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A Viral Antigen as a Marker for the Prognosis of Human Breast Cancer*

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Abstract—*An antigen present in human breast tumor cells, and which is immunologically related to the envelope protein (gp52) of murine mammary tumor virus, was used as a marker for the detection of breast cancer in an Israeli population. The results show that the antigen was detectable in* 128 *of* 204 *breast carcinomas tested (*62.7%*). The immunological reaction was not detected in normal breast tissue, benign breast tumors, ductal hyperplasia or in primary malignancies in other organs. A significantly higher percentage of cases with demonstrable antigen was found in Israeli women born in North Africa (*78%*) as compared to women of European origin (*60.6%*). The frequency of detection of the antigen was higher in stage IV (*80%*) as compared to stage I (*15%*), suggesting that the gp52 cross-reacting antigen is a marker for the severity of the disease. Moreover, a retrospective study of* 97 *cases of stage II breast cancer shows that if the antigen is detected at the time of mastectomy, one can usually predict an unfavorable prognosis. Survival data analysis indicates that patients without detectable antigen survived significantly longer than those with a detectable antigen.*

INTRODUCTION

BREAST cancer is the most common neoplasm in Israeli women. At present approximately 1000 new cases of breast cancer are diagnosed yearly. The highest incidence of this neoplasm is found in Ashkenazi Jews who immigrated from Middle and Eastern Europe, an intermediate incidence in the Sephardic Jews who originated from Southern European and Turkey, and the lowest in the North African and other Asian-born Jews [1].

Sacks, using the framework of the UICC study, found that European-born Israeli women have a higher incidence of breast cancer than those originating from North Africa or Asia, and infiltrating ductal carcinoma is the most frequent histological type in all ethnic groups [2, 4]. Melnik *et al.* [3] have recently described differences in the survival rates of breast cancer patients in Israeli women according to their country of birth. This publication suggests that Israeli patients of European origin survive longer than immigrants from Asia and North Africa.

A few years ago the presence of an antigen in human breast tumors was reported. This antigen cross-reacted immunologically with a protein of the mouse mammary tumor virus (MMTV gp52—an envelope glycoprotein with a molecular weight of 52,000 daltons). Using the indirect immunoperoxidase test, the reliability and sensitivity of the method for detecting the viral antigen in paraffin-embedded tissues of mouse mammary tumors was first established [5]. Not only was it possible to demonstrate the localization of the antigen in tumor cells, but the specificity of the immunological reaction was also confirmed by selective absorption experiments [5]. Furthermore, by this method the presence and specificity of the above-mentioned antigen in sections of human breast cancer was demonstrable [6, 7].

The purpose of this paper is to assess the presence of the gp52 cross-reacting antigen in human breast cancer, its distribution amongst the different Israeli ethnic groups and its possible role as a marker in the prognosis.

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MATERIALS AND METHODS

Tissues

Paraffin-embedded tissues from 204 cases were obtained from the pathology departments of hospitals in the Tel Aviv area. Serial sections 5 μm thick were used in the study. Most of the tissues had been fixed in 10% formalin and a few in Bouin's fixative. Ninety-seven cases received from the Sheba Medical Center were used in the follow-up study. All the cases were stage II, with 24.7% in the premenopausal age group (19–49 age group) at the time of mastectomy and the rest (50 yr and older) postmenopausal. The cases were staged according to UICC TNM classifications.

Antisera and IgG preparation

Antisera were prepared in white rabbits (local strain) using gp52 purified from MMTV isolated from the milk of Paris RIII mice (supplied by Dr. S. Spiegelman, Columbia University, NY). The gp52 was purified by affinity chromatography on concavalin-A-bound Sepharose, as described elsewhere [8]. The IgG fractions were purified by sodium sulphate fractionation followed by ion exchange chromatography on DEAE cellulose [5].

Characterization of antibodies

MMTV and gp52 were labeled with [^{125}I] using the Bolton–Hunter reagent [9]. The antibody preparations were characterized by using the *Staphyloccus aureus* (Cowan I strain) radioimmunoprecipitation assay [10]. Pelleted complexes were dissociated in 1% SDS, 1.5% β-mercaptoethanol, 10% glycerol, 0.125M Tris–HCl, pH 6.8, in a final volume of 50 μl and treated at 100°C for 2 min. The supernatants of a 5-min 10,000-rev/min centrifugation were loaded on an 11% SDS-polyacrylamide discontinuous gel and underwent electrophoresis and autoradiography as previously described [11] and shown in Fig. 1. The sera prepared against the purified gp52 were also tested by the indirect immunoperoxidase assay on mouse mammary tumor sections of the following: RIII tumor sections; GR and C3H mammary tumors (known as producers of B-type mature MMTV virions); and on X/Gf tumors, which produce mature B type particles *only* after induction by X-rays and urethan [12] (received from Dr. A. Goldfeder). Sections from the first three groups of tumors gave a positive immunohistochemical reaction with anti-gp52 sera, whereas the X/Gf sections were not stained unless the virus was induced. On the other hand, the X/Gf tumor sections were stained when the antisera against whole MMTV particles were used. This staining was not eliminated by preabsorbing the serum with gp52. The above information substantiates the notion that the antiserum is gp52-monospecific and does not react with either mouse tissue or MMTV components other than the envelope glycoprotein.

Absorption of IgG preparations

The immunoglobulin preparations used were preabsorbed as previously described [6]. The insolubilized absorbants used were: (a) a pool of normal human plasma; (b) fetal calf serum; and (c) human milk. For the demonstration of the specificity of the immunohistochemical staining, 25 μg IgG were preabsorbed with one of the following: (1) 40 μg of purified gp52; (2) 150 μg of MMTV purified from either Paris RIII milk or C3H MMTV grown in MM5T tissue culture cells; (3) 150 μg Rauscher leukemia virus (RLV) purified from BALB/c mice plasma; (4) 150 μg of avian myeloblastosis virus (AMV) purified from chicken plasma. The absorption was performed by stirring the antibodies with the absorbant for 30 min at 37°C; thereafter stirring was continued for 4 hr at 4°C. The incubation was continued at 4°C without stirring for a further 20 hr. To eliminate the presence of possible soluble complexes, the antibody–antigen mixture was centrifuged in a Beckman SW50.1 rotor at 45,000 rev/min for 45 min at 4°C. The supernatant was used for the immunoperoxidase staining reaction.

The indirect immunoperoxidase test

Ten serial sections (each 5 μm thick) were prepared from paraffin-embedded blocks and stored at room temperature until used. Sections were deparaffinized and dehydrated in xylene and graded alcohols. If the paraffin-embedded tissues were initially fixed in Bouin's fixative, the picric acid was removed by a saturated solution of lithium carbonate in 70% alcohol. Sections were rinsed in PBS (0.01M phosphate-buffered saline), pH 7.6, then incubated with hyaluronidase (Sigma, 600 U/ml) at 37°C for 1 hr. After rinsing, each section was incubated at 4°C overnight with 2.5 μg IgG. Thereafter sections were rinsed thoroughly in PBS and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Miles Yeda, 5-10 μg IgG) for 30 min at room temperature. Sections were rinsed again, treated with 0.04% diaminobenzidine (Sigma) and 0.003% H_2O_2 for 10 min, rinsed and counterstained with methylene blue in PBS. The sections were rinsed once more, dehydrated and mounted in permount.

Statistical evaluation of qualitative data analysis was based on the chi-square test. The

survival analysis was performed by the life-table method according to computer program BMDP 1L from the BMDP computer program using a CDC 6600 computer. The statistical difference between the curves (two curves representing life-table analysis) was calculated according to Mantel Cox formulas [13].

RESULTS

Sections from 204 cases of breast cancer from several Israeli hospitals were examined and 62.7% were found to contain gp52 cross-reacting antigen. The results have been subdivided according to region of birth and age. The first group of patients consists of women from 19 to 49 yr of age (premenopausal) and the second above 49 yr of age (postmenopausal). Most of the tumors were from patients of European origin (48.5%). The others were patients born in North Africa (20%), Asians (excluding Israelis: 21.5%) and Israeli-born (9.8%). Seventy percent were postmenopausal. Table 1 represents the detection of the gp52 cross-reacting antigen in the tumor cells of patients of different ethnic origin. The results show that this antigen was most frequently detected in North African-born patients (78%), the statistical significance compared with the European-born patients being $P<0.05$. The detection of the antigen in Asian-born patients was similar to that found in patients of European origin.

Table 2 shows the distribution of the histopathological types in the different ethnic groups and the frequency of detection of the antigen in them. In all ethnic groups most of the cases were infiltrating duct carcinoma. There was a high frequency of antigen detection in this histological type. In the five cases where metastatic tumor was obtained, the antigen was found in all. The number of the latter cases is small, thus one cannot be dogmatic about the incidence of detectable antigen in the metastatic tumor. It is evident that the gp52 cross-reacting antigen was detected in all the different histopathological types, but not with the same frequency.

Absorption of the antisera with either MMTV or purified gp52 completely abolished the immunological reaction. Figure 2 shows an example of the immunological reaction in a case of infiltrating duct carcinoma with tumor in small ductules and metastatic tumor in lymphatics. The immunological reaction appeared intracellularly only in the tumor cells (B). Preabsorption of the sera with gp52 eliminated the immunological reaction, indicating its specificity (C).

Table 1. Immunoperoxidase staining of carcinoma of the breast in various ethnic groups

Region of birth	No. of cases examined			Cases with a positive reaction for gp52 cross-reacting antigen					
	Premenopausal	Postmenopausal	Total	Premenopausal		Postmenopausal		Total	
				No.	%	No.	%	No.	%
Europe	17	82	99	10	58.8	50	61.0	60†	60.6
North Africa	11	30	41	8	72.7	24	80	32†	78.0
Asia*	20	24	44	11	55-0	16	66.7	27	61.4
Israel*	13	7	20	5	38.5	4	57.1	9	45.0
Total	61	143	204	34	55.7	94	65.7	128	62.7

*Asia–Israeli born excluded.
†Significant difference between European and North African groups, $P<0.05$.

Table 2. Immunoperoxidase staining-histological carcinoma of the breast

Histological type	Region of birth									
	Europe		N. Africa		Asia		Israel		Total	
	No. of cases	gp52-positive	No. of cases	gp52-positive	No. of cases	gp52-positive	No. of cases	gp52-positive	No. of cases	gp52-positive
Lobular invasive	3	1	0	0	1	1	0	0	4	2
Intraductal and infiltrating duct	87	55	32	26	36	21	17	8	167	110
Medullary	1	1	2	2	1	1	3	1	7	5
Colloid (mucin)	2	0	0	0	1	1	0	0	3	1
Others*	5	2	4	1	4	2	0	0	13	5
Metastatic†	1	1	3	3	1	1	0	0	5	5
Total	99	60	41	32	44	27	20	9	204	128

*Includes tubular and papillary invasive.
†Metastatic: 2 metastatic to lung; 2 to lymph nodes; 1 to ovary.

Eleven normal tissues (breast, lung, prostate, uterus), benign breast disease (23 fibrocystic disease, 14 fibroadenoma) and 20 cases of primary carcinoma in organs other than breast (kidney, colon, liver, ovary, skin, thyroid, lung) were investigated. All failed to show a positive antigen reaction.

The question arises: does a correlation exist between the stage of the disease and the frequency of detection of the antigen? Table 3 shows the existence of such a correlation. The higher the stage of the disease the more frequent is the detectability of the antigen.

Table 3. Immunoperoxidase staining of carcinoma of the breast according to stage of disease

Stage	No. of patients	gp52 cross-reacting antigen-positive cases	
		No.	%
I	40	6	15.0
II	103	57	55.3
III	21	13	62.0
IV	10	8	80.0

To assess whether the detectability of the gp52 cross-reacting antigen could be correlated with the prognosis, follow-up of a group of patients, all of them stage II, from the Sheba Medical Center was undertaken. Ninety-seven patients were included in this study, of which 24 were premenopausal. The distribution according to place of birth was as follows: Europe, 59; Israel, 9; Asia, 18; North Africa, 5; and 6 of unidentified countries. Table 4 shows the relationship between the detection of gp52 cross-reacting antigen, survival time and clinical status of the patient. Thirty-five percent with a positive gp52 antigen reaction were dead within 1 to 7 yr after mastectomy, whilst 13% with a negative reaction died during the same period of time. Also within the 1 to 7 yr period 23.5% who were positive for the gp52 antigen developed metastases, compared with 10.9% of the gp52 antigen-negative cases. Most significant was the third group, free of disease, during the observation period of 1–7 yr, as indicated in Table 4. Forty-one percent of patients with a gp52 antigen-positive reaction were free of disease, whilst 76% with a negative reaction appeared well and free of disease. Of the 97 cases, 51 had a positive reaction, and of these 2 were of North African origin. In the group of 46 negative cases 3 were of North African origin. Table 4 does not include any breast cancer patients who died from causes other than this disease.

The data obtained suggested a correlation between the detection of the antigen and prognosis, and this was also analysed by the life-table method [13]. When the age groups for survival rate were compared there were no statistical differences between the pre- and postmenopausal patients ($P = 0.824$, data not shown). This data is supported by Melnik *et al.* from a survey of 10,702 patients in Israel [3]. Hence the analysis was performed on the entire group of patients without separation according to age. The analysis was carried out on only 80 patients (those with advanced diseases excluded) according to the length each patient lived from the time of mastectomy. Figure 3 shows that cases with no detectable gp52 cross-reacting antigen in their tumor sections survived significantly longer than those with a positive immunological reaction ($P<0.05$).

Hence it would appear that tumors with positive gp52 antigen reaction at the time of mastectomy had a worse prognosis as far as survival time and dissemination of disease were concerned.

DISCUSSION

Carcinoma of the breast is one of the commonest malignancies of women in Israel. In a total population of 3.5 million, breast cancer accounts for more than 1000 new cases per year and 400 deaths per year [1]. Localized stage I, and

*Table 4. Immunoperoxidase assay for gp52 cross-reacting antigen in relation to patients status in a follow up study (1–7 yr)**

	Free of disease		Deceased		Advanced disease (metastatic)		Total	
	No. of positive cases	No. of negative cases	No. of positive cases	No. of negative cases	No. of positive cases	No. of negative cases	No. of positive cases	No. of negative cases
Premenopausal	16	9	6	1	1	1	13	11
Postmenopausal	15	26	12	5	11	4	38	35
All ages	21	35	18	6	12	5	51	46

*All the patients were at stage II at the time of mastectomy.
The positive and negative refers to the immunological staining with anti-gp52 IgG.

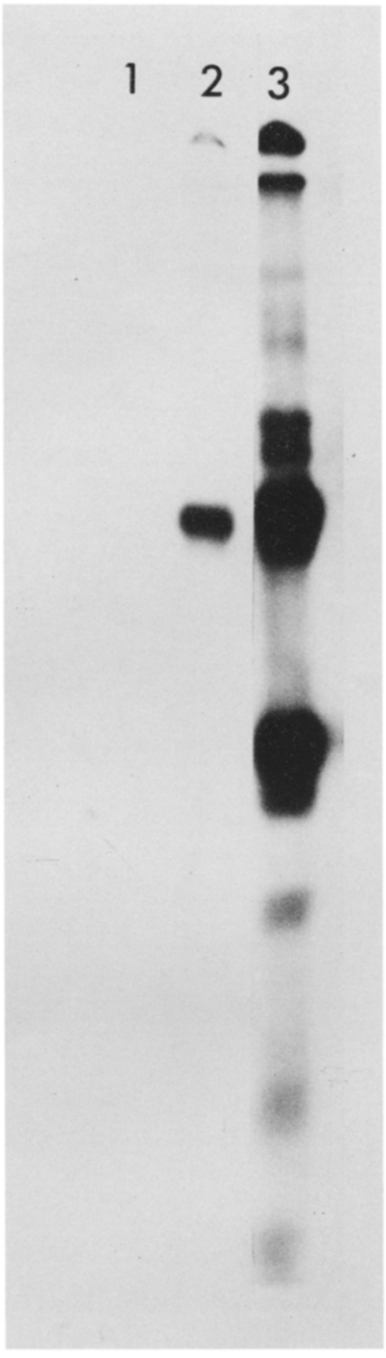

Fig. 1. *Radioimmunoprecipitation of* [^{125}I]-*MMTV proteins by the anti-gp52 serum.* [^{125}I]-*labeled MMTV proteins* (130,000 counts/min) *were immunoprecipitated either with control rabbit serum or with rabbit anti-gp52 serum (yielding pellets of* 2560 *and* 20,350 counts/min *respectively). The immunoprecipitates were dissociated and underwent electrophoresis on a polyacrylamide gel and autoradiography, as described in 'Materials and Methods'.* (1) *Precipitate of control serum;* (2) *pellet obtained with anti-gp52 serum;* (3) [^{125}I]-*labeled MMTV* (100,000 counts/min). *The direction of the protein migration during electrophoresis was from top to bottom.*

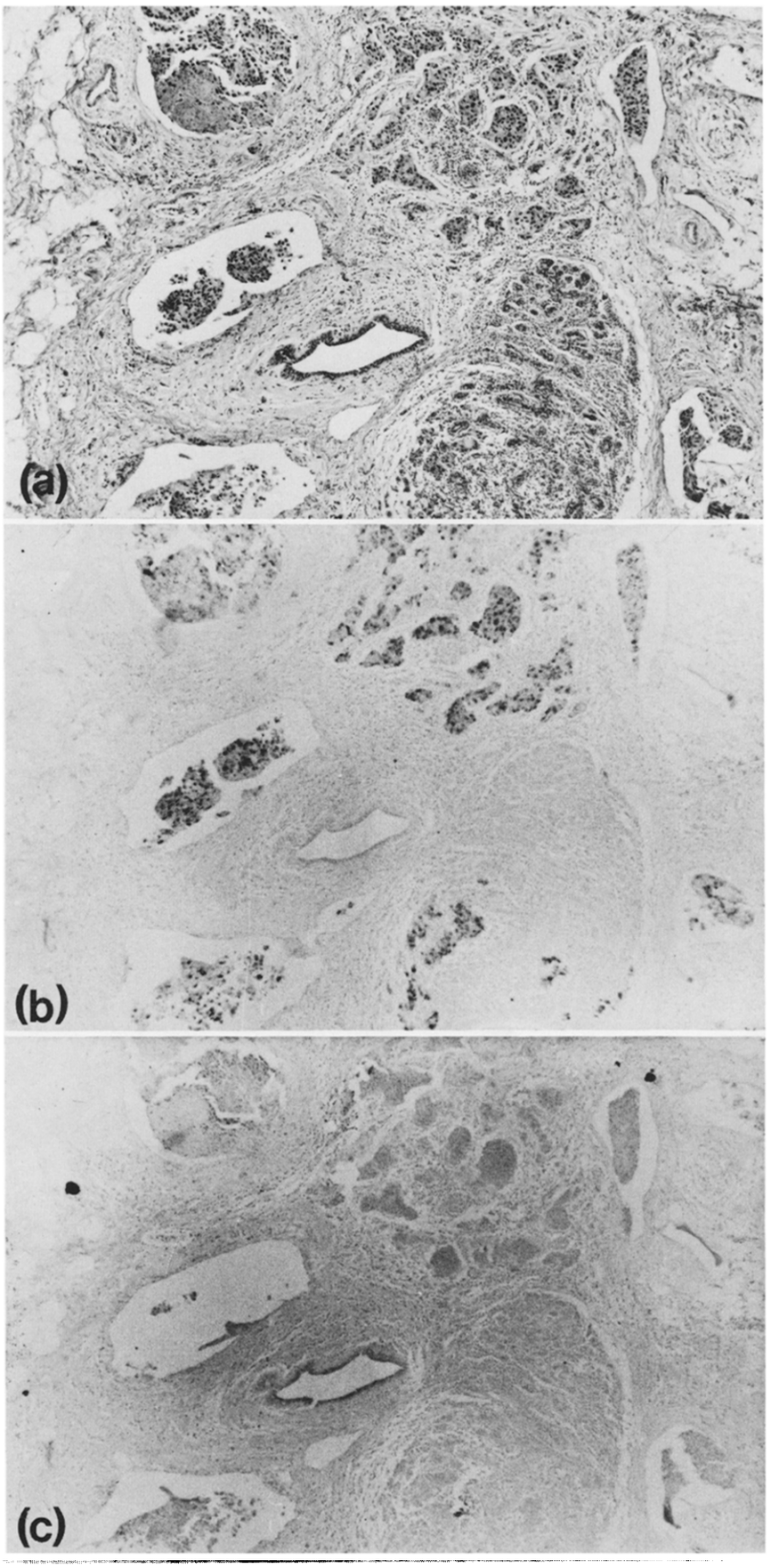

Fig. 2. Immunoperoxidase stain of human breast carcinoma. (A) *Intraductal and infiltrating ductal carcinoma as well as tumor metastases in lymphatic vessels (H&E., ×250);* (B) *the same field in an adjacent serial section stained with anti-gp52 serum showing the positive immunoperoxidase reaction in the malignant cells (methylene blue counterstain, × 250);* (C) *the same field after absorption of the serum by the gp52 antigen showing abolishment of the specific reaction (methylene blue counterstain, × 250).*

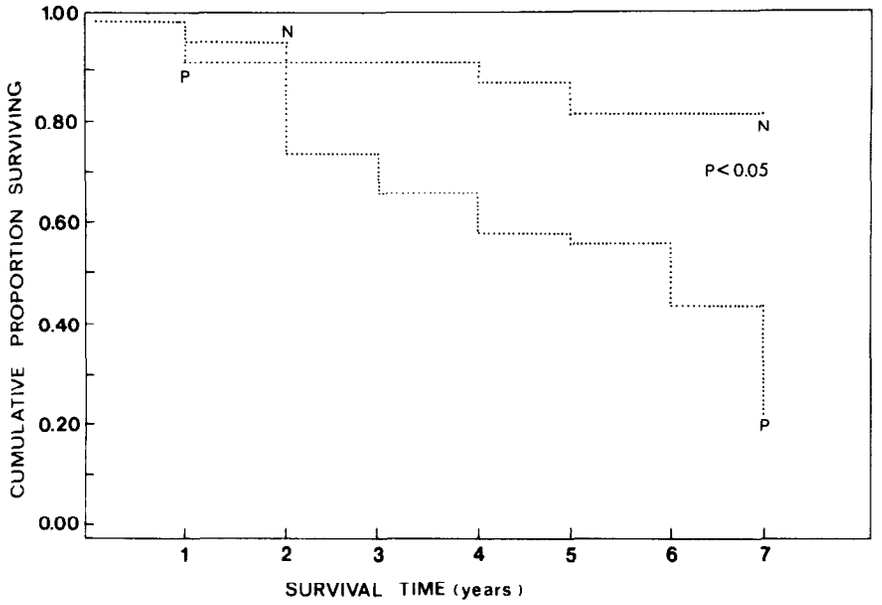

Fig. 3. Survival of breast cancer patients according to the gp52 cross-reacting antigen detection. Survival analysis of a group of 80 follow-up patients, all stage II, according to the period of time they lived following mastectomy (1–7 yr follow-up).

even stage II, breast cancer may be associated with prolonged survival. However, because of a lack of available markers the prognosis in many patients cannot be assessed. Therefore the demonstration of a virus-related protein as a marker for the severity of the malignancy may be of vital importance.

Incidence rates of breast cancer in Israel are similar to those in Western Europe and Caucasian women in North America [3]. There are, however, differences in the incidence of this disease in the different ethnic groups in Israel. The incidence in European-born Jews is 2–3 times higher than in those born in North Africa and Asia [3].

Melnik *et al.* [3] recently suggested that breast cancer is less aggressive in Israeli women of Western origin than in native-born Israelis or immigrants from Asia or North Africa. On the other hand, Sacks and Selzer [4] suggested that Israeli women born in North Africa and Asia should not be grouped together in studies, in spite of having the same low incidence rate, because of the differences that they found in the histological subtypes. The same authors also suggested that the difference in the aggressiveness of the disease seen in North African-born women compared with those born in Europe, as reported by Melnik *et al.* [3], might be due to the low frequency of circumscribed and medullary carcinomas of the breast.

The purpose of this research was to study: (a) the relative frequency of detectable gp52 cross-reacting antigen in breast cancer of Israeli patients from different ethnic groups, taking cognisance of age and histopathological type of the tumor; (b) analysis of the frequency of detection of antigen at different stages of disease; and (c) prognosis of the patient, at the time of mastectomy, assessed when the antigen is present.

Conclusions which can be drawn from our investigations are:

1. We have confirmed the observation that in normal tissues and malignancies other than breast, the gp52 cross-reacting antigen was not detected [6, 7].
2. Patients born in North Africa were found to contain a significantly higher presence of antigen ($P<0.05$) than European-born Jews.
3. It is well known that in advanced stages of the disease the prognosis of the patient is less favorable. The results of this investigation indicate that the frequency of the demonstration of the gp52 cross-reacting antigen is higher when the patients are in the advanced stage of the disease (Table 3), suggesting a correlation between the severity of the disease and detectability of the antigen. This is supported by the follow-up study where 97 patients, all in stage II of the disease, were investigated. The results showed that if antigen was detected at the time of mastectomy, an unfavorable prognosis could be predicted. The disease disseminated sooner and survival time was shorter (Table 4). For statistical assessment we decided to analyse only patients free of disease as compared to deceased in a follow-up study of 1 to 7 yr. Since the 97 cases tested in this study included only 5 of North African

origin, the question of the ethnic background cannot be assessed as a factor in the prognosis.

The fact that the antigen is not detected in a given tumor does not imply its absence. It might reflect a quantitive difference in the amount of antigen expressed when the disease is disseminating.

In conclusion, it appears that the presence of gp52 cross-reacting antigen in breast biopsies is a significant indication of the severity of the disease as judged from its frequency of detection in North African-born women and the higher rate of mortality in the group of antigen-positive patients. These conclusions are supported by the statistical significance of the data, although the number of patients tested was not high.

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REFERENCES

1. KATZ L, STEINITZ R, SELA T. Epidemological review of breast cancer in Israel. *Isr J Med Sci* 1981, **17**, 810–815.
2. SACKS MI. Cancer of the female breast in Israel. In: CORREA P, JOHNSON WD, eds. *An International Survey of Distributions of Hystological Types of Breast Cancer.* Geneva, UICC Technical Report Series, 1978, Vol. 35, 142–159.
3. MELNIK Y, SLATER PE, KATZ L, DAVIES AM. Breast cancer in Israel, 1960–1975—II. Effect of age and origin on survival. *Eur J Cancer* 1980, **16**, 1017–1023.
4. SACKS M, SELZER G. Breast cancer in Israel. Histopathologic types in different population groups. *Isr J Med Sci* 1981, **17**, 882–887.
5. KEYDAR I, MESA-TEJADA R, RAMANARAYANN M *et al.* Detection of viral proteins in mouse mammary tumors by immunoperoxidase staining of paraffin sections. *Proc Natl Acad Sci USA* 1978, **75**, 524–528.
6. MESA-TEJADA R, KEYDAR I, RAMANARAYANN M, OHNO T, FENOGLIO C, SPIEGELMAN S. Immunohistochemical detection of a cross-reacting virus antigen in mouse mammary tumors and human breast carcinomas. *J Histochem Cytochem* 1978, **26**, 532–541.
7. SPIEGELMAN S, KEYDAR I, MESA-TEJADA R *et al.* The presence and clinical implications of a viral-related protein in human breast cancer. *Cold Spring Harbor Conf Cell Prolif* 1980, **7**, 1149–1167.
8. RITZI E, BALDI A, SPIEGELMAN S. The purification of a gs antigen of the murine mammary tumor virus and its quantitation by radioimmunoassay. *Virology* 1976, **75**, 188–197.
9. BOLTON AE, HUNTER WM. The labeling of proteins to high specific radioactivity by conjugation to a ^{125}I-containing acylating agent. *Biochem J* 1973, **133**, 529–539.
10. KESSLER SW. Rapid isolation of antigens from cells with a staphylococcal protein-A antibody adsorbent: parameters of the interaction of antibody–antigen complexes with protein-A. *J Immunol* 1975, **115**, 1617–1624.
11. HIZI A, MCCRAE MA, JOKLIK WK. Studies on the amino acid sequence content of proteins specified by the gag and pol genes of avian sarcoma virus B77. *Virology* 1978, **89**, 272–284.
12. GOLDFEDER A. Induction of type B virions to bud into cytoplasmic vacuoles in a mammary tumor of an X-ray and urethan treated X/Gf mouse. *Cancer Res* 1976, **36**, 4190–4194.
13. BLACKWELL D, DIXON WJ, BROWN MB. *Biomedical Computer Programs.* Los Angeles, University of California Press, 1979.