared and characterized by radioimmunoprecipitation as previously described.

TABLE II
Immunoperoxidase Staining of Carcinoma of the Breast
Histopathologic Classification of Columbia-Presbyterian Cases

<table>
<thead>
<tr>
<th>Type</th>
<th>Cases</th>
<th>Positive</th>
<th>Percent Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraductal</td>
<td>22</td>
<td>8</td>
<td>36.3</td>
</tr>
<tr>
<td>Intraductal and invasive</td>
<td>61</td>
<td>39</td>
<td>63.9</td>
</tr>
<tr>
<td>Invasive*</td>
<td>101</td>
<td>47</td>
<td>46.5</td>
</tr>
<tr>
<td>Medullary</td>
<td>15</td>
<td>5</td>
<td>33.3</td>
</tr>
<tr>
<td>Metastatic†</td>
<td>39</td>
<td>11</td>
<td>28.2</td>
</tr>
<tr>
<td></td>
<td>238</td>
<td>110</td>
<td>46.2</td>
</tr>
</tbody>
</table>

*Includes all types of invasive carcinoma (e.g., tubular, lobular, small cell, etc.) except those with predominantly medullary features or associated with intraductal lesions.
†Includes metastases to lymph nodes, lungs, liver, adrenal gland and ovaries in proven cases of primary carcinoma of the breast.

Absorption of IgG Preparations

Numerous substances were used to absorb the antibody preparations to enhance or test the specificity of the immunohistochemical staining. The preparation of these immunoabsorbants and the conditions of absorption are detailed in a previous report.

Immunohistochemical Staining

An indirect immunoperoxidase method was used with conjugates prepared by the periodate procedure of Nakane and Kawaoi with some modifications. A detailed description of this procedure has previously been reported.

Results

A positive staining reaction has been observed in 171 (45.5 percent) of 376 cases of human breast carcinomas tested, including cases received from other institutions (table I). In table II are summarized positive staining reactions seen in 238 Columbia-Presbyterian breast cancer cases classified according to histopathologic type. The larger percentage of the positive cases in the intraductal and invasive group reflects a trend noted in our original report of 131 cases and suggests that the correlation seen in this larger series is indeed significant. It appears, therefore, that an invasive carcinoma associated with an intraductal component is more likely to contain crossreactive antigen than either a pure intraductal or invasive tumor.

The pattern of immunohistochemical staining in human breast tumors tends to be focal, intracellular and cytoplasmic with considerable variability even within the same tumor (figures 1 and 2), a situation not altogether unlike that encountered in mouse mammary tumors. For example, in the intraductal and invasive carcinoma illustrated in figure 1, only some of the cells in the intraductal lesion
FIGURE 1. Immunoperoxidase stain of intraductal and invasive carcinoma with anti-MMTV before (1a) and after (1b) absorption with gp52. Some of the cells in the intraductal lesion and most of the invasive cells contain reaction product. (These photomicrographs have been overdeveloped to enhance morphologic detail; the counterstain may therefore in some areas simulate positive staining.) (Methylene blue counterstain; × 778).
Figure 2. Immunoperoxidase stain of invasive breast carcinoma with anti-MMTV before (2a) and after (2b) absorption with gp52. Note intense stain of focus of invasive carcinoma and lack of reaction in neighboring morphologically benign ducts and acini (2a) as well as complete elimination of the former reaction after absorption with gp52 (2b). (Methylene blue counterstain; × 609).
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contain reaction product. Likewise, not all of the invasive malignant cells surrounding the intraductal lesions are stained. In other sections of tumor from this same case, however, staining was not universal and was even completely absent in some intraductal lesions which were surrounded by strongly reacting invasive cells. In figure 2, on the other hand, is illustrated an invasive tumor in which practically every malignant cell gave a strong staining reaction in all the secretions tested. The microscopic field illustrated depicts a focus of intensely stained invasive carcinoma surrounding morphologically benign ducts and nearby uninvolved acini which do not contain reaction product.

In figures 1b and 2b are shown serial sections adjacent to those illustrated in figures 1a and 2a, which have been treated identically except that the primary antiserum has been previously absorbed with purified gp52. Thus, absorption with purified gp52 is sufficient to eliminate the staining reaction in human breast carcinoma, while absorptions with numerous other related and unrelated substances (table III) do not produce any effect.

The specificity of the reaction for breast carcinoma was determined by testing 99 carcinomas from other organs and eight cases of cystosarcoma phyllodes (table IV). Only one of these 107 tumors, a mucoepidermoid carcinoma of the parotid gland, gave a positive reaction; two other parotid carcinomas of the same histopathologic type were negative.

Normal (resting and lactating) and benign (cystic disease, fibroadenoma, intraductal papilloma, gynecomastia) breast tissues from 137 patients were also tested with negative results (table V). The only exception to the absence of staining reaction in nonmalignant breast tissue was the staining of foci of apocrine metaplasia, one of the microscopic features of cystic disease. The different specificity of this reaction follows.

Discussion

The successful application of the immunoperoxidase technique to the localization of MMTV antigen in sections of paraffin-embedded mouse mammary tumor tissues13 made possible the extensive, primarily retrospective studies described in this paper. While eliminating the need for fresh tissues in our search for a crossreacting human antigen, we also had at our disposal the same tissues received by the Pathology Laboratory, the paraffin blocks of which could be used after the diagnostic sections had been cut.

TABLE III
Absorption Specificity Tests of Immunoperoxidase Staining of Human Breast Carcinomas with a-Mouse Mammary Tumor Virus

<table>
<thead>
<tr>
<th>Completely Eliminated by</th>
<th>Not Eliminated by</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMTV (RIII) from milk</td>
<td>Viruses</td>
</tr>
<tr>
<td>MMTV (C3H) from MM57 cell line</td>
<td>Rauscher leukemia virus, Simian sarcoma</td>
</tr>
<tr>
<td>MMTV (C3H) from CrFeK cell line</td>
<td>virus, Mason-Pfizer monkey virus, Baboon endogenous virus</td>
</tr>
<tr>
<td>gp52 (RIII) purified by concanavalin A</td>
<td>Human</td>
</tr>
<tr>
<td>gp52 (C3H) affinity chromatography</td>
<td>Normal plasma, normal leukocytes, collagen, actin, hyaluronic acid, milk, normal breast tissue</td>
</tr>
<tr>
<td>gp52 (RIII) purified by guanidium chloride chromatography</td>
<td>Bovine</td>
</tr>
<tr>
<td>gp52 (C3H) chloride chromatography</td>
<td>Mucin, fetal calf serum</td>
</tr>
<tr>
<td>gp52 (C3H)</td>
<td>Sheep erythrocytes</td>
</tr>
</tbody>
</table>

*The absorptions were performed by using these agents either in the soluble or insoluble form16.*
**TABLE IV**

<table>
<thead>
<tr>
<th>Malignancy*</th>
<th>Cases</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Stomach</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Pancreas</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Liver</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Lung</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Endometrium</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>Ovary</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Prostate</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Kidney</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Skin</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Thyroid gland</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Parotid gland</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Cystosarcoma phyllodes</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

*Primary sites of non-breast carcinomas stained with α-mouse mammary tumor virus; cystosarcoma phyllodes is also listed because it is the most common noncarcinomatous malignancy of the breast.

The staining reactions observed in human breast carcinomas with antibodies to MMTV differ from the reactions previously observed in the mouse mammary tumors with respect to variability and frequency of staining as well as with the results of absorption with gp52. These differences shall be considered in turn.

The variability of staining seen among and within the human breast carcinomas is greater than that observed in the mouse mammary tumors,15,16 which is not unexpected considering that the etiologic role of MMTV is well documented in the latter. Furthermore, tumors from highly infected mouse strains25 were deliberately chosen in these earlier studies in order to optimize our method of MMTV antigen localization. Nevertheless, it should be emphasized that cellular variability of antigen expression was not uncommon in the mouse mammary tumors and has also been frequently observed in human tumors with respect to other tumor-associated antigens.23

With respect to frequency, it is evident that there is considerable sampling error in our testing procedure and, therefore, the percentages given in tables I and II represent, at best, minimal values. This conclusion is based on the following facts: (1) diagnostic tissue blocks of a given case are usually representative, but seldom include the entire tumor; (2) these studies were limited to an average of less than three (one, in cases from other institutions) representative blocks per case, and as previously noted, a considerable variability in antigen localization was noted among and within sections of positive cases; and (3) in contrast to in vitro methods, where a given tissue can be assayed in bulk, our test is limited to 5 μm sections which represent only a minute fraction (less than 1/1000th) of the entire tumor.

Finally, in contrast to the mouse tumors, where only absorption with whole disrupted MMTV completely eliminates the staining reaction,15,16 absorption with purified gp52 alone was sufficient to obliterate the reaction in positively staining human tumors (figures 1b and 2b). The specificity of this staining reaction was further explored by absorption with the various preparations of MMTV and gp52 listed in table III. All of these blocked the staining reaction, indicating that the species differences that have been previously reported30,31 for gp52 of the CD8 and RIII MMTV do not play a role in this reaction. On the other hand, absorption with different virus
preparations (Rauscher leukemia virus, simian sarcoma virus, Mason-Pfizer monkey virus, baboon endogenous virus) and with several possible crossreacting substances, also listed in table III, fail to eliminate the staining reaction.

The specificity of the reaction for breast carcinoma is supported by the fact that only one other carcinoma of different origin has been found to be positive (table IV). In addition, the data presented in table V confirm the specificity of the reaction for malignant breast tissues. The troublesome reactivity observed in apocrine metaplasia, also shared by the morphologically and histochemically indistinguishable1,32 epithelium of apocrine glands of the axilla and perineum, has been found to differ in specificity from the reaction observed in the carcinomas. For example, absorption with gp52 blocks only a minimal part of the reaction in the apocrine glands and metaplasia while it completely eliminates the reaction in the carcinoma. A more complete discussion of experiments which further define the nature and differences of the reactions seen in the human tumor and the apocrine epithelium through separation of the sugar and protein moieties of the MMTV glycoprotein is in preparation, and will be reported elsewhere.

Our conclusion that at least some human breast carcinomas contain an antigen immunologically related to gp52 of MMTV is further supported by two recent studies,22,34 in addition to those previously cited. At the present time, it is clearly premature to speculate on the etiologic implications of this finding, just as it would be misleading to draw too close a parallel between the mouse mammary tumor model and the human disease. Our efforts, therefore, are directed rather toward the possibility that these findings can be used to generate clinically useful information. For example, preliminary evidence is available indicating that breast cancer patients with a family history of the disease have a greater probability of expressing the gp52 crossreacting antigen in their tumor as compared to patients with no such family history.

Finally, our laboratory has a well-established human breast carcinoma cell line, T47D12 which also expresses immunohistochemically detectable antigen(s) related to gp52.11 It is hoped that the availability of this source will simplify the task of obtaining the relevant human breast cancer antigen in adequate amounts and stages of purity. This, in turn, may provide the immunologic reagents needed to develop the heterologous radioimmunoassays that can hope to attain the sensitivities required for measurement of a systemic signal in the human disease.

Acknowledgments

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References


