Antibody Reacting with the Murine Mammary Tumor Virus in the Serum of Patients with Breast Carcinoma: A Possible Serological Detection Method for Breast Carcinoma

Walter D. Holder, Jr. and Samuel A. Wells, Jr.
Department of Surgery, Duke University Medical Center, Durham, North Carolina 27710

ABSTRACT

Sera from patients with Stages A and B infiltrating ductal carcinoma of the breast, benign breast disease, cancers other than breast carcinoma, and normal female controls were examined by indirect immunoelectron microscopy (IEM) and a viral agglutination test for evidence of antibodies directed against murine mammary tumor virus (MMTV). Sera from 41 (79%) of 52 patients with breast carcinoma and eight (19%) of 42 normal subjects or patients with benign breast disease (noncancer subjects) showed evidence of MMTV labeling by IEM. In the MMTV agglutination test, significant virus agglutination (2+ to 4+) was present in eight (13%) of 61 noncancer sera, 58 (86%) of 68 breast carcinoma sera, and two (11%) of 18 other cancer sera. The results of the more rapid MMTV agglutination test correlated well with IEM. Analysis of reacting antibody by IEM revealed no immunoglobulin A and significant immunoglobulin M and immunoglobulin G antibody. Serum reactivity against MMTV was completely absorbed by MMTV but not by the glycoprotein with a molecular weight of 52,000 of MMTV, Friend murine leukemia virus, avian myeloblastosis virus, or sheep erythrocytes. It is concluded that reactivity of human antibodies to MMTV is strongly associated with, but is not entirely specific for, breast carcinoma. It remains to be determined if normal persons with these antibodies will ultimately develop breast cancer and should therefore be considered at high risk. These tests may have potential usefulness as a diagnostic screen for breast cancer.

INTRODUCTION

The virus-induced breast cancer of the mouse has been an important model for the study of the etiology of human breast cancer. While murine breast cancer has a well-established etiology relative to MMTV (2, 3), firm evidence for a viral etiology in human breast cancer remains to be proven. During the past 10 years, a series of observations has been made which reveals an immunological relationship between mouse and human breast cancer (4–6, 9, 13–18, 22–27, 29–32). An earlier experiment showed the neutralization of the biological activity of MMTV by serum from some patients with breast cancer (10). A possible mechanism for this inactivation is the lysis of MMTV by serum from breast carcinoma patients which has been reported by Witkin et al. (30). The immunoperoxidase technique has been used to show the presence of a MMTV-like antigen in sections of human breast cancer tissue which is not present in that of normal controls (18). Serum antibodies in breast cancer patients have been detected which react against mouse mammary tumor cells by immunofluorescence (17, 29) and IEM (13, 14). In these studies, similar reactivity was found in some noncancer patients as well. Some investigators feel that this reactivity is nonspecific (16, 24); however, other studies appear to verify specificity of human antibodies reacting with MMTV-related antigens (4–6, 18, 25–27, 29, 32). There is a lack of consensus in regard to which MMTV components the human antibody is directed. Several studies suggest that the antibody might be reacting with the major coat glycoprotein (gp52) of MMTV (18, 26). This observation has not been substantiated by others, who suggest that the activity may be toward one or more core proteins (23, 25, 29, 32).

Utilizing IEM, we demonstrated previously anti-MMTV activity in the sera of 20 of 20 breast carcinoma patients and 2 of 20 control patients (13). The present study is designed to: (a) determine if the MMTV reaction with sera of breast cancer patients could be used as a screening method for the presence of breast cancer and (b) further elucidate the nature of the reacting components in the MMTV-human serum reaction. This study utilizes the IEM technique described previously, together with MMTV agglutination test done with negative stain electron microscopy to provide a semiquantitative evaluation of serum reactivity with MMTV.

MATERIALS AND METHODS

Cells. C3H/HeJ cells (12, 13) utilized for the IEM study were grown in Eagle’s minimal essential medium in 75-sq cm plastic flasks (Falcon). The medium was supplemented with 10% (v/v) heat-inactivated fetal calf serum and penicillin (100 units/ml), streptomycin (10 /g/ml), and amphotericin B (0.25 /g/ml) (Grand Island Biological Co., Grand Island, N. Y.). Cultures were incubated in a 5% CO2:95% air incubator at 37° and passed at 5 to 7-day intervals utilizing 0.25% trypsin and EDTA. Cultures were negative for Mycoplasma by thin-section and negative-stain electron microscopy when grown in the absence of antibiotics.

Patient Sera. For this study, sera from 68 women with breast carcinoma, 61 noncancerous subjects, and 18 women with cancers other than breast were examined. The various sera were obtained from the Special Virus Cancer Program and from patients at Duke University Medical Center and Columbia Presbyterian Hospital. The sera were stored frozen until used. Pathological diagnoses were obtained for
tissue specimens from all patients who had undergone a surgical procedure. All of the breast cancer patients selected for the study had infiltrating ductal carcinoma. Serum samples were coded and examined blindly and in duplicate.

Virus. Gradient-purified MMTV derived from mouse cells in tissue culture was supplied by Dr. G. P. Shipley. F-MuLV derived from the Evelope cell line (12), avian myeloblastosis virus (7), and gp52 purified from MMTV (19) were utilized in absorption studies.

Ferritin-labelling Studies. An indirect immunoferritin method was used to demonstrate human antibody on C3H/HeJ mouse cell surfaces. The details of this procedure have been described previously (12). Briefly, viable, washed C3H/HeJ cells were resuspended in human serum diluted 1:4 with PBS, pH 7.4. Following incubation for 30 min and washing in PBS, cells were then reacted with ferritin-conjugated goat anti-human IgG:lgA:lgM (Cappell Laboratories, Cochranville, Pa.), washed again, and processed for electron microscopy. In a control study, the ferritin conjugate alone was incubated with the C3H/HeJ cells and found to be nonreactive.

Electron Microscopy. Following immunoferritin labeling, cell pellets were immediately fixed in cold 2.5% glutaraldehyde in Millonig's buffer (20) for 1 hr. Pellets were then postfixed in 1% osmium tetroxide in Millonig's buffer, dehydrated in graded ethanol solutions and propylene oxide, and embedded in Epon. Thin sections cut on an LKB Ultratome with a diamond knife were stained with uranyl acetate and lead citrate and examined in a Siemens Elmsiakop model 1A electron microscope.

MMTV Agglutination. For the virus agglutination procedure, a serum dilution of 1:20 (with PBS, pH 7.4) was found to be the most advantageous to minimize natural clumping of the MMTV and to give a homogeneous dispersion. The stock gradient-purified MMTV preparation was diluted with PBS containing 1% albumin to yield a mixture containing 1.9 x 10^10 particles per ml or approximately 500 particles per 300-mesh grid square by the method of Monroe and Brandt (21).

Diluted serum was then mixed with the MMTV preparation in a 2:1 ratio using a 25-μl pipet and allowed to react in an ice bath for 1 hr, after which it was vigorously mixed with a pipet. The reaction mixture was then combined in a 1:2 ratio with 2% phosphotungstic acid, pH 4.2, and the resulting mixture was placed on Parlodion and carbon-coated grids, allowed to dry, and examined with the electron microscope. Each serum sample was given an agglutination score as shown in Table 1.

RESULTS

Thin-Section IEM. Figs. 1 and 2 show an example of C3H/HeJ cell surface and budding MMTV labeling by serum from a patient with breast cancer. In 5 samples, this activity was completely removed by absorbing the serum with MMTV (Fig. 3). Absorption with gp52, however, did not remove the activity (Fig. 4). The results of absorption studies with 5 reactive samples from breast cancer patients are shown in Table 2. Absorption with sheep erythrocytes, avian myeloblastosis virus, F-MuLV, and gp52 failed to remove significant reactivity from the tested sera, while all the reactivity was removed by MMTV.

<table>
<thead>
<tr>
<th>Serum</th>
<th>C. H.</th>
<th>V. S.</th>
<th>E. G.</th>
<th>V. P.</th>
<th>S. B.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unabsorbed</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sheep RBC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F-MuLV</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Avian myeloblastosis virus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>gp52</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* +, labeling of budding virus; - , no labeling of budding virus.

The results of thin-section IEM serum screening are shown in Table 3. Forty-two of the 68 serum samples from patients without breast carcinoma were examined by thin-section IEM for labeling of budding MMTV and C3H/HeJ cell surface membranes. Cell membranes were labeled with ferritin by 38 (83%) of 42 sera from noncancerous subjects and by 50 (96%) of 52 breast carcinoma sera. There was no significant difference in the incidences of staining between the 2 groups.

Ferritin labeling of MMTV budding from the C3H/HeJ cells was seen in 8 (22%) of 37 normal subjects but in none of 5 patients with benign breast disease. Compared to the 42 noncancerous subjects, MMTV labeling from breast carcinoma sera was much higher [41 (79%) of 52 sera; p < 0.001]. All of the sera from 20 Stage B patients showed reactivity, and 76% of 21 Stage A sera were positive for antibody against budding MMTV. The specific stage of disease was unknown in 11 serum samples where 5 (45%) showed reactivity.

MMTV Agglutination. Fig. 5 is an example of a negative stain preparation of MMTV, and Fig. 6 shows a viral aggregate produced by incubating serum from a breast cancer patient with MMTV. The results of the MMTV agglutination studies are shown in Table 4. “Normal” serum samples were examined from patients with the following diagnoses: (a) clinically free of disease, 47; (b) fibrocystic disease, 8; (c) fibroadenoma, 2; (d) papillomatosis, 3; and (e) ductal ectasia, 1. The results of the combined scores show that the majority of normal sera fall into the negative or 1+ category with only 8 of 61 (13%) having an agglutination score of 2+ to 4+. Examination of the “disease-free” subgroup shows only 4 of 47 (9%) with a score of 2+ or greater. The remainder of benign breast disease sera taken as a group show 4 of 14 (29%) with a 2+ or greater score.

The results of the MMTV agglutination test with serum samples from 68 breast carcinoma patients are also shown in Table 4. The combined scores in contrast to those of the normal serum group show 58 (85%) of 68 samples with a score of 2+ to 4+. Examination of the subgroups shows that 16 (80%) of 20 Stage A sera, 27 (87%) of 31 Stage B sera, and 15 (88%) of 17 of the unspecified stage sera were scored 2+ or greater. Scores are not statistically different in the subdivisions of the breast carcinoma group.

MMTV agglutination studies with sera from 18 patients with types of cancer other than breast showed 2 (11%) of the 18 with reactivity to MMTV in the 2+ to 4+ range. Both of these patients were females with metastatic colon carcinoma. This
sample is quite small, and it will be necessary to test additional samples before the significance of this finding can be determined.

Forty-one serum samples from patients with Stages A and B breast carcinoma and 42 serum samples from noncancer patients were examined by both IEM and MMTV agglutination studies. Table 5 shows a comparison of the scores from each study. Table 6 shows a comparison of the scores from each study.

Reactive Immunoglobulins. Five reactive human breast carcinoma serum samples were selected and examined by indirect IEM using either ferritin-conjugated anti-IgA, anti-IgM, or anti-IgG. The results are shown in Table 6. Both anti-IgM and anti-IgG show labeling of the budding MMTV and a +2 to +4 virus agglutination score. A (—) IEM score correlates well with a — or +1 virus agglutination score. A (+) IEM score correlates well with a +2 to +4 virus agglutination score.

DISCUSSION

These data support the findings of earlier investigations demonstrating the presence of antibody against MMTV in the sera of many patients with breast carcinoma, certain normal individuals, and certain patients with types of cancer other than breast. This finding suggests that the occurrence of these antibodies is strongly associated with, but not specific for, breast cancer. It remains to be determined if “normal” persons with these antibodies will ultimately develop breast cancer and should therefore be considered in a high-risk group. We have noted previously the development of anti-MMTV antibodies in 2 laboratory technicians who worked with tumor-bearing mice (13). It is unlikely that murine exposure is a significant factor in this process.

The MMTV antigen which is recognized by human serum is a topic of diverse opinion in the literature. Some investigators have failed to confirm this observation, while others have demonstrated its presence in human breast carcinoma. Still others have shown that the antigen can be removed by absorption with sheep erythrocytes or mouse tissue (29).

Specific human activity against MMTV has been demonstrated by cell-mediated immunity which is expressed to the greatest extent in carcinoma in situ (4, 5). Fluorescein-labeled humoral antibody against MMTV has been shown to localize in the cytoplasm of mouse mammary tumor cells (17, 23, 25, 27, 29) and, in particular, intracytoplasmic A particles. The data presented in this paper as well as our previous IEM data (13) and the IEM data of others (14) show that antibodies in the sera of breast cancer patients are not only directed against...
intracytoplasmic antigens but also to cell surface antigens, particularly budding MMTV.

We have shown previously that the major coat glycoprotein of the MMTV (gp52) and F-MuLV (gp71) can be detected by IEM on the surface of a variety of virus-producing and non-virus-producing mouse cells (12). This is a very sensitive technique that detects small quantities of surface antigen. Grant et al. (11) have shown that in addition to gp71, the internal proteins (M, 31,000 and M, 10,000) of F-MuLV are expressed on some mouse cell surfaces. These viral components are also found in a soluble form in virus-releasing cell cultures (8), and surface labeling of internal viral proteins may occur from passive absorption from disrupted virus or cells rather than from direct surface expression. Presently, however, this is not known. IEM studies are generally not useful for intracellular antigens due to a high degree of nonspecific binding of the ferritin conjugate. Efforts by our group to determine whether human antibody is directed at the same antigenic determinants on B particles as well as intracytoplasmic A particles using IEM have been unsuccessful.

An indirect immunoperoxidase technique has been used to detect human breast cancer antigen that appears to be related to gp52 of MMTV (16, 26). In the present study, the reactivity of human sera against MMTV could not be removed by absorptions with gp52, suggesting that serum antibody against gp52 is either not present in these sera or, if present, is combined with antibody against other MMTV component proteins. Tomana et al. (29) have also failed to absorb serum reactivity against MMTV with gp52 and also gp34 of MMTV but absorbed all activity with disrupted MMTV.

The human antibody:MMTV reaction may be due, at least in part, to cross-reactivities of a variety of nonviral substances with the carbohydrate moieties of the MMTV glycoproteins. Two recent reports (1, 28) present substantial data that human antibodies to the major glycoprotein (gp70) of Rauscher murine leukemia virus and simian sarcoma virus (C-type viruses) are the result of antibody directed at antigenic determinants which are not viral, but rather cellular, in origin. It is not known whether a similar reactivity exists with MMTV (a B-type virus), and this is presently being investigated.

Serum analysis by IEM suggests that antibody against MMTV is in the IgG and IgM fractions and absent from the IgA. This finding is in agreement with previous studies (29). In the course of the present study, the observation was made that serum:MMTV agglutination was sensitive to freezing and thawing with a rapid decrease in agglutination titer (20 to 30%) with repeated freeze-thaw cycles. The decrease in titer suggests that this may be the result of damage to IgM, and the recommendation is made that unfrozen serum or serum subjected to minimal freezing and thawing be utilized in future studies. The sera used in this study were not frozen or thawed more than twice.

Human breast carcinoma and MMTV appear to have one or more immunologically related antigens. This does not demonstrate that murine breast cancer and human breast cancer are etiologically related or that human breast cancer has an associated virus. These data are presented to show that serum labeling of budding MMTV correlates well with the much quicker and technically easier MMTV agglutination test and that both of these tests show correlation with the presence of Stage A or B breast cancer. The more sensitive IEM and MMTV agglutination tests reveal a higher incidence of activity in both breast cancer patient sera (79 to 85%) and normal sera (13 to 19%) than in immunofluorescent studies (17, 29), which show 41% of breast cancer sera reactive and 4% of normal sera reactive.

Long-term studies with a large number of patients will determine the usefulness of these techniques for the early detection of breast cancer, the serological monitoring of patients with breast cancer, and determining if antibody-positive patients should be considered in a high-risk category. Further research is necessary to show which of the MMTV antigenic determinants are being recognized by human breast cancer sera and to determine if these antigens are indeed virus specific or have cross-reactivity with cellular antigens.

ACKNOWLEDGMENTS

We would like to acknowledge the technical assistance of Gary Peer and Susan Curtas. We greatly appreciate the support of the study and manuscript review by Dr. Dani P. Bolognesi. Dr. Darrow Haagensen kindly provided sera from Columbia Presbyterian Hospital. Special Virus Cancer Program sera were acquired previously from Dr. Jack Gruber. We would like to thank Devona Stillwater and Linda Cusimano for the preparation of the manuscript.

REFERENCES

16. McCoy, J. L., Dean, J. H., Cannon, G. B., Jerome, L. J., Alford, T. C., Parks,


Antibody Reacting with the Murine Mammary Tumor Virus in the Serum of Patients with Breast Carcinoma: A Possible Serological Detection Method for Breast Carcinoma

Walter D. Holder, Jr. and Samuel A. Wells, Jr.

Cancer Res 1983;43:239-244.

Updated version Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/43/1/239

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.