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Properties of retrovirus-like particles produced by a human breast carcinoma cell line: Immunological relationship with mouse mammary tumor virus proteins

(T47D cell line/envelope glycoprotein gp52/immunocytochemistry)

I. KEYDAR*†, T. OHNO*‡, R. NAYAK*§, R. SWEET*¶, F. SIMONI†, F. WEISS†, S. KARBY†, R. MESA-TEJADA*||**, 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, NY 10032; and †Department of Microbiology, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel

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ABSTRACT **Clonal derivatives 8 and 11 of the T47D human breast carcinoma cell line release particles that have the biochemical characteristics of a retrovirus. Particles recovered from cultures of [^{3}H]uridine-labeled clone 11 had a density of 1.18 g/ml and contained 60–70S and 35S RNAs associated with reverse transcriptase activity. The production of these particles was steroid-dependent. Clone 8 particles had a higher density, 1.195 g/ml, and their production was independent of steroid hormone. By RIA, antigens crossreactive with the 52,000-dalton envelope glycoprotein gp52, the major external protein of mouse mammary tumor virus, were found associated with these particles and in the media. Most of the gp52-related antigen was in soluble form, but it was enriched in the particle preparation. A lesser amount of antigen was distributed within the cultured cells. Absorption of rabbit antibody to gp52 with clone 11 particle preparations eliminated the ability of this antibody to detect immunocytochemically a crossreactive antigen previously localized in tissue sections of human breast carcinoma. These results indicate that the particle isolates from T47D contain the same gp52-related antigen found in human breast carcinomas and constitute an excellent source for the purification and characterization of this antigen.**

Previous studies (1–4) have identified human breast carcinoma particles that exhibit many of the features characteristic of retroviruses. In addition to the expected size (600S) and density (1.16 g/ml), these particles have a double membrane surrounding a "core" containing a DNA polymerase (reverse transcriptase) complexed to a single-stranded (70S) RNA exhibiting detectable homology to the RNA of the mouse mammary tumor virus (MMTV). The latter finding suggests that an antigenic relationship might exist between the proteins of the human breast cancer particles and those of MMTV, an approach given further plausibility by numerous immunological studies from various laboratories (reviewed in refs. 5–7). Furthermore, an antigen immunologically related to the group-specific antigen gp52 (a 52,000-dalton envelope glycoprotein) of MMTV has been detected (8, 9) in paraffin sections of human breast cancer by peroxidase immunocytochemistry. The fact that it is the polypeptide, rather than the polysaccharide, portion of gp52 that is responsible for the immunological reactivity with the human breast cancer antigen (10) adds additional significance to the biological similarities between the human and the murine mammary neoplasias.

The etiologic implications of these findings are of obvious interest, but our immediate concern is the possibility that they might be used to generate clinical information that could aid in diagnosis and in monitoring the course of the disease. This expectation is supported by experiments with the murine model, which demonstrated that plasma levels of gp52 provide accurate diagnostic (11) and prognostic (12) information on mammary tumors.

To this end, it was of interest to resolve certain issues regarding the nature of the crossreactivity observed between gp52 and the unique antigens found in human breast cancers. Our experience indicated that it was unlikely that the supply of surgically removed human tumors would provide sufficient antigen for the necessary biochemical studies. Therefore, we focused on the T47D cell line, which had been established from the pleural effusion of a patient with intraductal and invasive carcinoma of the breast (13).

It is the purpose of the present paper to present data on the T47D cell line and its clonal derivatives that reproducibly secrete virus-like particles containing the gp52-related antigen. This situation solves the difficult logistic problem of making available a reliable source of material from which to purify the relevant breast cancer antigens with comparative ease.

MATERIALS AND METHODS

Cells. The cells were grown in RPMI 1640 medium containing fetal calf serum (10%), insulin (0.2 unit/ml), glutamine (2 mM), streptomycin (100 μg/ml), penicillin (100 units/ml), and mycostatin (25 μg/ml). When the cells were grown in the presence of steroid hormones, the fetal calf serum was dialyzed.

For virus collection, the cells were seeded in RPMI 1640 medium containing 10% fetal calf serum for 24 hr followed by medium containing 5% dialyzed fetal calf serum and 1 nM 17-estradiol. After one medium change, this medium was replaced by one containing 10 nM progesterone free of fetal calf serum (day 5 after passage). From day 6, medium was collected every day, and the cells were refed with progesterone-containing medium. The collected medium was clarified by centrifugation for 15 min (480 × g at 4°C) and stored at −70°C.

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Abbreviations: T47D, human breast carcinoma cell line; MMTV, mouse mammary tumor virus; gp52, 52,000-dalton envelope glycoprotein of MMTV; hLHβ, beta subunit of human luteinizing hormone.

‡Present address: Department of Microbiology, Tokyo Jikei University, School of Medicine 3-25-8 Nishi-Shinbashi, Minatoku Tokyo, Japan.
§Present address: Microbiology and Cell Biology Lab, Indian Institute of Sciences, Bangalore, India 560012.
¶Present address: Smith Kline & French Laboratories, 620 Allendale Road, King of Prussia, PA 19406.
††Deceased.
**To whom reprint requests should be addressed.

Isolation of Single-Cell Clones. The soft-agar technique for isolating single-cell clones was used. The agar plates contain an agar base layer and a soft-agar top layer containing cells. The base layer consisted of 0.5% of Bacto agar prepared in RPMI 1640 medium with 10% fetal calf serum, insulin, glutamine, and antibiotics (complete medium). For the top layer, the T47D monolayers were trypsinized and suspended in RPMI complete medium containing 0.33% Bacto agar. The concentration of cells used for cloning was 1–5 × 10^2 cells per plate. The plates were scanned for the presence of single cells, which were marked, and then incubated for 21 days. Each colony derived from a single cell was subcultured in RPMI complete medium containing 20% fetal calf serum and recloned on agar.

Purification of the T47D Particles from Culture Medium. Approximately 5 liters of cell-free culture supernatant was concentrated to 100 ml with the Pellicon cassette system (Millipore) and centrifuged (100,000 × *g* for 90 min at 4°C). The resulting pellets were resuspended in 5 ml of TNE buffer-(0.01 M Tris·HCl/0.15 M NaCl/3 mM EDTA, pH 7.6) and layered over linear 20–50% sucrose gradients in TNE buffer. The samples were centrifuged as above for 16 hr, and 25 fractions of equal volume were collected from below. The fractions with density between 1.16 and 1.20 g/ml were pooled, diluted, and centrifuged as above for 90 min. The resulting pellets were resuspended in 0.6 ml of phosphate-buffered saline (P_i/NaCl).

Assay of 60–70S RNA-Directed DNA Polymerase: Simultaneous Detection Test. Purified T47D clone particles (1 mg of protein) were resuspended in 100 μl of 0.01 M Tris·HCl (pH 8.3)/0.1% Nonidet P-40 and incubated at 0°C for 15 min. The disrupted particles were then added to an endogenous reverse transcriptase reaction mixture (final volume, 1 ml) containing 50 mM Tris·HCl (pH 8.3), 8 mM $MgCl_2$, 150 mM NaCl, 0.4 mM dithiothreitol, 0.004% sodium pyrophosphate, 4 μg of oligo$(dT)_{12-18}$, 50 nM [^{3}H]dCTP (1.5 × 10^4 cpm/pmol), and 0.2 mM each dATP, dGTP, and dTTP. After incubation at 37°C for 5 min, the reaction was terminated by the addition of NaCl and sodium dodecyl sulfate to final concentrations of 0.2 M and 0.1%, respectively. The sample was then incubated for 15 min at room temperature and centrifuged at 5000 × *g* for 5 min. The aqueous phase was layered over a preformed 5–20% linear sucrose gradient in TNE buffer containing 0.1% $NaDodSO_4$ and centrifuged (193,000 × *g* for 90 min). Fractions were collected from below, and trichloroacetic acid-precipitable radioactivity was counted.

Assay for RNA·[^{3}H]DNA Complexes by Cs_2SO_4 Density Gradient Centrifugation. After the endogenous DNA polymerase reaction (see above), the sample was divided into three aliquots. Aliquot 1 was extracted directly with phenol/cresol/isoamyl alcohol, 31:4 (vol/vol), and centrifuged (3000 × *g* for 10 min). Aliquots 2 and 3 were incubated with 10 μg of RNase A per ml and 0.4 M NaOH, respectively, for 2 hr at 37°C. After neutralization of aliquot 2, both samples were extracted with phenol as above. The aqueous phase of each sample was brought to 4.7 ml with 5 mM EDTA, and 5 ml of saturated Cs_2SO_4 was added. The samples were centrifuged in a 50 Ti rotor (45,000 rpm, 72 hr), fractions were collected from below, and aliquots were measured for trichloroacetic acid-precipitable radioactivity.

RIA Procedure. A blocking RIA that uses the delayed addition of the labeled antigen was used. An aliquot of sample was added to 200 μl of P_i/NaCl containing 1% bovine serum albumin and 500 kallikrein inactivator units of Trasylol (Mobay Chemical, New York, NY) per ml. Then 0.2–0.6 μg of rabbit anti-MMTV, anti-gp52, or anti-p27 total IgG was added. This amount of antibody was sufficient to precipitate approximately 40% of the ^{125}I-labeled MMTV antigen (see below) in the absence of blocking activity. The samples were incubated with the primary antibody for 20 min at 37°C and 18 hr at 4°C. Approximately 6,000 cpm of ^{125}I-labeled gp52 or ^{125}I-labeled p27 were then added to each tube. The samples were incubated for 20 min at 37°C and 6 hr at 4°C. Then 30 μg of normal rabbit IgG and a sufficient amount of second antibody (goat anti-rabbit IgG) were added and incubated for 20 min at 37°C. The samples were centrifuged (15 min at 3000 × *g*), and the radioactivity in both the supernatant and the precipitate were measured in a gamma counter.

Absorption of Rabbit Anti-gp52 with Clone 8 and 11 Particles and Immunocytochemical Staining. Under previously defined conditions (10), 200 μg of ether-extracted clone 8 and 11 viral region (density, 1.16–1.20 g/ml) were each used to absorb 10 μg of total IgG of R121, a rabbit antibody prepared against gp52 of MMTV, which recognizes the human cross-reactive breast carcinoma antigen (14). The final volume of each absorption mixture was 400 ml, resulting in a final R121 concentration of 25 μg/ml, its routinely used concentration in immunocytochemistry. Under identical conditions, an equal amount of rabbit antibody to the β subunit of human luteinizing hormone (hLHβ) was absorbed at the same concentration with the same amount of clone 11 viral region protein, as a control for the immunologic specificity of the absorption.

Absorbed and unabsorbed antibody preparations were used in a previously described indirect immunoperoxidase procedure (10) on adjacent serial paraffin sections of three breast carcinomas known to contain the crossreactive antigen. Sections of rat pituitary were used for the anti-hLHβ control.

RESULTS

T47D Cloned Sublines. Several different cloned sublines were isolated from the 47D cell line designated clones 5, 8, 11, and 19.

Characterization of the 47D Particles and Its RNA by Isopycnic Separation. Characterization of the nucleic acid in the particles was performed with [^{3}H]uridine labeling in culture. Fig. 1 represents the clone 11 banded particles on 20–60% sucrose gradient, where a single band was observed with a density of 1.18 g/ml. Fig. 1 *Inset* shows that the particles contained 60–70S RNA and a 35S RNA. No particles could be detected in clone 11 culture medium when the cells were grown in the absence of steroid hormones. However, the presence of steroid hormones did not influence the amount of particles released into the medium in the case of clone 8. The particles released by clone 8 were more dense (1.195 g/ml) than those from clone 11, suggesting that clone 8 releases immature particles. The particles from the sublines also contained high molecular weight RNA.

Detection of Intermediates in the RNA-Dependent DNA Polymerase Reaction from T47D Particles. One milligram of 47D clone 11 particles was used to obtain a DNA polymerase reaction. The results described thus far strongly support the existence of a reaction in which the RNA is used as a template to make complementary DNA. Because the RNA is single-stranded, an early intermediate should appear as a DNA·RNA hybrid structure. This, in fact, is shown in Fig. 2*A* in which the [^{3}H]DNA product isolated from a 5-min reaction was banded in Cs_2SO_4. The native product complexed to RNA, as indicated by its presence in the RNA density region of the gradient. Digestion with RNase A did not affect the hybrid (profile similar to that in Fig. 2*A*), whereas treatment with NaOH released the synthetic DNA from the complex, permitting it to band at its proper density position (Fig. 2*B*).

Identification and Fractionation of a MMTV gp52-Related Antigen in T47D Culture Fluid and Cells. We wished to examine the antigenic relationship between the particles produced by T47D clones 8 and 11 and MMTV. We have report-

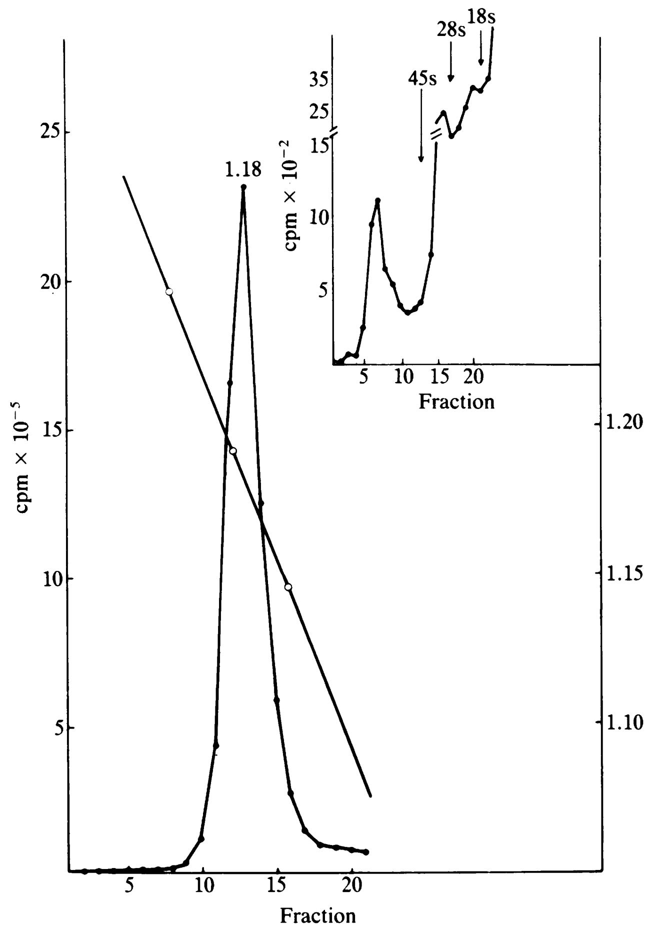

FIG. 1. Equilibrium centrifugation in a sucrose density gradient of the [^{3}H]uridine-labeled particle fraction in the supernatant of clone 11. (*Inset*) Velocity sedimentation of the [^{3}H]uridine-labeled RNA obtained from the banded particles of the supernatant of clone 11.

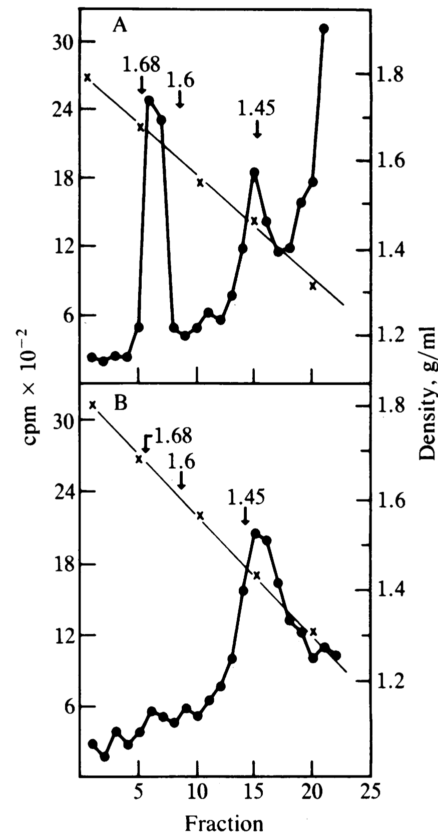

FIG. 2. Analysis by Cs_2SO_4 density centrifugation of 70S RNA·[^{3}H]DNA complex. Reverse transcription reaction was prepared as described. (*A*) Aliquot 1, untreated reaction mixture. (*B*) Aliquot 2, digested by adding RNase (3 μg) and incubated at 37°C for 2 hr. Both aliquots were extracted with phenol/cresol/isoamyl alcohol and submitted to Cs_2SO_4 density analysis.

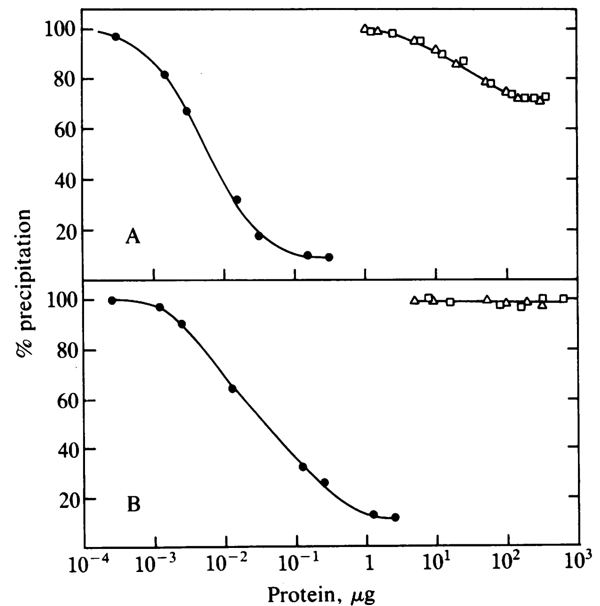

FIG. 3. (*A*) Standard blocking RIA using delayed addition of purified ^{125}I-labeled gp52 (approximately 6000 cpm per assay). Blocking to the anti-gp52 IgG (0.28 μg per assay) was done with material from the T47D clone 8 density region 1.16–1.19 g/cm^2 (□), clone 11 (△) culture fluid, and purified MMTV (●). (*B*) Standard blocking RIA with purified ^{125}I-labeled p27 (4500 cpm per assay). Blocking to the anti-p27 IgG (0.36 μg per assay) was done with particles from T47D clone 8 (□), T47D clone 11 (△), and MMTV (●).

ed previously the specific localization of an antigen related to the major MMTV envelope protein, gp52, in human breast tumors (8) and similar staining has been observed in T47D clone 8 and 11 cells (15). Therefore, we tested the 47D particles for antigenic crossreactivity with gp52 in a blocking RIA, and a partial blocking activity of about 40% was observed with particles from both clones (Fig. 3*A*). Clone 8 and 11 particles were essentially indistinguishable in terms of the relative amount and reactivity of the gp52-related antigen. They both gave the typical heterologous curve of reduced slope and plateau in comparison to the homologous blocking with MMTV. However, no crossreactivity was observed to the major MMTV internal protein, p27 (Fig. 3*B*).

The gp52-related antigen is also released into the culture fluid in soluble form. Supernatant from clone 8 cells grown in serum-free medium was centrifuged (100,000 × g for 90 min) to pellet virus particles, and the supernatant protein was precipitated with saturated ammonium sulfate. The pellet was dissolved and dialyzed overnight (Sup 100-AS). As shown in Fig. 4 for clone 8, this sample displayed the same partial blocking activity observed with the particles. The total amount of antigen in this fraction was about 10-fold greater than in the particle fraction (Table 1). However, comparison of the blocking curves for particles versus Sup 100-AS in Fig. 4 shows that the antigen in the particle fraction was about 15-fold greater. In either case, the supernatant fluid of clones 8 and 11 provided a convenient source of the crossreactive antigen.

We also examined the cells for the presence of the gp52-

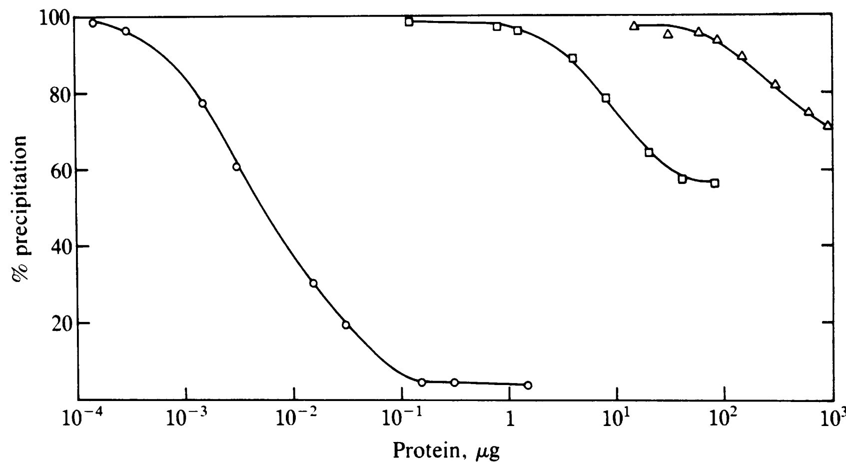

FIG. 4. Standard blocking RIA using delayed addition of purified ^{125}I-labeled gp52 (approximately 6000 cpm per assay). Blocking to the anti-gp52 IgG (0.28 μg per assay) was done with material from the T47D clone 8 culture fluid density region 1.16–1.19 g/cm^3 (□), Sup 100-AS from 47D clone 8 culture fluid (△), and MMTV purified through density gradient (○).

related antigen. About 2 g of tumor cells (both clone 8 and 11) were homogenized and centrifuged at 650 × *g* and then 10,000 × *g* to remove nuclei and mitochondria, respectively. Both pellet fractions were extracted with 4 M KCl overnight at 4°C and dialyzed ("PPT 1E" and "PPT 2E", respectively). The postmitochondrial supernatant was layered over a 25%/50% discontinuous gradient of sucrose in TNE buffer and centrifuged (100,000 × *g* for 90 min). The material in the 25% layer and that at the 25–50% interface were collected separately. The former (Sup 100) was added to an equal volume of saturated ammonium sulfate, stirred for 1 hr at 4°C, and centrifuged (10,000 × *g* for 20 min). The resulting pellet was dissolved and dialyzed in P_i/NaCl (Sup 100-AS). The material at the 25–50% sucrose interface was collected, diluted with TNE buffer, and layered on a linear 20–50% sucrose gradient in TNE buffer and centrifuged as above for 16 hr. Fractions were collected and pooled into three density regions: I, 1.09–1.16 g/ml; II, 1.16–1.19 g/ml (the viral region); and III, 1.19–1.24 g/ml. These samples were diluted and centrifuged to pellet particles that also were assayed as above for gp52-related antigen (Table 2). As observed above for the cell supernatants, crossreactive antigen was detected in both particulate (gradient pools I and II) and soluble (Sup 100-AS) forms. In the sucrose density gradient, most of the activity was found in the viral region (1.16–1.19 g/ml) with a lesser amount in the less-dense pool I region. Clone 8 antigen shows a greater distribution into pool II than does that from clone 11, which may reflect the higher density of clone 8 particles observed in the [^{3}H]uridine labeling assays. The total amount of soluble antigen was again about 10-fold greater than the particle-associated activity. We were unable to detect the antigen in other samples from the fractionation or, it should be particularly noted, in the crude homogenate (Table 2).

The numerology of the distribution of the gp52-related protein between clone 8 cell and culture supernatant is described in Table 1. More than 85% of the related protein was found in the culture fluid, of which 10% appears in particles having the density and other characteristics of retroviruses.

Table 1. Distribution of the gp52-related protein between the cells and supernatant of the culture fluid of T47D clone 8

	Total protein, mg	gp52-related protein, ng	% of related protein
Culture fluid (5.6 liter)			
Virus region	3.1	155	10.9 } 88.5
Sup 100-AS	240	1104	77.6 }
47D cell (2 g)			
Virus region	0.035	17.5	1.2 } 11.5
Sup 100-AS	7.6	146	10.3 }

Twelve percent of the antigen was found in the cells, mostly in the particle fraction.

Absorption of the Immunocytochemical Stain by Clone 8 and 11 Particles. In all three breast carcinomas tested, absorption of rabbit anti-gp52 with clone 11 viral region completely eliminated the ability of this antibody to detect the gp52-related protein. The intracellular stain observed in many cells of this invasive carcinoma (Fig. 5 *A* and *B*) was not seen in a semi-serial section incubated with the same antibody absorbed with clone 11 viral region (Fig. 5*C*). The control absorption of the anti-hLHβ antibody with clone 11 viral region did not at all interfere with its ability to stain gonadotropin-producing cells in the section of rat pituitary, eliminating the possibility of a nonimmunologic blocking of the immunocytochemical reaction.

Under the absorption conditions described above, only partial blocking of the immunocytochemical reaction was obtained with clone 8 viral region. Use of amounts greater than 200 μg resulted in a greater degree of absorption but, under such conditions, less intense staining also was observed by anti-hLHβ in the section of rat pituitary.

DISCUSSION

Our primary purpose is to characterize the human breast cancer antigen and the nature of its biochemical relatedness to MMTV gp52. The logistical feasibility of attaining this goal has been achieved by the establishment of a cell line from the pleural effusion of a patient with intraductal and invasive breast carcinoma (13). The experiments described in this report confirm our initial observations (15) that this cell line, T47D, and its clonal derivatives express the gp52-related antigen. In various blocking RIAs using rabbit anti-gp52 and ^{125}I-labeled gp52, the crossreactive antigen was

Table 2. Fractionation of clone 8 and 11 cells for gp52 crossreactive proteins

Fraction	Total protein, mg		gp52-related protein, ng	
	Clone 8	Clone 11	Clone 8	Clone 11
Crude homogenate	2160	2650	UD	UD
PPT 1E	3.8	3.1	UD	UD
PPT 2E	4.2	3.4	UD	UD
SUP 100-AS	7.6	6.8	146	133
Pool I*	0.270	0.160	1.5	5.0
Pool II†	0.035	0.060	17.5	10.0
Pool III‡	0.016	0.008	UD	UD

UD, undetectable.
*Density, 1.01–1.16 g/cm^3.
†Density, 1.16–1.19 g/cm^3 (viral region).
‡Density, 1.19–1.24 g/cm^3.

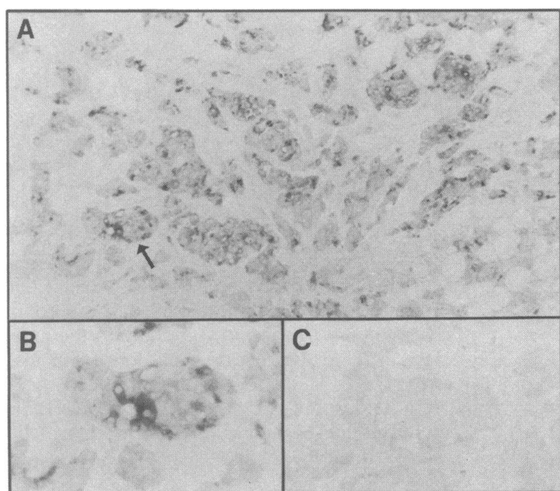

FIG. 5. Immunoperoxidase stain of section of human breast carcinoma. Most of cells indicated by the arrow in *A* can be seen in greater detail in *B* and *C*; *C* is from a semiserial section incubated with the same antiserum used in *A* and *B* (Rb anti-gp52) after absorption with T47D clone 11 viral region; note the complete absence of reaction product in *C*. (Methylene blue counterstain; *A*, ×41; *B* and *C*, ×99.)

found not only in the T47D cells but also, most importantly, in the culture medium. Our data indicate that about 10% of the antigen is shed into the supernatant in particulate form and close to 70% as a soluble protein, making its isolation relatively convenient (Table 1).

The elimination of the immunohistochemical reaction in breast carcinomas by absorption of the anti-gp52 with clone 11 viral region supports the above findings and confirms the presence of the crossreactive antigen in these T47D derivatives. Although a comparatively large amount of antigen was needed to obtain complete absorption, the possibility of a nonimmunologic blocking effect was eliminated through the parallel identical absorption in the hLHβ rat pituitary system. In fact, this control prevented the misinterpretation of results obtained in our attempt to attain complete absorption with the clone 8 viral region preparation. When more than 200 μg of this antigen was used to absorb 10 μg of IgG, a decrease in the staining was also noted in the rat pituitary, indicative of nonspecific blocking. If one considers that clone 8 and clone 11 viral region preparations behave identically in RIA, the inability of clone 8 to absorb completely in the immunohistochemical reaction is surprising. Such an outcome could perhaps be due to different concentrations of specific crossreactive protein in the preparations used for absorption. The isolation and purification of the specific crossreactive antigen is, therefore, of great importance and remains one of our primary objectives. Preliminary results of radioimmunolocalization in polyacrylamide gels (16) of disrupted clone 8 and 11 particles with anti-gp52 reveal three bands with estimated M_rs of 54,000–83,000 (unpublished observations).

The present study also provides evidence that the particles isolated from the culture supernatants of T47D clones 8 and 11 have the physical and biochemical characteristics of retroviruses, as has been shown previously for particles from solid breast tumors (1–4). The [^{3}H]uridine-labeled particles of clone 11 band sharply at a density of 1.18 g/ml, whereas the particles from clone 8 are more dense and may represent immature virus. The RNA extracted from these particles consistently gave a peak of about 70S in sedimentation gradient. Finally, reverse transcriptase activity giving rise to 70S RNA·DNA complexes was identified in both clone 8 and 11 particles.

It was important, of course, to establish that the cell lines were not in fact producing MMTV itself. Protein analysis of the particles showed only a gp52-related antigen with no evidence of either p27 or gp36 of MMTV. In addition, RNA extracted from clone 8 and 11 particles does not hybridize with MMTV cDNA or with cloned segments of an endogenous MMTV genome under stringent hybridization conditions; likewise, total cellular DNA from either clone showed no evidence of MMTV sequence by solution hybridization on Southern blot analysis (unpublished observations). Under much less stringent conditions, sequences weakly related to MMTV have been identified and cloned from normal human DNA (17).

The availability of the T47D culture and its derivatives facilitates purification of the human breast cancer antigen and the design of experiments to elucidate the nature of this unique protein. Furthermore, it has allowed us to produce monoclonal antibodies, via hybridomas against this and other antigens, that will be needed to develop the homologous immunoassays that can hope to attain the sensitivities required for the detection of a systemic blood signal for human breast cancer.

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