Detection in human breast carcinomas of an antigen immunologically related to a group-specific antigen of mouse mammary tumor virus

(immunoperoxidase staining/paraffin sections/cancer antigen)

R. MESA-TEJADA*†, I. KEYDAR*, M. RAMANARAYANAN*, T. OHNO*, C. FENOGLIO†, AND S. SPIEGELMAN*

* Institute of Cancer Research and † Department of Pathology, College of Physicians and Surgeons of Columbia University, 701 West 168th Street, New York, New York 10032

Contributed by S. Spiegelman, December 27, 1977

ABSTRACT An antigen immunologically related to a group-specific antigen (gp52, a 52,000-dalton glycoprotein) of the mouse mammary tumor virus has been identified in paraffin sections of human breast cancers by means of the indirect immunoperoxidase technique. The specificity of the reaction with antibody against mouse mammary tumor virus was examined by absorption of the IgG with the following: (a) purified gp52; (b) a number of virus preparations (mouse mammary tumor virus, Rauscher leukemia virus, simian sarcoma virus, baboon endogenous virus, and Mason-Pfizer monkey virus); (c) normal plasma, leukocytes, breast tissue, milk, actin, collagen, and hyaluronic acid, all of human origin; (d) sheep erythrocytes and mucin. Only mouse mammary tumor virus (from C₃H or Paris RIII strains and grown in either murine or feline cells) and purified gp52 eliminated the immunohistochemical reaction in the human breast tumors.

Positive reactions were seen in 51 of 131 (39%) breast carcinomas of various histologic types, a minimal estimate in view of the limited number of sections from each tumor that could be examined. Negative reactions were obtained in all 119 benign breast lesions (cystic disease, fibroadenoma, papilloma, gynecomastia) and in all 18 normal breast tissues. With one exception, 99 carcinomas from 13 organs other than breast and 8 cystosarcomas were all negative.

Earlier investigations (1) have revealed key similarities between human breast cancer and the mouse mammary tumor experimental model. Human breast carcinomas contain particles with many of the characteristic biochemical and biophysical features of the RNA tumor viruses. These include the characteristic size and density and possession of outer membranes as well as central cores encapsulating an RNA-dependent DNA polymerase (reverse transcriptase) complexed to a 70S RNA molecule. Further, we (2-4) and others (5) have demonstrated a partial homology between RNA molecules found in human breast tumors and the RNA genome of the mouse mammary tumor virus (MMTV).

It is at present futile to dwell on the etiologic implications of these findings, because no hard conclusions can be drawn until more definitive experiments become possible. However, whether they are the cause or the consequence of the human malignancy, the presence of these particles provides a novel opportunity to generate information of potentially practical importance for the diagnosis and the management of the disease. Thus, if one or more of the particle proteins appear in the circulation, they could serve as systemic signals of the presence of the corresponding tumor. The feasibility of this approach was explored and established with the mouse mammary tumor

model. We showed (6) by radioimmunoassays that the plasma level of a 52,000-dalton viral glycoprotein (gp52) is an excellent diagnostic device and also provides reliable prognostic indications of the ultimate effectiveness of surgical and adjuvant therapy. Any attempt at an extension of this approach to the human disease must begin by answering the following questions: (a) What kind of antigen should be sought in the human disease? (b) Where and how should one initially look for this antigen?

Before embarking on a blind quest for an antigen specific for the human tumor it would seem prudent to take advantage of the clues supplied by the information gained from the murine mammary tumor model. The fact that some homology has been shown between the RNAs of the human and murine tumor particles suggests that an antigenic relation might conceivably exist between one or another of the proteins of these particles. The plausibility of this expectation is supported by serological investigations with sera from breast cancer patients, studies that have identified antibodies interacting with MMTV components (7-11). Most recently (12), migration inhibition studies of leukocytes from breast cancer patients provided evidence of crossreactivity with gp52 of MMTV.

In considering the question of where and how to look for such antigens in humans, it is important to recognize logistical and quantitative limitations in transferring certain technologies from mice to humans. Thus, the temptation to set up radioimmunoassays for crossreacting proteins in human plasma is likely to meet with disappointment due to the 1000-fold blood volume difference between mice and humans. Even if a human tumor is every bit as effective on a gram basis as a mouse tumor in producing a protein antigen in the blood, one would still have to cope with the fact that the signal would be diluted 1000-fold in the human as compared with the mouse. For this and other reasons we decided to focus on the tumor itself as the most plausible site to initiate the search for a crossreactive protein in the human. We again turned to the murine model to develop a convenient, reliable and sensitive microscopic method for identifying such an antigen. We found that of all the immunohistochemical procedures available, immunoperoxidase staining provides the most reliably interpretable reactions, and in addition, possesses the overriding advantage of being applicable to paraffin sections (13).

We present evidence here that immunoperoxidase staining of paraffin sections can be used to detect a breast cancer antigen that crossreacts with gp52, the group-specific antigen of the mouse mammary tumor virus.

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Abbreviations: MMTV, murine mammary tumor virus; α -MMTV, antibody to MMTV.

FIG. 1. (Legend appears at bottom of the next page.)

MATERIALS AND METHODS

Tissues. Paraffin blocks of tissues used for diagnostic purposes were obtained from the files of the Divisions of Surgical, Gynecologic, and Anatomic Pathology of the College of Physicians and Surgeons of Columbia University. Blocks of normal, benign, and malignant breast tissues were selected from specimens from biopsies, resections, mastectomies, and reduction mammoplasties. Disseminated carcinomas from four autopsies were also used. Samples from non-breast malignancies included biopsy and resection specimens. Whenever available, multiple blocks from each specimen were used. All of these were initially fixed in Bouin's fixative or, in the case of larger specimens, in 10% buffered formalin followed by Bouin's fixation.

In all cases 5- μ m serial sections were cut for immunohistochemical staining, and at least one section from each block was stained with hematoxylin and eosin for routine histopathologic examination. Sections of mammary tumors from Paris RIII or CD8F₁ mice were always included as positive controls.

Immunohistochemical Staining. Except for one modification, the indirect immunoperoxidase method used on the human material was the same as the one previously described (13). A preliminary step was introduced involving an incubation with hyaluronidase (600 units/ml, Sigma, type 1) at 37° for 1 hr. This enzymatic pretreatment was found to minimize non-specific background staining of collagenous stroma. It did not interfere with the specific staining of the human or murine malignant cells as determined by the use of parallel nontreated controls.

IgG Preparations. The indirect immunoperoxidase technique requires the use of two antibodies. The primary one detects the antigen being sought and the second antibody, to which the peroxidase is conjugated, is directed against the first antibody. In the present study the primary IgG (α -MMTV) is derived from a rabbit immunized with MMTV isolated from the milk of Paris RIII mice. The second antibody is goat anti-rabbit IgG that is conjugated to peroxidase. All of these reagents were prepared and purified as described previously (13).

Immunoabsorbants and Their Preparation. A rigorous test of the specificity of any staining reaction observed in the human tissues requires absorption with relevant viral antigens as well as with a variety of substances containing antigens that might be detected by the antibodies being used and that would be irrelevant to the presence or absence of virus-related proteins. The absorbants were used in either the insoluble or soluble state, depending upon convenience and the amounts of material available. Insoluble absorbants were prepared by crosslinking the proteins with glutaraldehyde (14) from the following: (a) pooled normal human plasma; (b) fetal calf serum; (c) pooled normal human breast tissue extracts; (d) normal human milk; and (e) human lung collagen. In addition, sheep erythrocytes and pooled human lymphocytes were treated with glutaraldehyde (15). Insoluble absorbants were used at a concentration of 30 mg of lyophilized powder per ml containing 1 mg of the IgG.

Viruses used as absorbants included: (a) MMTV (RIII) from the milk of Paris RIII mice; (b) MMTV (C₃H) grown in feline kidney cells (CrFeK); (c) MMTV (C₃H) grown in C₃H mouse cells (MM5T); (d) Rauscher leukemia virus grown in the mouse JLSV9 cell line; (e) Rauscher leukemia virus purified from the

plasma of BALB/c mice; (f) Mason-Pfizer monkey virus grown in the human NC-37 cell line; (g) simian sarcoma virus grown in the NC-37 cell line; and (h) baboon endogenous virus grown in the canine thymus cell line (BKCT). Viruses disrupted by freezing and thawing (10 times) were used as absorbants at a concentration of 1 mg of protein for each ml containing 50 μ g of IgG. The following soluble immunoabsorbants were used at 1 mg/ml for each 50 μ g of IgG: (a) mucin from bovine submaxillary gland; (b) hyaluronic acid from human umbilical cord; and (c) actin prepared from human platelets. In addition, gp52 was purified to homogeneity from MMTV (RIII) and MMTV (C₃H) via affinity chromatography (16) or guanidinium chloride columns (17). The purified protein was used at 40 μ g for each 50 μ g of IgG in 1 ml.

Absorption of the Antisera. Absorption of the primary antibodies, α -MMTV (RIII), and separation of the soluble immunoabsorbants were done as previously described (13). The insoluble immunoabsorbants were separated by spinning at 8000 $\times g$ for 15 min at 4° twice, using the clear supernatant as the reagent. The secondary goat anti-rabbit IgG conjugated to peroxidase was always absorbed with insolubilized human plasma.

RESULTS

It is useful to begin by illustrating positive and negative reactions with representative instances of normal, benign, and malignant samples. Normal breast tissues, whether lactating (Fig. 1B) or not (Fig. 1A), do not show evidence of a reaction with α -MMTV. Similarly, there is no response seen in the two benign lesions, fibroadenoma (Fig. 1C) and fibrocystic disease (Fig. 1D). On the other hand, the breast carcinomas illustrated, which include an intraductal and invasive type (Fig. 1E) and a metastatic tumor in the ovary (Fig. 1G), both show clear evidence of a positive reaction.

The specificity of the positive reactions observed in this study was examined by selective absorptions with a variety of absorbants. Comparison of Fig. 1F and H with their positive counterparts (Fig. 1E and G, respectively) reveals that after absorption with purified gp52, the α -MMTV is unable to evoke a reaction in the carcinomas. Results of absorptions with other materials are discussed below.

The pattern of staining is quite well exemplified in Fig. 1E and G and may be briefly noted. The reaction observed is primarily focal, intracellular and cytoplasmic. The considerable staining variability that was encountered among different tumors, and also amongst individual cells within a given tumor, is well illustrated in these two examples. Furthermore, in instances in which many sections were cut from the same block, some levels showed more stained cells than others, and in some instances not all blocks from the same tumor gave a positive reaction.

Table 1 summarizes 131 cases of various types of adenocarcinomas of the breast that were examined by the immunoperoxidase method. Of these, 51 or 39% gave clear evidence of positive reactions. With the possible exception of the 52% positive tumors with intraductal and invasive features, there does not appear to exist any special relationship between the presence of detectable antigens and the histopathologic type of tumor.

FIG. 1 (on preceding page). Immunoperoxidase stain of normal breast, benign lesions, and primary and metastatic breast carcinoma with α -MMTV. Note complete absence of brown reaction product in resting (A) and lactating (B) mammary gland as well as in a fibroadenoma (C) and in an area of cystic disease (D). In the intraductal and invasive tumor (E) some of the cells in the intraductal lesion at right, and most of the invasive cells, are stained. Likewise, in the metastatic lesion of the ovary (G), not all of the tumor cells are stained. F and H are serial sections adjacent to E and G in which the reaction has been eliminated by prior absorption of α -MMTV with gp52. (Methylene blue counterstain. A, B, $\times 228$; C, D, $\times 140$; E, F, $\times 566$; G, H, $\times 1402$.)

Table 1. Immunoperoxidase staining of carcinomas of the breast

Type	Cases	Positives	% positive
Intraductal	20	6	30
Intraductal and invasive	31	16	52
Invasive*	49	19	39
Medullary	12	4	33
Metastatic†	19	6	32
Total	131	51	39

* 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.

None of 137 of the normal and benign breast tissues examined showed evidence of a positive reaction (Table 2). It should be noted that included in this group are 74 cases in which the benign lesions coincided with carcinoma in the same breast, and some of these carcinomas gave a positive staining reaction.

One apparent exception to the absence of reactions in non-malignant tissues was a positive response observed in foci of apocrine metaplasia, one of the microscopic hallmarks of cystic disease, and in apocrine glands from the axilla and perineum. However, this reaction is apparently different from that observed in malignant cells, because the apocrine cell response is virtually completely removed by absorption of the α -MMTV with mucin, a treatment that does not eliminate the reaction of malignant breast cells.

It was of obvious interest to see whether malignancies other than breast carcinomas contain this antigen, and Table 3 summarizes the results of testing 99 carcinomas from other organs and eight cases of cystosarcoma phyllodes (a primarily mesenchymal breast malignancy). Of these 107 tumors, only one, a mucoepidermoid carcinoma of the parotid gland, gave a positive reaction of uncertain specificity. Two other parotid carcinomas of the same histologic type were negative. It is clear that the antigen being detected by the α -MMTV in human breast carcinomas is confined principally to this neoplastic disease.

We have thus far discussed studies that tested the specificity of the reaction by preabsorption of the α -MMTV with gp52. Table 4 summarizes a more extensive examination of this question by pretreatment of the primary antibody with a number of different virus preparations and a variety of antigenic substances that might also be detected by this antibody. The only materials that suppressed the positive reaction of the

Table 2. Immunoperoxidase staining of benign and normal breast tissues

Type	Cases	Associated with breast carcinoma*	Positives
Cystic disease†	81	60	0
Fibroadenoma	19	4	0
Intraductal papilloma	10	10	0
Gynecomastia	9	0	0
Resting gland (normal)	9	0	0
Lactating gland (normal)	9	0	0
Total	137	74	0

* In the same breast.

† Excluding foci of apocrine metaplasia; absorption studies indicate that an antigen different from the one localized in breast carcinoma is responsible for this reaction.

Table 3. Immunoperoxidase staining of malignancies other than breast carcinomas

Malignancy	Cases	Positive
Colon	12	0
Stomach	3	0
Pancreas	3	0
Liver	4	0
Lung	9	0
Endometrium	22	0
Ovary	20	0
Prostate	8	0
Kidney	5	0
Urinary bladder	4	0
Skin	2	0
Thyroid gland	4	0
Parotid gland	3	1
Cystosarcoma phyllodes	8	0
Total	107	1

Primary sites of non-breast carcinomas stained with α -MMTV. Cystosarcoma phyllodes is also listed because it is the most common noncarcinomatous malignancy of the breast.

human breast cancers were the MMTV preparations and the purified gp52 derived from them. It will be noted that RIII and C₃H viruses and the gp52 prepared from both by either affinity chromatography or guanidinium chloride were equally effective. Further, it did not matter whether the C₃H virus was grown in mouse cells or feline cells. It is evident that the species differences that have been reported (18) for the group-specific antigen of the C₃H and RIII viruses are not affecting the reactions being studied here. The fact that C₃H virus grown in feline cells can also block the reaction indicates that the relevant antigen is determined by the virus and not by the cell in which it is grown.

The list of reagents in Table 4 that do not remove the reactivity from α -MMTV serves further to define the specificity of the reaction observed with malignant breast cells. None of the other viruses tested, including Rauscher leukemia virus, simian sarcoma virus, Mason-Pfizer monkey virus, and baboon endogenous virus, contained an antigen sufficiently related to the one in MMTV to block the reaction being observed with breast

Table 4. Absorption specificity tests of immunoperoxidase staining of human breast carcinomas with α -MMTV

Completely eliminated by	Not eliminated by
MMTV (RIII) from milk	Rauscher leukemia virus,
MMTV (C ₃ H) from MM5T	simian sarcoma virus,
MMTV (C ₃ H) from CrFeK	Mason-Pfizer monkey
gp52 (RIII) } Purified by concanavalin A affinity chromatography	virus, and baboon endogenous virus
gp52 (C ₃ H) } Purified by guanidinium chloride chromatography	Erythrocytes (sheep)
gp52 (RIII) }	Normal plasma (human)
gp52 (C ₃ H) }	Normal leukocytes (human)
	Collagen (human)
	Actin (human)
	Mucin (bovine)
	Hyaluronic acid (human)
	Milk (human)
	Normal breast tissue (human)

The absorptions were done using these agents either in the soluble or insolubilized form.

cancer cells. The same conclusion can be drawn from the negative results obtained with the other materials listed in Table 4. These included sheep erythrocytes as a source of heterophile antigens, normal human plasma, leukocytes, milk, normal breast tissue, collagen, actin, mucin, and hyaluronic acid. Finally, the remote possibility that we were observing the carcinoembryonic antigen (CEA) (19) was eliminated by the failure of anti-CEA to stain α -MMTV-positive breast tumors under conditions in which CEA is readily found in paraffin sections of colon carcinomas (20).

DISCUSSION

Detailed comparisons of the reactions observed in the human and mouse carcinomas must be interpreted with caution in view of the wider spectrum of histopathological variability of human breast cancer compared to its murine counterpart. Certain similarities and differences may, however, be worthy of note. Considering their origin from virus-infected mice, it is not surprising to find that the expression of MMTV antigens in the mouse mammary tumors is both more intense and more frequent (13) than is seen in human cancer. However, it should be noted that in both cases one observes instances of malignant cells that do not exhibit staining reactions. This cellular heterogeneity in antigen expression is a common phenomenon, which has been seen in immunoperoxidase staining of other tumor-associated antigens (21). One striking difference between the human and murine breast cancer cells is that absorption of the α -MMTV with purified gp52 completely eliminates the reaction of the human and not of the murine tumors. It is clear that the latter are expressing MMTV antigens not observed in the human lesions.

Because only two to three sections were stained from each tumor, the 39% positives found for the breast carcinomas examined (Table 2) must be a minimal value. A more realistic estimate will require a patient series in which a more exhaustive examination is made of the several thousand sections that can be produced from each tumor.

The tissue and immunological specificities of the reaction observed with the human breast cancer are supported by the following findings: (a) none of 137 normal and benign breast tissues show evidence of a positive reaction (Table 2); (b) with one exception, none of the 99 carcinomas from a variety of organs other than breast and none of eight cystosarcomas were positive (Table 3); (c) of all the viruses and nonviral preparations tested, only the mouse mammary tumor viruses blocked the reactions (Fig. 1 F and H, Table 4); (d) among the proteins examined, only purified gp52, the group-specific antigen of MMTV, eliminated the response to the α -MMTV (Table 4).

It is of course gratifying to have extended to the protein level a relation between human breast cancer and the mouse mammary tumor virus that was discovered (2, 3) in terms of nucleic acid sequence homology. But again, the etiologic implications are not our immediate concern, but rather the possibility that these findings can be used to generate clinically useful information. To this end, the resolution of the following issues assumes first priority: (a) Is the sugar or the protein moiety of the gp52 glycoprotein responsible for its ability to block the immunological reaction with the breast cancer cells? (b) What is the chemical nature of the gp52 crossreacting antigen found in human breast cancer cells? (c) Can additional specific anti-

gens be detected by using antisera to other oncornaviruses (e.g., Mason-Pfizer monkey virus) with suspected (22, 23) cross-reactivities?

The first question can be answered by examining the effect of specific glycosidases on the ability of gp52 to block the reaction with breast cancer tissue. A resolution of the second question will require purification to homogeneity of the relevant human breast cancer antigen. Its availability will also make possible the production of the immunologic reagents needed to develop the heterologous radioimmunoassays that can hope to attain the sensitivity levels required for the measurement of a systemic signal in the human disease.

We are extremely grateful to Dr. Raffaele Lattes, Director of the Division of Surgical Pathology, for his invaluable advice and cooperation. We thank Dr. Gabriel Godman and Dr. Inez Mandl for the actin and lung collagen, respectively. The invaluable technical assistance of Peggy Moy, Elizabeth Osze, and Marianne Cenno is gratefully acknowledged. This investigation was supported by Grant CA-02332 and Contract NO1-CP7-1016 awarded by the National Cancer Institute.

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