

## Antibodies reactive with murine mammary tumor virus in sera of patients with breast cancer: Geographic and family studies

(viral antibody/geographic distribution)

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**ABSTRACT** Sera of patients with breast cancer, of healthy women from the United States, East India, East Africa, and China, and of healthy women of American and Parsi families in which breast cancer occurred in several family members were assayed for levels of antibody reactive with the murine mammary tumor virus (MuMTV) by an enzyme-linked immunosorbent assay. Increased levels of antibody to MuMTV (absorbance  $\geq 0.4$ ) were found in sera of 18.6% of American patients with breast cancer and of 2.8% of healthy American women and in 38% of patients from India and 61.9% from East Africa (healthy, 26.9%). In contrast, antibody reactive with MuMTV was found in <5% of women with breast cancer from mainland China (healthy Chinese, 5.0%). Differences in serum MuMTV antibody levels between breast cancer patients in the four groups were found to be significant ( $P < 0.0001$ ). Studies of two families from the United States and of one Parsi family from India with genetic propensity to breast cancer showed that high levels of antibody to MuMTV were found in 33%, 71%, and 23% of healthy family members, respectively. The antibody to MuMTV was readily absorbed with purified MuMTV and gp52. In contrast, fetal calf serum, murine type C retroviruses, or erythrocytes from various species failed to absorb the antibody.

A relationship between human breast cancer and murine mammary tumor virus (MuMTV) has been suggested from several observations. RNA partially homologous to the MuMTV genome was identified in human breast cancer tissue (1). Lymphocytes from individuals with breast cancer were shown to proliferate after stimulation by MuMTV (2, 3). Sera from breast cancer patients decreased the infectivity of MuMTV in mice (4), suggesting the presence of antibodies against the virus. More recently, using immunoperoxidase staining, Mesa-Tejada *et al.* (5) found that antigen reacting with antibody to the envelope glycoprotein of MuMTV (gp52) could be detected in breast cancer tissue. Ohno *et al.* (6) have now demonstrated that this crossreactivity is directed to the protein moiety of gp52 and not the carbohydrate.

In prior studies using a virolytic assay with complement and antibody, we found that antibodies reactive with MuMTV are present in sera of approximately 20% of American women with breast cancer (7). Sera of age-matched healthy women and of patients with other cancers had such antibodies in lower frequency. We developed a method to detect these antibodies directly by using MuMTV and an enzyme-linked immunosorbent assay (ELISA) (8). With this method also we found that sera of approximately 25% of American women with breast cancer had antibodies to MuMTV. Approximately 10% of women with benign cystic disease of the breast also had such antibodies in

their sera (8). Reactivity was inhibited by the membrane proteins of MuMTV (gp52 and gp34) but not by the core antigen (p28) (9).

We now report results on the specificity of the human antibody for MuMTV, the geographic distribution of the antibody in breast cancer patients, and analyses of families in which breast cancer occurred in high frequency.

### MATERIAL AND METHODS

**Patient Population.** The breast cancer patients used for our study were Caucasian women from the United States, Black women from Kenya (East Africa), Chinese women from Peking (People's Republic of China), and Muslim, Parsi, Sindhi, and Maharashtrian women from Bombay (India). In addition, we studied individuals from American and Parsi families with high propensity of breast cancer. The distribution of patients with breast cancer was as follows: 145 from the United States, 53 from India, 24 from China, and 21 from East Africa.

**Patient Sera.** Blood samples (5–10 ml) without anticoagulants were drawn under sterile conditions and the sera were prepared by established procedures (10), divided into 100- $\mu$ l aliquots, and stored at  $-70^{\circ}\text{C}$ . Sera from abroad were shipped to the United States in dry ice.

**Viruses and Purified Preparation of the Virus.** MuMTV was purified from cultures of MuMT-73 murine mammary epithelial cell line as described (11). gp52, the purified membrane antigen, was prepared according to published procedures (12). AKR and Rauscher murine leukemia viruses were purified as described for the MuMTV.

**Determination of Antibody to MuMTV by ELISA.** Sera were assayed by ELISA for presence of antibody reactive with MuMTV by described methods (8). Briefly, sera were diluted 1:30 in phosphate-buffered saline containing 0.05% Tween 20 ( $\text{P}_i/\text{NaCl}/\text{Tween}$ ) and incubated in microtiter plate wells containing 0.5  $\mu\text{g}$  of fixed MuMTV. After 2 hr, the wells were washed with  $\text{P}_i/\text{NaCl}/\text{Tween}$ , and alkaline phosphatase-conjugated swine anti-human IgG (Orion Diagnostics, Helsinki, Finland) was added to the wells. After 120 min, the wells were again washed with  $\text{P}_i/\text{NaCl}/\text{Tween}$  and the alkaline phosphatase substrate *p*-nitrophenyl phosphate was added. After 30–60 min, the reaction was stopped with 3 M NaOH and the absorbance of the yellow color was determined at 400 nm in a spectrophotometer. The observed absorbance is proportional to viral protein over the range 0.1–10  $\mu\text{g}$ , and rabbit antibody to MuMTV is readily detectable at dilutions up to 1:3000 (8).

**Absorption Studies.** To 50  $\mu\text{l}$  of purified MuMTV (0.1 mg/ml) was added 50  $\mu\text{l}$  of 1:10 dilution ( $\text{P}_i/\text{NaCl}$ ) of heated ( $56^{\circ}\text{C}$ ,

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Abbreviations: MuMTV, murine mammary tumor virus; ELISA, enzyme-linked immunosorbent assay;  $\text{P}_i/\text{NaCl}/\text{Tween}$ , phosphate-buffered saline containing 0.05% Tween 20.

30 min) test serum with positive antibody titer determined by the ELISA. The reaction mixture was incubated at 37°C for 60 min. The mixture was centrifuged at  $10,000 \times g$  for 5 min and half of the supernatant was removed. This process was repeated three times and the supernatants were analyzed by the ELISA. The above procedure was repeated with the AKR and Rauscher murine leukemia and purified gp52.

Absorption with erythrocytes from human, sheep, bovine, equine, and guinea pig sera was carried out as above with equal volumes of washed (three times) erythrocytes of various species ( $1 \times 10^8/\text{ml}$ ) and serum.

**Blocking Studies.** High-titered antisera were incubated with MuMTV or AKR murine leukemia virus (0.5 mg/ml) or various carbohydrates (1 mg/ml) in a final volume of 150  $\mu\text{l}$  and incubated at 37°C for 60 min and at 4°C for 18 hr. After centrifugation, the supernatants were assayed by the ELISA for binding to MuMTV.

**Analysis of Results.** The results are expressed (a) as the percentage of patients in each group with increased levels of antibody to MuMTV (background absorbance of sera of 50 healthy women  $\geq 0.4$ ) at 1:30 dilution and (b) as the mean of individual values of antibody to MuMTV. The two-sample *t* test was used to detect differences between mean MuMTV antibody levels of breast cancer and healthy groups of each nationality. One-way analysis of variance was used to detect differences among mean MuMTV antibody levels of healthy groups of three nationalities, among breast cancer groups of four nationalities, and among breast cancer groups of three East Indian communities in the Bombay area. Multiple comparisons by Fisher's least significant difference procedure were made when appropriate.

## RESULTS

**Specificity of Antibody in Serum of Patients with Breast Cancer.** Fig. 1 is representative of results of absorption studies carried out with diluted (1:10) sera from several patients with

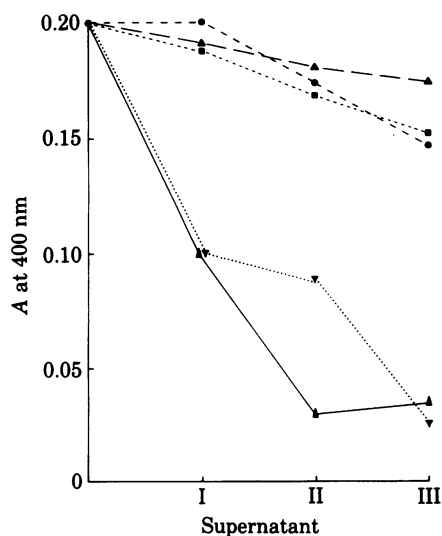


FIG. 1. Absorption of human antibodies reactive with MuMTV. Aliquots of MuMTV were incubated with fetal calf serum (▲), sheep erythrocytes (■), Rauscher murine leukemia virus (●), MuMTV (▲), or the MuMTV surface glycoprotein gp52 (▼). The supernatants from each of three consecutive such absorptions (I, II, III) were assayed for IgG reactivity with MuMTV by ELISA. A at 400 nm refers to the absorbance of the yellow color formed by the reaction of alkaline phosphatase-conjugated swine anti-human IgG with *p*-nitrophenyl phosphate. The decreases in absorbance resulting from three absorptions were: fetal calf serum, 4%; sheep erythrocytes, 24%; Rauscher murine leukemia virus, 26%; MuMTV, 85%; gp52, 96%.

breast cancer who have high levels of antibody reactive with MuMTV. The antibody was readily absorbed with purified MuMTV and the envelope glycoprotein of the virus. In contrast, fetal calf serum or sheep erythrocytes [or erythrocytes from cows, horses, guinea pigs, pigs, or humans (not shown)] failed to absorb the antibody in this way.

Preincubation of the serum with MuMTV prevented binding of the antibody to MuMTV coated on microtiter plates (Fig. 2). In contrast, preincubation with glycogen or another murine retrovirus, the type C AKR leukemia virus, did not prevent the binding of the antibody to MuMTV. Similar results were obtained when D-galactose, *N*-acetyl-D-galactosamine, L-fucose, or D-mannose were substituted for glycogen (data not shown). Furthermore, no significant changes in background binding were observed after absorption of normal serum with MuMTV, gp52, or various erythrocytes.

**Geographical Study of Antibody in Sera of Patients with Breast Cancer.** Fig. 3 summarizes the binding to MuMTV of specific IgG from sera of breast cancer patients from several different geographical regions. Increased levels of antibody to MuMTV (absorbance  $\geq 0.4$ ) were found in sera of 27 of 145 (18.6%) patients from the United States, 20 of 53 from India (37.7%), and 13 of 21 from Kenya (61.9%). In striking contrast to the high frequency of positive antibody levels observed in sera of patients from Kenya, less than 5% of sera from Chinese patients had antibody reactive with MuMTV. Sera of the Chinese breast cancer patients who were positive for MuMTV exhibited antibody levels lower than those in the American, East African, and East Indian patients. Hence, differences in MuMTV antibody levels among breast cancer patients of these four nationalities were found to be significant (*F*, with 3 and 239 degrees of freedom, = 9.12; *P* < 0.0001).

Distributions of individual MuMTV antibody levels in sera of breast cancer patients from three different communities from the Bombay region are summarized below. The Sindhi group was omitted from the statistical considerations because only three Sindhi women with breast cancer were included in the study (only one had an increased level). Increased levels of MuMTV antibody were found in 1 of 14 Parsi women (7.1%),

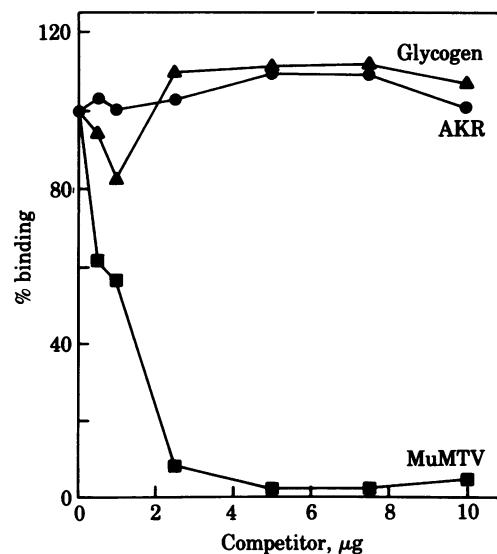


FIG. 2. Effect of MuMTV, AKR leukemia virus, and glycogen on the reactivity of human breast cancer serum with MuMTV. Aliquots of sera from breast cancer patients were incubated with 0–10  $\mu\text{g}$  of MuMTV (■), AKR virus (●), or glycogen (▲) and the supernatants were assayed for IgG binding to MuMTV, as described in Fig. 1.

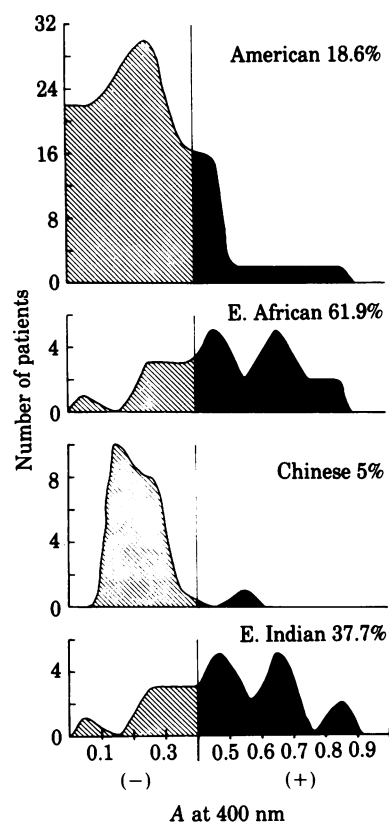


FIG. 3. Antibody reactive with MuMTV in sera of breast cancer patients from the United States, East Africa, China, and India.  $A \geq 0.4$  was considered to be significant.

5 of 12 Muslim women (41.7%), and 13 of 24 Maharashtrian women (54.2%). Differences in the mean of individual antibody levels among the three groups were found to be significant ( $F$ , with 2 and 4.7 degrees of freedom, = 3.396;  $P = 0.04$ ). The mean level was 0.2115 in the Parsi group, 0.4809 in the Maharashtrian group, 0.3407 in the Muslim; the first two means were significantly different from each other but the third was not significantly different from either ( $P = 0.05$ ; Fisher's least significant difference procedure).

Sera from healthy women of several nationalities were also compared. Increased levels of antibody to MuMTV were found in 1 of 22 healthy Chinese women (4.5%), 1 of 36 healthy American women (2.8%), and 7 of 26 healthy East African women (26.9%) (Fig. 4). The mean levels of antibody reactive to MuMTV in sera of healthy Chinese, American, East African, and East Indian women were also compared. The four groups were found to be significantly different ( $F$ , with 3 and 104 degrees of freedom, = 11.9;  $P < 0.0001$ ). Specifically, the East Indian group had significantly higher mean levels of MuMTV antibody than the other three groups (mean, 0.5580). Moreover, the American group had significantly lower mean level of antibody to MuMTV (mean, 0.1851) than the East African group (mean, 0.3157); the mean of the Chinese group (mean, 0.2267) was between the American and East African means but was not significantly different from either ( $P = 0.05$ ; Fisher's least significant difference procedure). No significant difference was found between levels of antibody to MuMTV in sera of healthy Chinese women and Chinese women with breast cancer (means, 0.2267 and 0.2424, respectively). In contrast, significant differences were found between healthy American women and American women with breast cancer (means, 0.1832 and 0.2568, respectively;  $F$ , with 179 degrees of freedom, = 2.43;

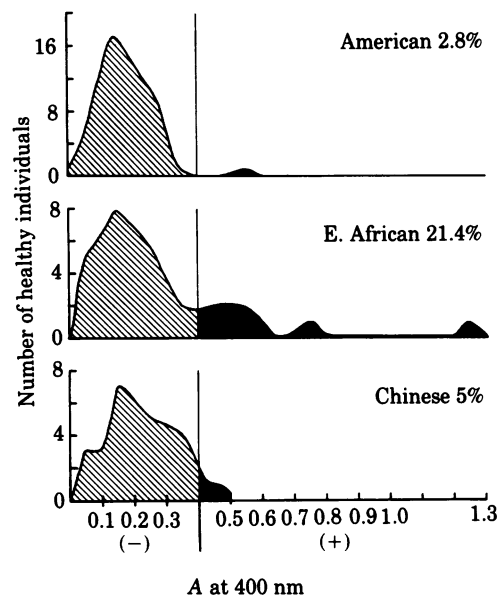


FIG. 4. Antibody reactive with MuMTV in sera of healthy individuals from the United States, East Africa, and China.

$P = 0.016$ ) (Fig. 5) and between healthy East African women and East African women with breast cancer (means, 0.3157 and 0.4694, respectively;  $F$ , with 45 degrees of freedom, = 2.11;  $P = 0.04$ ) (data not shown).

**Family Studies of Antibody to MuMTV.** Fig. 6 shows a representative American family pedigree in which breast cancer occurred in several family members. Breast cancer had occurred in both maternal and paternal relatives. Neither the

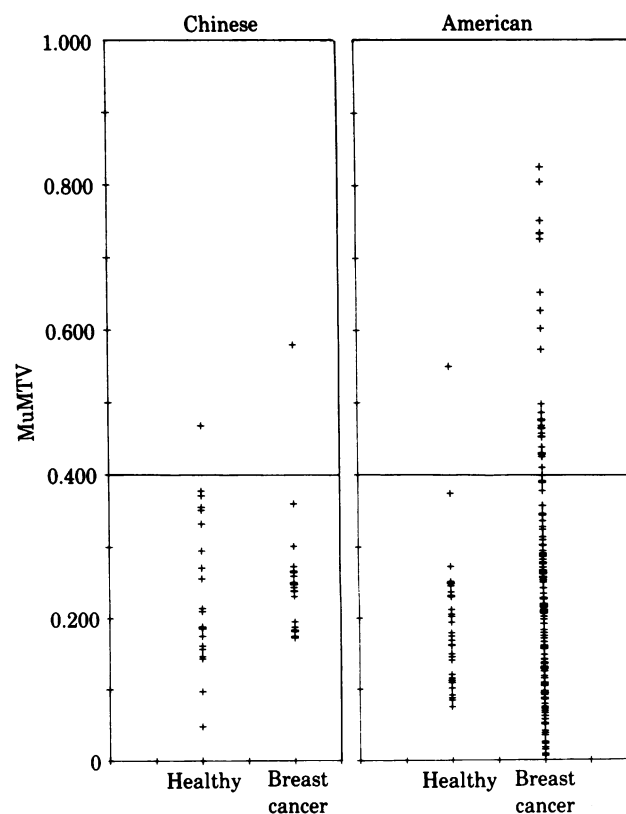


FIG. 5. Comparison of serum levels of antibody to MuMTV in healthy Chinese and American women and breast cancer patients.

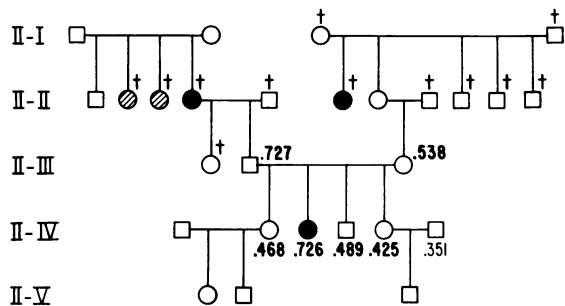


FIG. 6. Antibodies reactive with MuMTV in a family with a history of breast cancer. Sera from three generations of a family with a history of breast cancer were assayed for antibody to MuMTV by the ELISA. The numbers refer to the absorbance at 400 nm (see Fig. 1 for explanation);  $A \geq 0.4$  is positive for MuMTV antibody,  $\circ$ , female;  $\bullet$ , female with breast cancer;  $\oslash$ , female with other cancer;  $\square$ , male;  $\dagger$ , deceased.

father nor the mother of the propositus had breast cancer but both had significantly increased titers of antibody to MuMTV. Furthermore, in each of these the antibody was absorbable with MuMTV and gp52. All siblings (both male and female) of the propositus had increased titers of antibody and in each case the antibody was absorbable with MuMTV and gp52.

In two other American families with high frequency of breast cancer (Fig. 7), 33% of the members of family I and 71% of members of family II showed an increased titer of antibody to MuMTV. In family I the patients' sera did not show increased antibody levels whereas in family II, the patients' serum did.

Similar observations of frequently increased levels of antibody reactive to MuMTV were observed in family members of Parsi women with a high frequency of breast cancer. A representative Parsi family is illustrated in Fig. 8. In this family, 23% of healthy relatives had increased levels of antibody reactive with MuMTV.

## DISCUSSION

Much evidence links immune responses in human breast cancer to antigens represented in MuMTV. The findings from our present geographical and family study support the hypothesis that a relationship exists between antibodies reactive with MuMTV and breast cancer in humans. Some patients with breast cancer react to antigens that are at least crossreactive with antigens

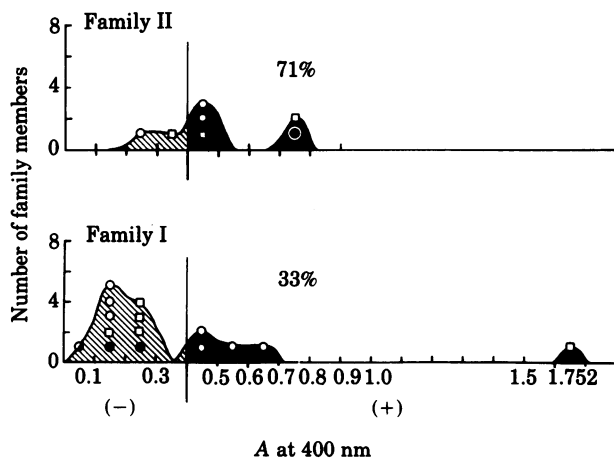


FIG. 7. Antibody to MuMTV in sera of two American families with genetic propensity to breast cancer in family members.  $\square$ , Healthy male;  $\circ$ , healthy female;  $\bullet$ , female with breast cancer;  $\oslash$ , female with benign breast disease.

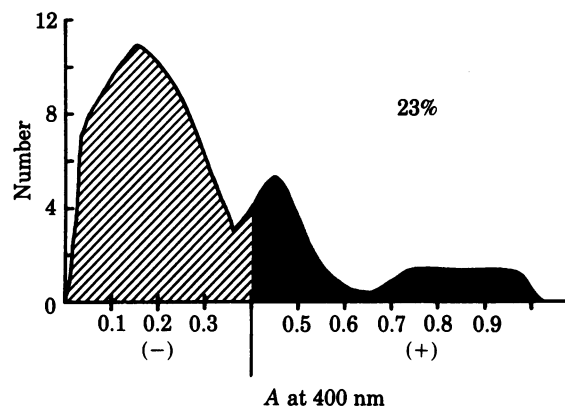


FIG. 8. Antibody to MuMTV in sera of one Parsi family with genetic propensity to breast cancer in family members.

represented on MuMTV, especially the envelope glycoprotein gp52. Various erythrocytes and other murine tumor viruses did not yield significant absorption of the antibody under study. Furthermore, blocking of binding of antibody to MuMTV adherent to microtiter plates was not inhibited by glycogen or other sugars found in cell membranes but was completely inhibited by MuMTV.

These findings do not exclude the possibility that antibodies against heterophile antigens related to the virus may also occur in sera of patients with breast cancer. Antibodies in human sera to the glycoprotein of type C retroviruses appear to be directed to the sugar moiety (13).

Several possibilities must be considered to explain our findings, of which one is that a virus or viruses related to MuMTV play a role in some patients with breast cancer. More likely is the possibility that gene sequences related to those determining MuMTV proteins are expressed in some patients with human breast cancer and that antibodies or autoantibodies to these antigenic determinants are sometimes made in humans with certain forms of breast cancer.

Perhaps the most striking of the findings in this report is that major differences in these antibody responses were found when sera of breast cancer patients from different parts of the world were investigated. Sera of women from Kenya with breast cancer had these antibodies in highest frequency and highest titers followed, in turn, by sera of breast cancer patients from East India, America, and China. The Chinese women with breast cancer had both the lowest titers and the lowest frequency of increased titers. The simplest interpretation of these data is that there exist multiple (at least two) separate forms of breast cancer and in one, antigens stimulate production of the anti-MuMTV antibodies. It is of interest in this regard that Levine *et al.* (14) recently demonstrated, by immunoperoxidase assay, an antigen that crossreacts immunohistochemically with gp52 of the MuMTV in large quantities in 70% of biopsy specimens (23/33) from Tunisian breast cancer patients compared with 47% of American breast cancer biopsy specimens. In contrast, even significantly less antigen was present in Japanese breast cancer biopsies (Sol Spiegelman, Columbia University, New York, personal communication). The parallel with our observations, and thus the complementarity of the two findings, is obvious.

That genetic factors or factors in the immediate environment play a role in the expression of the antigenic stimulation that leads to the antibody response under study is suggested by the reported observations that breast cancer patients who are members of different communities in the Bombay area of India express these antibodies in different frequencies. These findings require more penetrating analysis.

The findings from our studies of American and East Asian families in which breast cancer occurs in high frequency are also challenging. They show that increased titers of antibody against MuMTV can occur in healthy female family members and even in some males. The latter antibodies also were absorbable with the virus and gp52. These family studies must be pursued in greater depth but they suggest the possibility that members at risk for breast cancer in such families may be identifiable by immunological analyses prior to the development or full expression of the breast cancer.

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