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Characterization of a protein, released by the T47D cell line, immunologically related to the major envelope protein of mouse mammary tumor virus

(human breast carcinoma cell line/T47D cell line/gp52)

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ABSTRACT **The T47D human mammary adenocarcinoma cell line *in vitro* releases viral particles as well as soluble proteins. Both fractions were shown to contain antigens that immunologically crossreact with the major glycoprotein (gp52) of mouse mammary tumor virus. The crossreacting antigens are located on polypeptides with apparent molecular weights of about 68,000 and 60,000. The larger one is present in viral particles whereas both are found in the soluble fraction. Both proteins are glycosylated. The human tissue culture proteins differ from gp52 not only in molecular weight but also in charge heterogeneity and in polypeptide profiles obtained after partial proteolysis. The results suggest that there is a restricted similarity between MMTV gp52 and the immunologically related T47D proteins.**

Evidence for an association of viral proteins and viral nucleic acid sequences with human mammary carcinomas has been accumulated increasingly over the past few years. Key similarities between the human malignancy and mouse mammary tumors have focused attention on the possible involvement of mouse mammary tumor virus (MMTV)-like retroviruses in the human disease (1, 2). An antigen immunologically related to the protein moiety of gp52 (a 52,000 M_r glycoprotein that constitutes the major envelope protein of MMTV) was detected in sections of human breast carcinoma tissue and in particulate fractions of human milk (3–5). The antigen also has been detected in established cell lines derived from pleural effusions of patients with breast cancer (6, 7). In addition, these cell lines, MCF-7 and T47D, have been shown to possess DNA sequences with partial homology to the genome of MMTV (8).

The T47D cell line, which was established and subcloned in our laboratory (9), releases retrovirus-like particles (10). Therefore, it was of interest to determine whether these particles manifested proteins antigenically related to MMTV gp52. Since MMTV gp52 is shed from tumor cells *in vivo* in the mouse and enters the circulation, the tissue culture system also might serve as a model in the search for crossreacting antigens *in vitro*. An MMTV gp52 crossreacting antigen was recently found in both retrovirus-like particles and soluble proteins released by the T47D cell line (10).

The present study was designed to identify and characterize these gp52-related proteins and also to determine their degree of similarity to MMTV gp52.

MATERIALS AND METHODS

Cells and Viruses. The T47D cell line (sub-line Cl-11) was used in this study. The cells were grown in RPMI 1640 medium containing 10% fetal calf serum, insulin (0.2 IU/ml), 2 mM glutamine, and antibiotics (complete medium). When cells were grown in the presence of steroid hormones, serum was omitted. For virus collection, the cells were seeded in RPMI 1640 with 10% fetal calf serum and incubated 24–48 hr; the medium then was replaced with RPMI 1640 with 5% dialyzed serum and 1 nM 17β-estradiol. After 24 hr, the medium was replaced with RPMI 1640 containing the same amount of 17β-estradiol without serum. The next day and each day subsequently, medium was replaced with RPMI 1640 containing 10 nM progesterone only. Medium was collected from the second day onward, clarified by centrifugation (5 min at 500 × g and 10 min at 1000 × g, both at 4°C) and stored at −70°C. The stored medium was pooled and then centrifuged in a Beckman 19 rotor at 18,000 rpm for 3 hr at 4°C. The pelleted particles were purified by isopycnic sucrose gradient (20–60%, wt/wt) centrifugation in a Beckman SW27 rotor at 25,000 rpm for 18 hr at 4°C. The regions corresponding to 1.16–1.22 g/cm^3 were collected and centrifuged in a Beckman SW41 rotor at 40,000 rpm for 60 min at 4°C to sediment the particles. These particles were designated human mammary tumor virus (HMTV). The soluble proteins from the virus-free tissue culture medium (SP) were precipitated with ammonium sulfate at 65% saturation. The precipitated proteins were dissolved in and dialyzed against phosphate-buffered saline, pH 7.6.

MMTV, isolated from the milk of Paris RIII mice, and MMTV gp52, purified by affinity chromatography on concanavalin A-Sepharose (11), were kindly supplied by the late Dr. S. Spiegelman of Columbia University.

Preparation of Antisera and Affinity-Purified Antibodies. Anti-MMTV gp52 sera were prepared in rabbits as described (11, 12). The antisera were always adsorbed with insolubilized normal human plasma and fetal calf serum prior to experiments, to eliminate traces of activity against nonspecific antigens (12, 13). Only anti-gp52 antisera reacting with human breast tumor tissue in the immunoperoxidase assay (12–14) were selected for further studies. The antibodies were purified by immunoaffinity adsorption to virus proteins or SP covalently bound to crosslinked agarose (Affi-Gel 10, Bio-Rad). The agarose columns were loaded with preadsorbed rabbit anti-gp52 serum and, after 1 hr at 37°C, washed extensively with 10 mM Tris Cl/0.15 M NaCl, pH 7.4. The bound antibody molecules were eluted with 5 M $MgCl_2$ and dialyzed against 10 mM Tris Cl/0.15 M NaCl/1 mM EDTA, pH 7.4.

Iodination of Proteins. Proteins were labeled with ^{125}I according to Greenwood *et al.* (14). The T47D virus (HMTV) and MMTV were precipitated with acetone and then dissoci-

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Abbreviations: MMTV, mouse mammary tumor virus; HMTV, human MTV; SP, soluble proteins obtained from tissue culture medium after removal of virus particles; PAS, periodic acid/Schiff reagent.
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ated in 0.5% sodium deoxycholate/0.5% Triton X-100/0.2 M potassium phosphate, pH 7.4, prior to iodination. The specific radioactivity of the labeled proteins was 2–6 × 10^6 cpm/μg of protein.

Immunoprecipitation of Radiolabeled Proteins. [^{125}I]-labeled proteins (1.5–4 × 10^6 cpm) were immunoprecipitated as follows: The antigens were preadsorbed by incubation with 5 μl of concentrated unadsorbed normal rabbit serum for 1 hr at 37°C, followed by a 15-min incubation at room temperature with 10 μl of a 10% (vol/vol) suspension of formalin-fixed *Staphylococcus aureus* Cowan I strain (15) and centrifugation for 4 min at 10,000 rpm. To the preadsorbed antigens in the supernatant, the antisera or affinity-purified antibodies were added. After incubation for 1 hr at 37°C and then 15 min at room temperature with fixed *S. aureus*, the mixtures were centrifuged through 1-ml cushions of 1 M sucrose/10 mM Tris Cl, pH 7.4/0.15 M NaCl/1 mM EDTA/0.5% Nonidet P-40. The thoroughly washed pellets were analyzed for radioactivity with a γ scintillation counter and subjected to $NaDodSO_4$/PAGE.

Discontinuous $NaDodSO_4$/PAGE. $NaDodSO_4$/PAGE was done as described (16). The gel acrylamide concentration was 11%, unless otherwise stated. Gels were either stained for protein with Coomassie brilliant blue R-250 or autoradiographed on x-ray films with the aid of image-intensifying screens.

Peptide Mapping. Labeled proteins were eluted from gel bands and then subjected to limited proteolysis with *S. aureus* V8 protease (Sigma) as described (16). Polypeptides derived from T47D virus or SP were cleaved with protease (either 50 μg/ml or 5 μg/ml) for 1 hr at 37°C. MMTV gp52 was digested with the V8 protease at either 100 μg/ml or 10 μg/μl for 1.5 hr at 37°C. Peptides produced were electrophoresed on 12.5% acrylamide gels.

Presence of Carbohydrate Moieties. Carbohydrate analysis of proteins was achieved by two methods: (*i*) periodic acid/Schiff reagent (PAS) staining after $NaDodSO_4$/PAGE (17) and (*ii*) binding to concanavalin A-Sepharose. One-milliliter columns of concanavalin A-Sepharose 4B (Sigma), equilibrated in 10 mM Tris Cl, pH 7.4/0.1 M NaCl, were loaded with ^{125}I-labeled HMTV proteins or SP. The columns were washed extensively with the same buffer but including 0.2% Nonidet P-40, until no radioactivity emerged from the columns. The carbohydrate-containing material was eluted with 0.1 M α-methyl-D-mannoside in the washing buffer. The eluted material was immunoprecipitated and then was analyzed by $NaDodSO_4$/PAGE and autoradiography.

RESULTS

Protein Composition of HMTV and SP from T47D Cell Cultures. Purified T47D HMTV and SP, obtained as described in *Materials and Methods*, were analyzed by $NaDodSO_4$/PAGE (Fig. 1). The viral particles yielded a major protein band corresponding to a molecular weight of about 68,000, whereas SP contained mainly two polypeptides with mean apparent molecular weights of about 68,000 and 60,000 (p68 and p60). Other proteins also were detected in both the viral and SP preparation. Molecular weights of 45,000–95,000 were obtained in the case of viral preparations, whereas molecular weights of 15,000–95,000 daltons characterized the SP. The protein designated p60 was detected mainly in SP but also occasionally was identified in viral preparations.

Since p68 of both viral and SP origin exhibited electrophoretic mobility very close to that of bovine serum albumin, it was conceivable that the protein might, in fact, be albumin. The "albumin" could be either of bovine origin (since the cells were grown for some time in medium containing fetal calf serum) or human—if the T47D human mammary cells

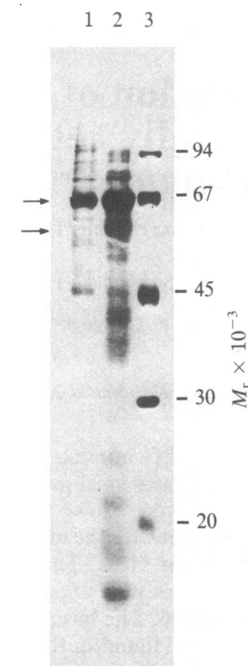

FIG. 1. $NaDodSO_4$/PAGE analysis of the proteins of the viral particles and of SP. The 11% acrylamide gel was stained with Coomassie brilliant blue. Lane 1, 80 μg of HMTV protein; lane 2, 64 μg of SP; lane 3, protein markers of known molecular weights. The protein markers are (from top to bottom) phosphorylase A (M_r 94,000), bovine serum albumin (M_r 67,000), ovalbumin (M_r 45,000), DNase (M_r 30,000), trypsin inhibitor (M_r 20,000), and α-lactalbumin (M_r 14,000). Arrows indicate p68 and p60.

are able to synthesize albumin. Both possibilities were ruled out by enzyme-linked immunoadsorbent assay (ELISA) and radioimmunoprecipitation experiments employing anti-bovine serum albumin and anti-human serum albumin antibodies to probe the viral and SP preparations (data not shown).

Proteins Immunologically Related to MMTV gp52 Are Present in HMTV and SP Preparations. Disrupted viral particles and SP of T47D origin were labeled with ^{125}I and immunoprecipitated with anti-MMTV gp52 rabbit serum. $NaDodSO_4$/PAGE electrophoresis of immunoprecipitated labeled HMTV proteins gave a major radioactive band corresponding to the p68 component (Fig. 2*A*, lane 2). Immunoprecipitated SP yielded major labeled bands in the p68 and p60 regions of the gel (Fig. 2*B*, lane 2). Preimmune serum did not precipitate iodinated antigens, and no radioactive bands were observed after gel electrophoresis [Fig. 2 *A* (lane 5) and *B* (lane 1)]. The observed immunological crossreactivity between human and mouse viral proteins was substantiated further by immunoaffinity purification of the rabbit anti-gp52 antibodies by adsorption onto immobilized human antigens of either viral or SP origin. Purified antibodies gave identical results to those obtained with antiserum, when used to immunoprecipitate human material [Fig. 2 *A* (lanes 3 and 4) and *B* (lanes 3 and 4)]. These results showed that the MMTV gp52 antiserum contained antibodies that recognized HMTV and SP proteins. However, specific crossreactivity with MMTV gp52 remained to be proven.

Demonstration of the Specific Antigenic Similarity Between HMTV and gp52 MMTV. Rabbit antisera directed against purified MMTV gp52 precipitated mainly gp52 molecules. However, prolonged exposure of gels to x-ray film brought out two minor ^{125}I-labeled protein bands in addition to the major one characteristic for gp52. These bands appeared at positions corresponding to gp36 and to p28 of MMTV (Fig. 3*A*, lane 3). Accordingly, it was possible that observed crossreactivity between human and mouse viral proteins did

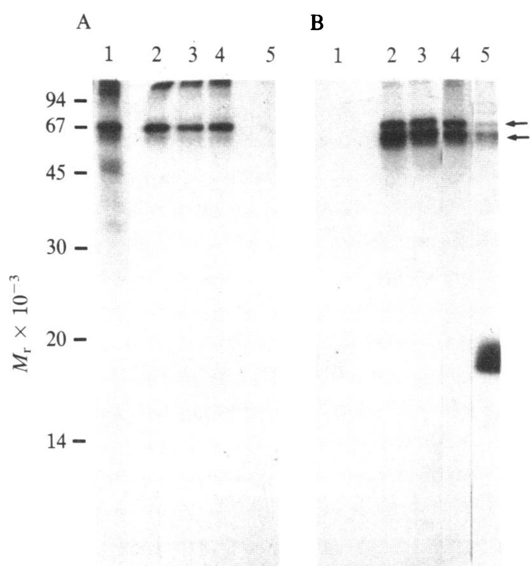

FIG. 2. Radioimmunoprecipitation of ^{125}I-labeled HMTV proteins and SP by anti-MMTV gp52 serum. Immunoprecipitated HMTV proteins (*A*) and SP (*B*) were subjected to $NaDodSO_4$/PAGE and autoradiography. Antisera used: normal rabbit serum (lane 5 in *A* and lane 1 in *B*); rabbit serum raised against HMTV (lane 1 in *A* and lane 5 in *B*); rabbit anti-MMTV gp52 serum (lanes 3); the same anti-MMTV gp52 serum, affinity-purified on HMTV proteins-agarose (lanes 4); anti-gp52 serum affinity-purified on SP-agarose (lanes 2). Positions of marker proteins are shown on the left. Arrows at right indicate p68 and p60.

not pertain to gp52 at all. Such a possibility was not substantiated by further investigations.

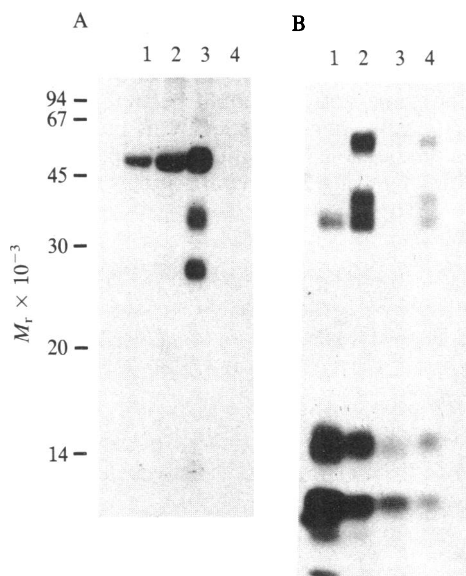

FIG. 3. Immunoprecipitation of MMTV proteins by anti-gp52 serum. (*A*) ^{125}I-labeled MMTV was immunoprecipitated with normal rabbit serum (lane 4), rabbit anti-MMTV gp52 (lane 3), or the same anti-gp52 serum after immunoaffinity purification on either SP-agarose (lane 1) or on HMTV proteins-agarose (lane 2). (*B*) Autoradiography of the peptides generated by limited proteolysis of the gp52 bands. The gp52 bands immunoprecipitated with the different anti-gp52 sera (as in *A*) were eluted, cleaved with *S. aureus* V8 protease, and then analyzed by $NaDodSO_4$/PAGE. Lanes 1 and 2: MMTV gp52 immunoprecipitated with rabbit anti-MMTV gp52, isolated by $NaDodSO_4$/PAGE, eluted, and digested with V8 protease at 100 μg/ml (lane 1) or 10 μg/ml (lane 2). Lanes 3 and 4: MMTV gp52 precipitated with affinity-purified (SP-agarose) anti-gp52 antibodies, isolated, eluted, and digested with V8 protease at 100 μg/ml (lane 3) or 10 μg/ml (lane 4).

When standard anti-gp52 serum was purified by affinity chromatography on immobilized proteins of HMTV origin (soluble as well as particulate) and the eluted antibodies were immunoprecipitated with iodinated MMTV proteins, $NaDodSO_4$/PAGE analysis of precipitates showed only single bands at the gp52 position regardless of the duration of film exposure (Fig. 3*A*, lanes 1 and 2). Since eluted antibodies were still capable of precipitating HMTV and SP p68 and p60, there is little doubt about the antigenic similarity of these two proteins to MMTV gp52. When we compared the immunoprecipitation of antigens with the standard anti-gp52 sera vs. the affinity-purified antisera, we found a significant decrease in the amount of gp52 antigen precipitated with the affinity-purified antibodies (9- to 19-fold difference). No significant changes were observed in the extent of immunoprecipitation of HMTV proteins (Table 1). Nevertheless, to make certain that the HMTV proteins recognized by the anti-MMTV gp52 were related to gp52 of MMTV origin, we immunoprecipitated MMTV proteins with either antiserum or affinity-purified antibodies against MMTV gp52 (see above). The precipitated material was resolved by $NaDodSO_4$/PAGE, the recovered gp52 bands were subjected to limited proteolysis with staphylococcal V8 protease, and the resulting peptide profiles were compared by $NaDodSO_4$/PAGE. Since both profiles were identical (Fig. 3*B*), it could be concluded unambiguously that specific crossreaction between MMTV gp52 and the p68 and p60 fractions of HMTV and SP does occur.

Molecular Characterization of HMTV and SP p68 and p60. *Two-dimensional gel electrophoresis.* In addition to its molecular weight difference relative to MMTV gp52 (see Fig. 2), the p68 protein from HMTV exhibited less charge heterogeneity than gp52 when immunoprecipitates of MMTV and HMTV were compared by two-dimensional isoelectric focusing/$NaDodSO_4$/PAGE (Fig. 4 and ref. 19). Similar results were obtained for p68 and p60 from SP (data not shown).

p68 and p60 polypeptides are glycosylated. Electrophoretically resolved p68 and p60 bands were tested for carbohydrate by PAS staining. Of the many bands that stained with Coomassie brilliant blue, only those corresponding to p68 and p60 reacted with PAS. The p68 band from viral particles also gave a positive response with PAS (data not shown). The ability to bind an immobilized lectin (concanavalin A) also was examined. Specific binding of p68 and p60 to Seph-

Table 1. Immunoprecipitation of ^{125}I-labeled proteins of HMTV and of MMTV by anti-gp52 sera and affinity-purified antibodies

	^{125}I, cpm		
		Immunoadsorbent-purified antibodies prepared on	
Antigen	Anti-gp52 serum	HMTV proteins-agarose	SP-agarose
Experiment 1			
HMTV	55,100	51,300	60,300
MMTV	106,200	5,500	7,800
Experiment 2			
HMTV	66,100	65,200	71,600
MMTV	191,800	19,900	21,100

The radiolabeled proteins were precipitated in the presence of 100 μl of a 1:10 dilution of the whole anti-gp52 serum or of anti-gp52 antibodies (20 μg/ml) prepared from the same serum by immunoaffinity chromatography on either HMTV proteins-agarose or SP-agarose. The results of two separate experiments (with different batches of labeled viruses and affinity-purified antibodies) are expressed as cpm detected in the washed immunoprecipitates.

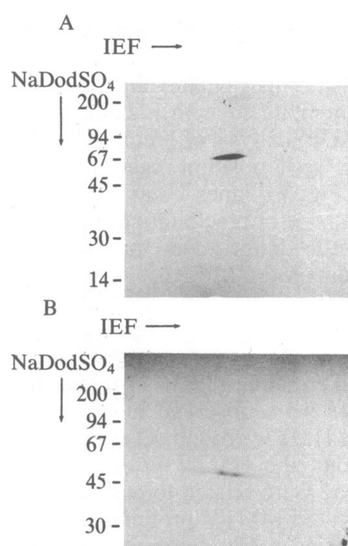

FIG. 4. Two-dimensional gel electrophoresis of anti-gp52-immunoprecipitated MMTV proteins (*A*) and T47D virus proteins (*B*). Immunoprecipitates were subjected to two-dimensional isoelectric focusing (IEF)/NaDodSO$_4$/PAGE (18). The pH gradient in the first dimension was from 4.5 to 7.0. Positions of marker proteins are at left ($M_r \times 10^{-3}$).

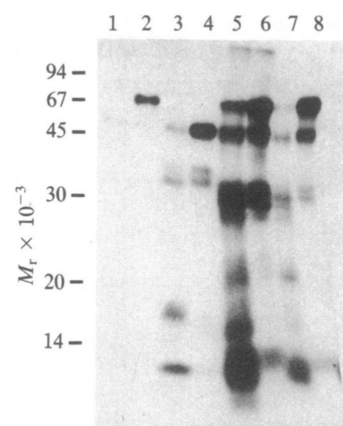

FIG. 5. Peptide pattern after proteolytic cleavage by staphylococcal V8 protease. The preparation and digestion of the ^{125}I-labeled protein bands were described in *Materials and Methods* and in the legend to Fig. 3*B*. Lane 1, undigested MMTV gp52; lane 2, undigested T47D HMTV p68; lane 3, MMTV gp52 digested with V8 protease at 100 μg/ml; lane 4, MMTV gp52 digested with protease at 10 μg/ml; lane 5, T47D HMTV p68 digested with protease at 50 μg/ml; lane 6, HMTV p68 digested with protease at 5 μg/ml; lane 7, T47D SP p68 digested with protease at 50 μg/ml; lane 8, T47D SP p68 digested with protease at 5 μg/ml. The reason for using higher protease concentrations in the case of MMTV gp52 (lanes 3 and 4) stems from the fact that unlabeled heavy chains of the immunoglobulin molecules present in the immunoprecipitates comigrate with gp52.

arose-linked concanavalin A was observed (data not shown), suggesting that α-D-mannopyranose and α-D-glucopyranose residues likely are present at the ends of the oligosaccharide chains of these glycoproteins. In any case, it is probably appropriate to redesignate the p68 and p60 components of HMTV and SP as gp68 and gp60, respectively.

Peptide profiles of gp68 from HMTV particles and culture supernatants. The polypeptide maps obtained after limited proteolytic cleavage of gp68 purified from viral particles and from T47D SP were identical. Therefore, the two fractions probably contain the same viral glycoprotein. In contrast, HMTV and SP gp68 polypeptide maps differ markedly from that of MMTV gp52 (Fig. 5).

Immunoperoxidase Staining of Human Mammary Carcinoma Cells with Affinity-Purified Anti-MMTV gp52. Substantial evidence has accumulated in the past few years for the presence of MMTV gp52-related proteins in human mammary carcinomas (3, 4) and in human mammary carcinoma cell lines such as T47D or MCF-7 (6, 7). We have shown that such antigens also are present both in the viral particles released by the T47D cell line and among the soluble proteins that are shed by these cells (10). Therefore, it was of interest whether the same antigens could be detected in paraffin blocks of human mammary adenocarcinomas by the affinity-purified antibodies (Fig. 6). Anti-MMTV gp52 antibodies, affinity-purified by using SP or viral proteins, recognized spe-

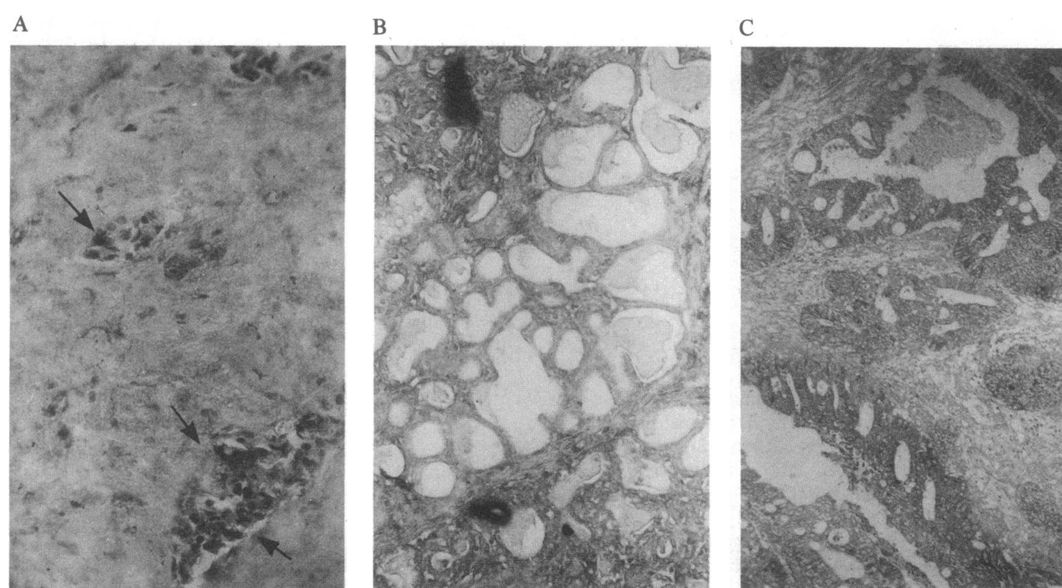

FIG. 6. Indirect immunoperoxidase staining of sections from paraffin-embedded blocks of human tissues. The test was carried out as described in detail elsewhere (4, 12, 13). The immunoperoxidase assay was performed with anti-gp52 sera, affinity-purified on HMTV proteins-agarose. (*A*) Invasive breast carcinoma. (*B*) Cystic breast. (*C*) Adenocarcinoma of the colon. Note intense stain (positive reaction products) in the cytoplasm of the breast tumor cells (marked by arrows). No reaction is seen in normal and benign tissue or in the adenocarcinoma of the colon. (Methylene blue counter-stain, ×200.)

cific antigens in sections from human mammary adenocarcinoma but not from normal breast tissue, benign breast tumor tissue, or carcinomas of other organs. These findings suggest that the antigen detected *in vivo* in human tumor tissues by anti-gp52 sera (4) is immunologically related to the p68 and p60 proteins of the T47D/HMTV *in vitro* system.

DISCUSSION

Three main lines of research have suggested that oncornaviruses might be associated with human mammary adenocarcinomas: (*i*) Retrovirus-like particles possessing RNA-dependent DNA polymerase activities have been identified in human milk samples (13). (*ii*) Antigens immunologically related to the major envelope protein (gp52) of MMTV were detected in human mammary tumors (3, 4). (*iii*) MMTV-related sequences have been found in human DNA as well as in DNA from breast cancer cell lines (8, 17).

To further characterize T47D HMTV proteins, which immunologically crossreact with MMTV gp52 (10), it seemed important to us to determine whether T47D HMTV differs from MMTV, though both share some similar antigenic determinants. We have shown that T47D HMTV does not immunoreact with sera directed against MMTV gp36 and p27 proteins. Furthermore, Keydar *et al.* (10) have used radioimmuno-competition assays to show that the homology between those two viruses is quite limited as judged from the different slopes of the competition curves and from the partial competition exhibited by the human particles (10).

In the present paper, we have shown the following: (*i*) The crossreacting antigen is found on glycosylated polypeptides with apparent molecular weights of about 68,000 and 60,000. (*ii*) These glycoproteins differ from gp52 not only in size but also in charge heterogeneity (two-dimensional gel electrophoresis) and in polypeptide profiles after partial proteolysis. (*iii*) Even the antigenic relationship is limited. As indicated in Table 1, only a small fraction of the entire anti-gp52 antibody population was retained on affinity columns of immobilized HMTV or SP. That is to say, the majority of the antibodies directed against different determinants of the gp52 molecule do not recognize any part of the human antigens. Compatible with this result are molecular hybridization studies with cloned MMTV *env* gene and DNA from the T47D line. A very low but significant degree of sequence homology was found (8).

It already has been shown that antigen from human breast carcinomas immunologically crossreacts with the protein moiety of gp52 and not with the carbohydrate portion of this MMTV glycoprotein (3). Perhaps a similar situation holds in the present case. Nevertheless, further research will be required to elucidate the common peptide sequences in the human and mouse viral proteins discussed here.

Shedding of protein molecules related to MMTV gp52 was evident in the T47D model system described herein. The virus-free tissue culture medium contained proteins that crossreact with anti-gp52 serum (10). Two glycosylated species with apparent molecular weights near 68,000 (gp68) and 60,000 (gp60) were found. As deduced from their antigenic crossreactivity and the similarity of the peptide profiles after partial proteolysis, gp68 from supernatant fluids and the gp68 component in HMTV particles are, if not identical, very similar. The smaller polypeptide (gp60) coprecipitates with the gp68 of SP even when treated with several detergents before or after immunoprecipitation (data not shown). In fact, gp60 is occasionally detected in viral preparations as well. Therefore, it might be derived from gp68 by limited proteolysis of the larger protein and not represent some unrelated molecular species. Be that as it may, the fact that these gp52-related proteins also are expressed on the cell membranes of T47D cells renders this *in vitro* system appropriate for studying the modulation and expression of tumor antigens *in vitro*. In particular, results with the affinity-purified gp52 antibody preparation suggest that anti-gp52 antibodies specifically detect gp68-like molecules in human adenocarcinomas of the breast.

We gratefully acknowledge the pioneering contributions of the late Dr. Sol Spiegelman to the field of viruses and human neoplasia. We thank Dr. A. Ben Zeev for assistance in two-dimensional gel electrophoresis, Dr. D. Serban for his help with affinity chromatography, and Prof. S. Yankofsky for his critical comments on the manuscript. N.S. was a fellow in cancer research supported by the Imperial Cancer Research Fund and the Montreal Chapter of Israel Cancer Research Fund and a Research Associate of the Israel Cancer Association.

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