

Isolation from patients with breast cancer of antibodies specific for antigens associated with breast cancer and other malignant diseases

(cancer immunodiagnosis/tumor-specific antibodies)

YORAM GORSKY*, FARKAS VANKY†, AND DOV SULITZEANU*

* Lautenberg Center for General and Tumor Immunology, Hebrew University, Hadassah Medical School, Jerusalem, Israel; and † Department of Tumor Biology, Karolinska Institute, Stockholm, Sweden

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ABSTRACT Antibodies were specifically purified, with the use of pleural fluid antigens entrapped in a polyacrylamide gel as immunoabsorbent, from a pool of sera from patients with breast cancer and from the pleural effusion of an individual patient. The purified antibodies were radioiodinated and tested for capacity to bind to the pleural fluid adsorbent. Binding of the radiolabeled antibodies was inhibited by many of the sera from women with breast cancer to a much greater extent than by sera of healthy women. With purified antibodies originating from the serum, 52% of the breast cancer sera and 16% of sera from patients with other malignancies were more inhibitory than were 95% of the sera of healthy women. In the tests with antibodies eluted from pleural fluid, 24% of the breast cancer sera and 25% of the sera from patients with other neoplastic diseases were more inhibitory than were 95% of the normal sera. We concluded that sera of patients with breast cancer may contain antibodies against antigens associated with breast cancer as well as additional antibodies against antigens that are also found in patients with other neoplastic diseases or in normal individuals. Individual breast cancer sera may inhibit binding of the labeled antibodies against breast cancer owing to their content of antibodies, antigen(s), or both.

We described in a recent publication a test for inhibition of binding of radioiodinated antibody for the detection of tissue antigens circulating in human serum (1). In the present work, we used a similar test to demonstrate the existence of antibodies against breast cancer and other tumor-associated antigens in the sera of patients with breast cancer. Antibodies specifically purified from such sera and radioiodinated were shown to bind to an immunoabsorbent consisting of pleural effusions from patients with breast cancer entrapped in a polyacrylamide gel. Binding of the radiolabeled antibodies was inhibited by a considerable proportion of the sera of patients with breast cancer and other malignant diseases.

MATERIALS AND METHODS

Sera. Sera obtained from patients with breast cancer 3-4 weeks after mastectomy were used as the source of standard antibody (Ab). Sera assayed in the tests for inhibition of radioactive Ab binding were obtained from: (a) patients with breast carcinoma at various stages of the disease; (b) patients with other malignant diseases; and (c) healthy women. Sera were tested either fresh or after storage at -20° . Normal rabbit serum was used as a control in the initial experiments, and normal sheep serum in the later experiments.

Exudate Fluids. Pleural effusions from patients with breast cancer and ascitic fluids from patients with ovarian cancer were centrifuged for 15 min at $500 \times g$ in a refrigerated centrifuge, to remove the cells. The supernates were centrifuged at $12,000 \times g$ for 3 hr to remove debris, and stored at -20° .

Glutaraldehyde-Fixed Human Cells and Tissues. Spleen

and kidney specimens removed at surgery were teased apart to disperse the cells; muscle tissue was cut into small pieces; and placenta and ovarium were homogenized with a tissue homogenizer. Cells and organ homogenates were washed in phosphate-buffered saline (pH 7.2) until the supernatant fluids were clear, then fixed with glutaraldehyde (1). Phosphate-buffered saline was also used to suspend and wash the immunoabsorbents.

Gel Filtration. Pleural fluid obtained from one patient (1.5 liters) was treated with 33% ammonium sulfate. The precipitated protein (5.2 g) was dissolved in phosphate-buffered saline and dialyzed. Forty milliliters of this solution (122 mg/ml) were filtered through a large Sephadex G-200 column (4.5×120 cm). Elution was carried out with phosphate-buffered saline. Most of the protein was recovered in two large peaks, which were separately concentrated by ultrafiltration under reduced pressure. The first, large molecular weight fraction, containing, among others, antigen-Ab complexes (pleural fluid "complexes", see *Results*) served as immunoabsorbent for the isolation of Ab. The second fraction, rich in IgG, as ascertained by diffusion in agar against a rabbit antiserum against human IgG, was used as one of the sources of the antibodies against breast cancer. A large pool of normal human serum was filtered through the same column, and the large molecular weight fraction was used to prepare an immunoabsorbent containing "normal" antigen-Ab complexes.

Human Serum Polymer. A pool of human sera was polymerized by treatment with glutaraldehyde (2). A polymer was similarly prepared from a pool of 10 ascitic fluids removed from patients with ovarian cancer.

Polyacrylamide Gel Immunoabsorbents. Immunoabsorbents were prepared by a modification of earlier techniques (1, 3): 4 g of acrylamide (Canalco, Rockville, Md.) and 1 g of *N,N'*-methylenebisacrylamide (Serva, Heidelberg, Germany) were dissolved in 19 ml of distilled water. Before use, 2.5 ml of this solution were diluted with 2.5 ml of distilled water. One milliliter of protein solution and 0.15 ml of a riboflavin suspension (10 mg in 50 ml of distilled water) were added and the mixture was photopolymerized for 1 hr. Occasionally, small variations in the amount of riboflavin (found by trial and error) were needed to speed up polymerization and obtain gels of adequate consistency. The gel was broken up and fixed with glutaraldehyde (1).

The following adsorbents were prepared: pleural fluid adsorbent, containing 0.2 ml of fluid per ml of gel, was used as antigen in the radioimmunoassays; ascitic fluid adsorbent, prepared from a pool of ascitic fluids from patients with ovarian carcinoma; normal "antigen-antibody complexes" and pleural fluid "antigen-antibody complexes" adsorbents, each containing 10 mg/ml of protein (see *Gel Filtration* above).

Iodination of Specifically Purified Ab with 125 I. Iodination

Abbreviation: Ab, antibody.

Table 1. Inhibition of binding of ^{125}I -labeled Ab by pooled normal and breast cancer sera

Tube no.	Inhibition	^{125}I -Labeled Ab (cpm)		% Binding	Mean % binding	% Inhibition	% Specific inhibition*
		Added	Bound				
1	None (NRS)	259,809	5,256	2.02			
2		253,184	5,579	2.20	2.11		
3	NHS undiluted	268,981	4,440	1.65			
4		272,639	4,952	1.81	1.73	18.0	
5	BCS undiluted	253,965	3,110	1.22			
6		282,195	3,064	1.08	1.15	45.5	27.5
7	NHS 1:10	264,884	4,398	1.66			
8		264,646	4,548	1.71	1.68	20.4	
9	BCS 1:10	274,675	3,988	1.45			
10		273,839	3,887	1.41	1.43	32.2	11.8

Normal human serum (NHS) or breast cancer serum (BCS) were pools of 10 sera each. One-tenth milliliter of the undiluted serum or of the serum diluted 1:10 in 10% normal rabbit serum (NRS) was mixed with ^{125}I -labeled Ab and kept overnight in the cold. It was then added to the pleural fluid immunoadsorbent and rotated overnight on a tissue culture rotator.

* Specific inhibition: difference between percent inhibition by BCS and NHS.

was carried out with 0.5–1 mCi of carrier-free ^{125}I (4). Radioactivity was measured in a well-type autogamma scintillation counter.

Radioactive Assays for Ab Binding Inhibition. Two types of assays were used: a competition assay, to test for the presence of inhibitors (Ab and/or antigen) in the sample, and a saturation assay, to confirm the Ab nature of the inhibitor (3).

Competition Assay. Mixtures were prepared in duplicate and consisted of 0.1 ml of each of the following: normal rabbit serum (or normal sheep serum), ^{125}I -labeled Ab (diluted in 10% normal rabbit serum, $50\text{--}250 \times 10^3$ cpm) and the test sample. The mixtures were kept overnight at 4° , then added to tubes containing 0.25 ml of the washed, packed pleural fluid adsorbent. The tubes were rotated overnight (or, occasionally, for 4 hr) in a tissue culture rotator, the radioactivity was measured, and the contents were washed three times. The radioactivity bound to the adsorbent was determined and the percent inhibition was calculated with reference to the control tubes containing normal rabbit serum.

Saturation Assay. Mixtures, in duplicate, containing 0.1 ml of normal rabbit serum and 0.1 ml of the test sample were added to tubes containing 0.25 ml of pleural fluid adsorbent and rotated overnight in the cold, to saturate the binding sites in the adsorbent. The contents were washed three times; 0.1 ml of ^{125}I -labeled Ab mixed with 0.1 ml of normal rabbit serum was added; and the tubes were rotated for a further 12 hr. The radioactivity was measured, the contents were washed, and the percent radioactivity bound was determined as before.

Isolation of Ab from Serum. A pool of sera (18 ml) obtained from patients with breast cancer 3–4 weeks after mastectomy, was serially absorbed as follows: the serum was mixed with an equal volume of packed adsorbent, stirred gently for 4 hr, and centrifuged for 20 min at $12,000 g$. The supernate was transferred to the next adsorbent and the procedure was repeated. Three absorptions were performed with the polymer from normal human serum and one absorption each with ovarian cancer ascitic fluid in polyacrylamide gel; the same fluid polymerized with glutaraldehyde; a mixture of muscle, ovary, and placental tissues; glutaraldehyde-fixed spleen cells; and glutaraldehyde-fixed kidney cells. The supernate was centrifuged at $12,000 g$ for 3 hr, and an equal volume of saturated ammonium sulfate was added to precipitate the globulin fraction. The precipitate was dissolved in 2 ml of phosphate-buffered saline, dialyzed against phosphate-buffered saline, and added to 2 ml of pleural fluid adsorbent. The mixture was

stirred for 2 hr; the polymer was washed five times with phosphate-buffered saline, resuspended in 2 M NaSCN in phosphate-buffered saline, and stirred for 20 min. After centrifugation, the supernate was dialyzed against phosphate-buffered saline concentrated by ultrafiltration under reduced pressure to 0.2 ml, centrifuged for 3 hr at $12,000 \times g$ and stored at -20° . The entire procedure was carried out at $0\text{--}4^\circ$.

Isolation of Ab from Pleural Fluid. The rationale underlying the procedure detailed below was as follows: we assumed that pleural fluid was likely to contain antibodies to antigens associated with breast cancer, which are released as a result of tumor cell breakdown and/or turnover, and antigen–Ab complexes. The pleural fluid was consequently fractionated on Sephadex G-200 with the expectation that the first peak eluted from the column would be enriched in antigen–Ab complexes. This fraction could then be used to prepare an immunoadsorbent for the purification of the Ab likely to be found in the second peak, i.e., the IgG-rich fraction. However, the IgG fraction could be expected to also contain Ab directed against “self antigens” (3). To remove these autoantibodies as far as possible, we first absorbed the pleural fluid IgG with the “normal complexes” adsorbent.

Twenty-eight milliliters of the pleural fluid-IgG (29 mg/ml of protein) were absorbed four times (2 hr at room temperature) with equal volumes of the “normal complexes” adsorbent. The supernate was then adsorbed onto an equal volume of the “pleural fluid complexes” adsorbent. The latter was washed five times with phosphate-buffered saline, and the bound material was eluted by stirring with 30 ml of 2 M NaSCN, for 30 min at room temperature. The eluate was dialyzed against phosphate-buffered saline, concentrated to 0.5 ml, and stored at -20° .

Protein Determinations. Protein concentrations were determined spectrophotometrically at 280 nm, with human IgG as standard.

Statistical Analysis. Significance of differences between groups of sera was determined by the Fisher's exact test for 2×2 tables or its normal approximation corrected for continuity.

RESULTS

Reactivity of purified, radioiodinated Ab in competition assays

Experiments with Ab Isolated from Serum of Patients with Breast Cancer. A pool of sera from 10 patients with breast cancer and a pool of sera from 10 normal women were tested

Table 2. Reactivity of different Ab preparations with different pools of normal human serum (NHS) and breast cancer serum (BCS) in the competition test

Exp. no.	Inhibitor	% Binding	% Inhibition	% Specific inhibition
1	None (NRS)	2.48		
	NHS (pool no. 2)	1.71	31	
	BCS (pool no. 2)	1.15	53	22
2	None (NRS)	2.11		
	NHS (pool no. 3)	1.73	18	
	BCS (pool no. 3)	1.15	45	27

¹²⁵I-Labeled Ab used in Exps. 1 and 2 were iodinated separately. Each serum pool was a mixture of 10 sera.

undiluted and diluted 1:10 in a competition assay. The results are presented in full in Table 1, which shows the extent of binding of the antibodies to the pleural fluid immunoabsorbent and the percent inhibition obtained with the two serum pools. The amount of radioactivity bound was very small, presumably due to the small concentration of the reactants, but the reproducibility within duplicates was excellent in the vast majority of cases. Results were accepted as valid if duplicates deviated from the mean value by no more than 10%. As seen in Table 1, both the pools of breast cancer sera and normal human sera inhibited binding of the radioactive Ab. However, the undiluted breast cancer serum gave 45.5% inhibition, as compared to the 18% given by the normal serum. The inhibitory activity of the normal sera was attributed to the presence in the preparation of ¹²⁵I-labeled Ab of Ab reacting with normal antigens in pleural fluid (3). Addition of 20% normal human serum to the labeled Ab partly blocked the activity of these autoantibodies and thus enhanced the difference between the breast cancer and normal sera. Therefore, most of the experiments carried out subsequently with the Ab enriched from the serum were performed with ¹²⁵I-labeled Ab mixed with normal human serum.

Table 2 shows the results of two experiments carried out with two different pools of normal human and breast cancer sera and with Ab iodinated at different times. Similar differences in inhibitory capacity between breast cancer and normal human sera were found, thus attesting to the reproducibility of the assays.

Sera from 31 patients with breast cancer, 26 normal women, and 69 patients with various malignant diseases were tested individually in the competition test. The results are shown in Fig. 1. Sixteen of the breast cancer sera (52%) inhibited the binding of the Ab by 60% or more, whereas only one of the 26 normal sera (4%) gave a comparable degree of inhibition. Eleven of the 69 (16%) sera from patients with other malignancies also gave a high degree of inhibition, although the percent of strongly inhibitory sera was lower than that of the breast cancer sera (with the possible exception of sera from patients with cancer of the uterus). We concluded that the labeled preparation contained Ab directed against antigens associated with breast cancer, against antigens associated perhaps with other malignant diseases, and against normal antigens.

In several instances, binding of ¹²⁵I-labeled Ab was enhanced rather than inhibited by preincubation with the serum. This was attributed to the presence in the serum of antigens in concentrations too low to completely block the labeled Ab. In such instances, complexes of the type antigen-¹²⁵I-labeled Ab would be formed (1). These complexes would interact to yield larger complexes (antigen-¹²⁵I-labeled Ab - antigen-¹²⁵I-labeled

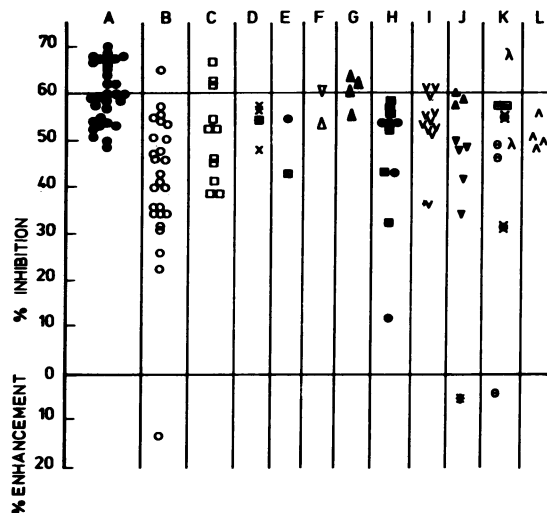


FIG. 1. Competition tests with individual sera of patients with breast cancer, of patients with other neoplastic diseases, and of healthy women. All sera were tested undiluted. Each point represents one individual serum from patients with the following diagnoses: (A) breast cancer; (C) melanoma; (D) carcinoma of rectum (■) and colon (×); (E) carcinoma of pancreas (▣) and ovarium (⊙); (F) hepatoma (▽) and basal cell carcinoma (Δ); (G) cancer of the uterus; (H) osteosarcoma (⊕) and other types of sarcoma; (I) Burkitt's lymphoma; (J) cancer of antrum (▲) maxilla (▼) and zygoma (*); (K) cancer of the prostate (λ), stomach (⊖), thyroid (⊞), lung and nasopharynx (⊗); (L) Hodgkin's disease. (B) Sera of healthy women.

Ab-), which would then bind to the immunoabsorbent, resulting in the observed enhancement.

As mentioned, addition of normal human serum to the labeled Ab increased the differences in the inhibitory capacity between the breast cancer and the normal sera. The results of several experiments performed with ¹²⁵I-labeled Ab adsorbed with normal human serum are shown in Fig. 2. Four different pools of breast cancer and normal human sera were tested, as well as five individual breast cancer and normal sera. The individual sera were also tested at 1/10 dilution. There was no overlap in this case between the normal and the breast cancer sera.

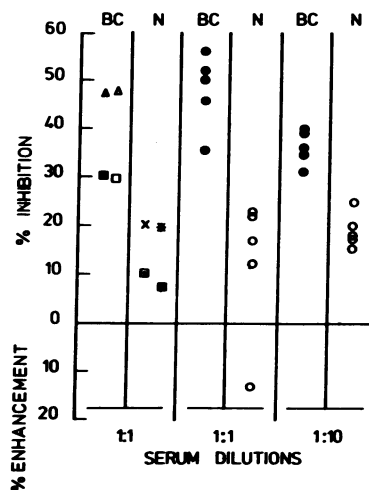


FIG. 2. Competition test with ¹²⁵I-labeled Ab mixed with normal human serum. Four pools of sera (10 sera in each pool) from patients with breast cancer (BC) and from normal women (N) were tested, as well as five individual sera (not included in the pools) from each group. The individual sera were assayed at 1/10 dilution.

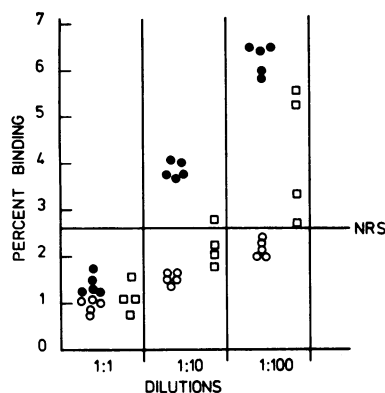


FIG. 3. Enhancement of binding of ^{125}I -labeled Ab in the presence of pleural fluids from patients with breast cancer. Five pleural fluids from patients with breast cancer (\bullet), five ascitic fluids from patients with ovarian carcinoma (\circ), and four normal sera (\square) were tested in a competition assay, at 1/1, 1/10, and 1/100 dilution. The labeled Ab were first mixed with normal human serum. NRS, normal rabbit serum.

Competition tests with pleural fluids from patients with breast cancer, were carried out in parallel with competition tests with ascitic fluids from patients with ovarian carcinoma and with normal sera. The pleural fluids were found to enhance rather than inhibit the binding of the labeled Ab, even in dilutions as high as 1/100 (Fig. 3). This is believed to be due to the presence in the pleural fluids of complexes of antigens (associated with breast cancer) with the corresponding Ab.

Saturation Tests. Inhibition obtained with the breast cancer sera in the competition tests could be due to (a) Ab competing with ^{125}I -labeled Ab for binding sites in the adsorbent; (b) antigen blocking the labeled Ab; or (c) both. To determine whether Ab were involved in the inhibitory activity of the sera, saturation assays were carried out. The pleural fluid adsorbent was treated with the test serum, and then washed and tested for capacity to bind ^{125}I -labeled Ab.

The results of tests performed with sera from patients with breast cancer, from patients with other malignant diseases, and from normal individuals are depicted in Fig. 4. None of the 15 normal sera blocked the binding of the labeled Ab by more than 10%. There was no difference in the blocking activity between the breast cancer sera and the other malignant sera when tested undiluted. At 1/10 dilution, however, nine of 14 breast cancer sera (64%) blocked by more than 15%, whereas only one of the 10 sera from patients with other neoplastic diseases (10%) gave a comparable degree of inhibition, thus confirming further the breast cancer specificity of the labeled Ab.

Experiments with ^{125}I -Labeled Ab Isolated from the Pleural Fluid. A large experiment in which 40 sera from healthy women, 68 sera from patients with breast cancer, and 88 sera from patients with a variety of neoplastic diseases, were tested, is shown in Fig. 5. Both the extent of binding to the adsorbent and the percent inhibition were higher with the pleural fluid antibodies (4.40% and over 70%, respectively). Only two of the 40 normal sera (5%) inhibited binding by 66% or more, as compared to 16 of 68 (24%) breast cancer sera and 22 of 88 (25%) sera from patients with other neoplastic diseases. The pleural fluid antibodies appeared therefore to be directed against widely occurring "tumor associated," rather than breast cancer associated antigens.

DISCUSSION

There have been a number of investigations aimed at demonstrating the existence of antibodies against antigens associated

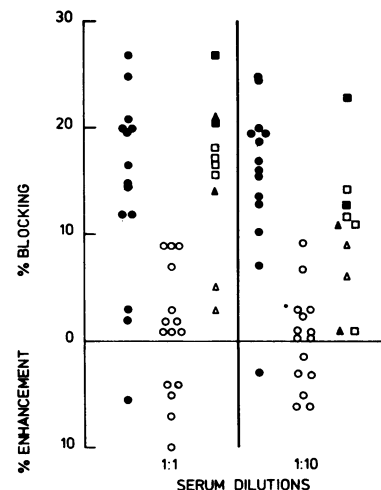


FIG. 4. Saturation assays. Pleural fluid adsorbent was treated with the test sera and washed, then tested for capacity to bind the labeled Ab. Percent blocking was calculated with respect to the adsorbent treated with normal rabbit serum. The labeled Ab were first mixed with normal human serum. \bullet , Breast cancer serum; \circ , normal human serum; \blacktriangle , osteosarcoma; \square , melanoma; \triangle , Burkitt's lymphoma; \blacksquare , colon and tumor of unknown type.

with breast cancer in recent years. Techniques used have included immunodiffusion (5, 6), immunofluorescence (7, 8), and complement-mediated cytotoxicity (9, 10). Antibodies were indeed found in these studies, but their specificity for breast cancer was not conclusively established. In at least one case (5) the antibodies were specific for a new oncofetal antigen (gamma fetal protein-2) distributed in a variety of tumors.

In the present work, antibodies showing a remarkable degree of specificity for breast cancer were isolated from the pool of sera of patients with breast cancer. These antibodies, after radioiodination, bound to an immunoadsorbent prepared from a pool of pleural fluids (presumed to contain the antigens associated with breast cancer). Binding could be blocked specifically by more than 50% of the sera of patients with breast cancer. The antibodies obtained from the pleural fluid appeared to be directed mainly against more broadly distributed "tumor associated" antigens. Since these antibodies were isolated from the IgG fraction of an individual pleural fluid with use of another fraction of that same fluid as antigen, the possibility that alloantigens were involved could be ruled out. The "saturation assays" provided strong indication that the inhibitory substances in the patients' sera included antibodies, but whether or not circulating antigens also contributed to the inhibition is not yet clear.

In spite of the extensive absorptions with normal antigens, the preparations recovered after elution from immunoadsorbent still contained antibodies to normal components, as shown by the lower but still significant inhibition obtained with sera of normal individuals. This inhibition must be ascribed to the presence in normal sera of autoantigens and autoantibodies of the type reported in our recent publication (3). It would seem therefore, that the labeled preparation contained three types of antibodies: antibodies against normal antigens, against antigens associated with breast cancer, and against antigens associated with other neoplastic diseases. Antibodies against antigens associated with neoplastic diseases were found by Edynak *et al.* (5) in a very small proportion (0.25%) of patients with various neoplastic diseases. These antibodies reacted in agar diffusion tests with an antigen (gamma fetal protein-2) extracted from cancer of the breast, colon, ovary, and stomach.

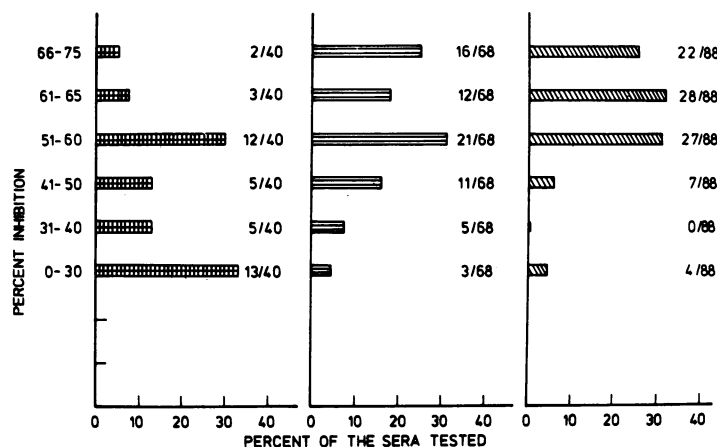


FIG. 5. Competition tests with antibodies purified from pleural fluid. Sera of healthy women (left), of patients with breast cancer (center), and of patients with other tumors (right) are grouped according to percent inhibition. Details of the sera inhibiting by 61% or more are as follows: breast cancer, 16/68 (68 = total tested); colon carcinoma, 5/24; lung carcinoma, 4/12; melanoma, 3/11; stomach cancer, 1/12; ovarian cancer, 0/4; leukemias, 4/8; Hodgkin's disease, 1/4; hepatoma, 1/3; squamous cell carcinoma, 0/3; other neoplastic diseases, 2/7; sera of healthy women, 5/40. Differences between percent of sera of healthy women and of patients with either breast cancer or other neoplastic diseases inhibiting by 61% or more or by 66% or more are significant at the 1% level.

We do not know whether similar or identical antibodies are detected in our tests, but with a higher frequency due to the sensitivity of the assay.

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