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Human breast carcinoma antigen is immunologically related to the polypeptide of the group-specific glycoprotein of mouse mammary tumor virus

(breast cancer/tumor antigen/immunohistochemistry/deglycosylated glycoprotein)

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ABSTRACT **We have shown [Mesa-Tejada, R., Keydar, I., Ramanarayanan, M., Ohno, T., Fenoglio, C. & Spiegelman, S. (1978) *Proc. Natl. Acad. Sci. USA* 75, 1529–1533] that an antigen immunologically related to gp52, a 52,000-dalton glycoprotein of the mouse mammary tumor virus, can be identified in sections of human breast cancer by means of an indirect immunoperoxidase technique. The specificity of the reaction was established by absorption experiments which revealed that only purified gp52, or material containing it, served to eliminate the IgG molecules responsible for the immunohistochemical reaction in the human breast tumors. We show here that the cross-reactivity between the human and murine tumor antigens is due to the polypeptide rather than the polysaccharide components of gp52. Sugar-free gp52 prepared by deglycosylation with a mixture of glycosidases was as fully effective as the intact gp52 in removing from anti-MMTV the IgG responsible for the reaction with the human tumor antigen. In contrast, the isolated polysaccharide of gp52 was unable to exert blocking activity.**

Molecular and virological comparisons of human breast cancer with murine mammary tumors have revealed certain key similarities. In particular, human breast carcinomas have been found (1–3) to contain particles with many of the biochemical and biophysical features characteristic of RNA tumor viruses. These include size, density, the possession of outer membranes and of central cores encapsulating a large (70S) RNA molecule complexed to a RNA-directed DNA polymerase (reverse transcriptase). Furthermore, a partial homology has been established (2–4) between RNA molecules found in human breast cancers and the RNA genome of the mouse mammary tumor virus (MMTV). The latter finding suggested that an antigenic relationship might conceivably exist between one of the proteins of the human breast cancer particles and those of MMTV, an approach given further plausibility by serological and cellular studies (5–11) of breast cancer patients that have identified antibodies interacting with MMTV antigens. In any event, this possibility was explored by using antisera raised against the proteins of MMTV as reagents to search for crossreactive components in human breast cancer tissue and this search was crowned with apparent success. An antigen immunologically related to the group-specific antigen, gp52 (a 52,000-dalton glycoprotein) of MMTV was identified (12, 13) in paraffin sections of human breast cancers by means of an indirect immunoperoxidase procedure.

The specificity of the reaction with anti-MMTV sera was checked by absorptions with the following: (*i*) purified gp52; (*ii*) a number of virus preparations [MMTV, Rauscher leukemia virus (RLV), simian sarcoma virus, baboon endogenous virus, and Mason–Pfizer monkey virus]; (*iii*) normal plasma, leukocytes, breast tissue, milk, actin, collagen, and hyaluronic acid, all of human origin; (*iv*) sheep erythrocytes, bovine mucin, and fetal calf serum. Only absorptions with MMTV (derived from either milk or tissue culture) or purified gp52 eliminated the immunohistochemical reaction with the human breast tumors. Further support for the specificity of the reactions observed in breast cancers came from examination of other tissues for the presence of crossreactive antigen. There was no evidence of a positive reaction in any of 119 benign breast lesions (cystic disease, fibroadenoma, papilloma, gynecomastia) or in any of 18 normal breast tissues (lactating or resting). Furthermore, with one possible exception (a mucoepidermoid carcinoma of the parotid gland), none of 98 carcinomas of 13 other organs showed any evidence of a positive reaction and neither did eight cystosarcoma phyllodes of the breast. In contrast, 45.5% of 376 breast cancer cases of various histopathological types have shown clear positive reactions (14). Because at most five sections are stained from each breast tumor, the percentage of positives detected must be an underestimate.

It was of course gratifying to have extended to the protein level the relationship between human breast cancer and MMTV that was initially discovered in terms of nucleic acid sequence homology. The etiologic implications of these findings are of obvious interest but our immediate concern is the possibility that they might be used to generate clinically useful information. To this end, it was of interest to resolve certain issues regarding the nature of crossreactivity observed between the gp52 and the unique antigen found in the human breast cancers. In particular, it was of some importance to know whether the sugar or the protein moiety of the gp52 glycoprotein was responsible for its ability to block the immunological crossreaction with the antigen found in the breast cancer cells. Aside from its genetic implications, the outcome could materially influence the nature of our attempts to isolate and characterize the human tumor-specific antigen.

It is the purpose of the present paper to present data that resolve this issue. The results establish that the antigenic crossreactivity between human breast cancer and gp52 glycoprotein resides in the polypeptide portion and not in the sugar residues of the latter protein.

MATERIALS AND METHODS

Enzymes. The enzymes used were proteinase K (fungal) at 20 milliAnson units/mg (E. Merck) [1 Anson unit is the amount of enzyme that liberates under standard conditions 1 mmol of folin-positive amino acids (calculated as tyrosine) per min] and endoglycosidase H (from *Streptomyces griseus*) (30 units/mg). Mixed glycosidases were [units/mg]: α-mannosidase [166],

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Abbreviations: gp52, 52,000-dalton glycoprotein; MMTV, murine mammary tumor virus; RLV, Rauscher leukemia virus; PAS, periodic acid Schiff; $NaDodSO_4$, sodium dodecyl sulfate; P_i/NaCl, phosphate-buffered saline.

β-mannosidase [166], α-glucosidase [3.2], β-glucosidase [20], α-galactosidase [24], β-galactosidase [125], α-L-fucosidase [58], β-xylosidase [12], α-*N*-acetylglucosaminidase [2.5], β-*N*-acetylglycosaminidase [383], α-*N*-acetylgalactosaminidase [46], and β-*N*-acetylgalactosaminidase [30] (from *Turbo cornutus*) (Seikagaku Kogyo Co., Tokyo, Japan).

Virus. MMTV (RIII) was purified from the milk of RIII mice, MMTV (C3H) was obtained from cultured fluids of the MM5T mouse mammary tumor cell line, and RLV was purified from the plasma of RLV-infected BALB/c mice, as described (15).

Preparation of Purified Viral Components. Isopycnically banded and pelleted virions were resuspended (approximately 10 mg of viral protein per ml) in 0.1 M phosphate-buffered saline (P_i/NaCl) and solubilized at 0°C for 15 min by the addition of KCl (to 0.4 M), dithiothreitol (to 0.01 M), and urea (to 3 M). The sample, approximately 0.9 ml, was sonicated four times for 15 sec at 50 watts at 30-sec intervals at 0°C and applied to a 0.9 × 56 cm column of polyacrylamide agarose gel (Ultrogel AcA34) equilibrated with 3 M urea, 0.1 M P_i/NaCl (pH 7.8), and 2 mM dithiothreitol. The column was eluted with the same buffer at a flow rate of 2 ml/hr. The column effluent was monitored by absorption at 280 nm and sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis as described (16). gp52 from MMTV or gp69 from RLV fractions was pooled, dialyzed against 0.1 M P_i/NaCl, and rechromatographed on a column equilibrated with 0.1 M P_i/NaCl buffer lacking urea. Fractions (0.4 ml) were collected and monitored as above.

Internal Labeling of Virus. C3H mouse cell cultures were grown in Eagle's minimum essential medium with 3 μg of dexamethasone containing 50 μCi (1 Ci = 3.7 × 10^{10} becquerels) of D-[6-^{3}H]glucosamine (38 Ci/mmol, Amersham, England) per ml. Virus was also labeled with leucine-free medium containing 25 μCi of L-[4,5-^{3}H]leucine (48 Ci/mmol, Amersham) per ml. Virions were harvested every 24 hr over a period of 2 days from medium containing radioisotope. Medium lacking [^{3}H]leucine was then added and virus was collected for an additional 24 hr. MMTV was concentrated and purified as described (16) by pelleting and subsequent centrifugation in discontinuous sucrose gradients.

External Labeling of Virus and Viral Components. Gradient-purified MMTV was disrupted with 0.2% Triton X-100 (New England Nuclear) and externally labeled with ^{125}I (New England Nuclear) by using the chloramine-T method (17).

Purified gp52 and sugar-free gp52 from MMTV was iodinated with *N*-succinimidyl 3-(4-hydroxy-3-[^{125}I]iodophenyl)-propionate (Bolton and Hunter Reagent) (1860 Ci/mmol, Amersham), followed by separation on a column of polyacrylamide/agarose gel (Ultrogel AcA 34, LKB). Iodination and purification was performed according to the procedure of Bolton and Hunter (18) as described (16).

Treatment of Viral Components with Mixed Glycosidases. MMTV gp52 (500 μg) or RLV gp69 (300 μg) was incubated with 50 μg (for gp52) or 30 μg (for gp69) of the mixed glycosidases for 16 hr at 37°C in 0.05 M sodium citrate buffer, pH 4.0. [^{3}H]Glucosamine-labeled MMTV was mixed with cold MMTV to a total of 100 μg of viral protein, then 10 μg of the mixed glycosidases was added in the same buffer containing 0.2% Triton X-100, and the mixture was incubated as above.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ was performed by a modification of the method of Shapiro *et al.* (19). Gels containing 7.5% polyacrylamide plus 0.375% methylene bisacrylamide or 5% acrylamide plus 0.25% methylene bisacrylamide, 0.5% $NaDodSO_4$, and 0.1 M sodium phosphate (pH 7.6) were used. Protein was detected by staining with Coomassie brilliant blue (0.25%) in 7% acetic acid and 50% methanol. For polysaccharide staining, the periodic acid Schiff (PAS) reaction was employed according to the procedure of Zacharius and Zell (20). When the gels were assayed for ^{3}H radioactivity, they were sliced in 2-mm segments and dissolved in 5 ml of 3% Protosol containing Econofluor (New England Nuclear) by shaking overnight at 37°C. ^{125}I radioactivity was determined by counting in a gamma counter (Searle). In each case, the radioactive samples were electrophoresed with unlabeled MMTV, and the gel was split and stained with Coomassie brilliant blue and PAS to determine the position of the viral proteins and glycoproteins. The relative migration of the labeled peaks was determined with reference to known viral component markers.

Measurements of Carbohydrate and Protein Contents. Except for a few modifications, estimation of the carbohydrate content of viral components was performed according to the procedure of Krystal and Graham (21). For the standard ferricyanide assay, 100 μl of reaction mixture contained 20 μl of 1:1 diluted ferricyanide reagent (1.25 μg of potassium ferricyanide/70 μg of K_2HPO_4/2.1 μg of K_2PO_4) and varying amounts of sample sugar or hydrolyzed protein dissolved in distilled water. The reactions were carried out at 100°C for 10 min and then cooled by immersion in an ice bath. Ice cold distilled water (400 μl) was added and the absorbance at 237 nm was measured. The sugars α-D-glucose, β-D-glucose, D-galactose, D-glucosamine, and D-mannose (Calbiochem) were dissolved in glass-distilled water each at 10 mg/ml as a standard stock solution. Protein was determined by the Lowry procedure (22).

Absorption of Antisera. Rabbit anti-MMTV (RIII) purified total IgG fraction was prepared and absorbed with the following soluble immunoabsorbants, as described (12, 13), by using the indicated quantities per each 0.5 ml containing 25 μg of total IgG: (*i*) gp52 from MMTV (20 μg); (*ii*) deglycosylated gp52 (25 μg); (*iii*) isolated sugar moiety from gp52 (3 μg); (*iv*) gp69 from RLV (30 μg); (*v*) mixed-glycosidase-treated gp69 (30 μg); (*vi*) isolated sugar moiety from gp69 (2 μg). The amounts of absorbants used were determined by our previous experience (12, 13) with these antibody preparations. The carbohydrate moieties were used in excess (1.5-fold) to obviate the possibility of incomplete absorption.

Immunohistochemical Staining. Adjacent serial sections (5 μm) were cut from paraffin blocks of four human breast carcinomas in which the gp52 related antigen had previously been localized and the specificity of the staining reaction confirmed by immune absorption studies. These were deparaffi-

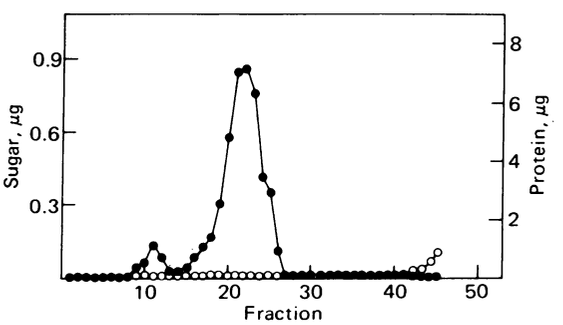

FIG. 1. Gel filtration profile of viral components. Purified gp52 (400 μg) was treated with proteinase K (50 μg) for 16 hr at 37°C. Cold trichoroacetic acid was added to the sample to a final concentration of 20%, and the mixture was incubated at 0°C for 15 min. The enzyme-treated sample was centrifuged, and the supernatant fraction was neutralized and then fractionated through the Sephadex G-50 column (0.7 cm × 28 cm). Aliquots of each column fraction were assayed for carbohydrate (20 μl) (●) and for protein (25 μl) (○) contents.

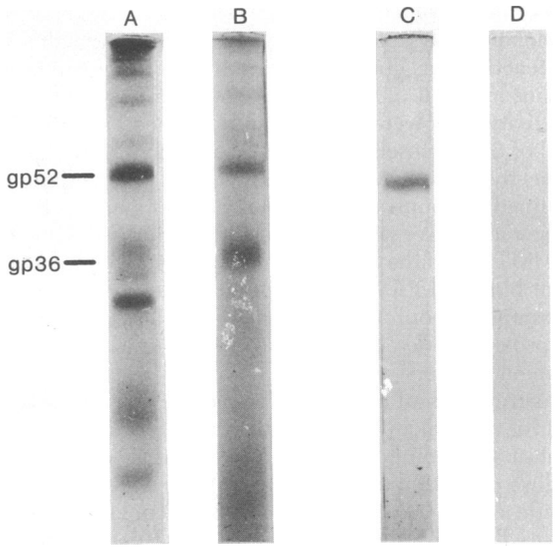

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis of glycosidase-treated gp52. Purified gp52 was incubated with glycosidase and electrophoresed on $NaDodSO_4$/7.5% polyacrylamide gels. The control MMTV was electrophoresed on a separate gel. The gels were sliced longitudinally and stained with Coomassie brilliant blue (protein) and PAS (carbohydrate). Gels: A, MMTV stained with Coomassie brilliant blue; B, MMTV stained with PAS; C, glycosidase-treated gp52 stained with Coomassie brilliant blue; D, glycosidase-treated gp52 stained with PAS.

nized and stained with the unabsorbed and absorbed antibody preparations by using an indirect immunoperoxidase technique (12, 13). The staining reaction after the specific absorptions described above was always compared to that seen in the adjacent serial section treated with unabsorbed antibody in which essentially the same cells could be identified.

RESULTS

Preparation of Sugar Moiety from Viral Glycoproteins. Samples of purified gp52 from MMTV and gp69 RLV were each divided into two aliquots containing 400 μg of protein, and 10 μl of proteinase K (5 mg/ml) in 0.1 M P_i/NaCl was added to one of each pair and a similar amount of buffered saline was added to the other to serve as controls.

A 400-μg aliquot in 300 μl of 0.1 M P_i/NaCl was incubated with 50 μg of proteinase K for 16 hr at 37°C. Cold trichloroacetic acid was then added to a final concentration of 20%, and the mixture was incubated at 0°C for 15 min and then centrifuged at 11,000 × *g* for 20 min at 4°C. The supernatant was neutralized with 6 M NaOH and fractionated through a Sephadex G-50 column. Fractions were assayed for carbohydrate and protein. Fig. 1 shows the profile of the carbohydrate and protein contents of the fractionated proteinase K-treated gp52. The bulk (37 μg) of the carbohydrate moiety was recovered in fractions 17–26 and this corresponds to better than 90% of the starting material. It is further apparent that no protein can be detected in the region of the carbohydrate and for many fractions beyond.

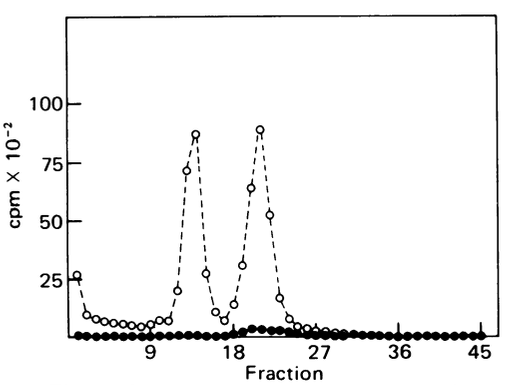

FIG. 3. Electrophoretic pattern of effect of mixed glycosidase treatment on [^{3}H]glucosamine-labeled gp52 and gp36. Labeled MMTV was solubilized by the addition of KCl (to 0.4 M), Triton X-100 (to 0.02%), and dithiothreitol (to 0.01 M) and disrupted by sonication (four times for 15 sec at 0°C). The sample was dialyzed against 0.05 M sodium citrate buffer, pH 4.0, and treated with mixed glycosidases (●), and a corresponding amount was similarly incubated but without enzymes (○). Thereafter, each sample was electrophoresed on a $NaDodSO_4$/7.5% polyacrylamide gel.

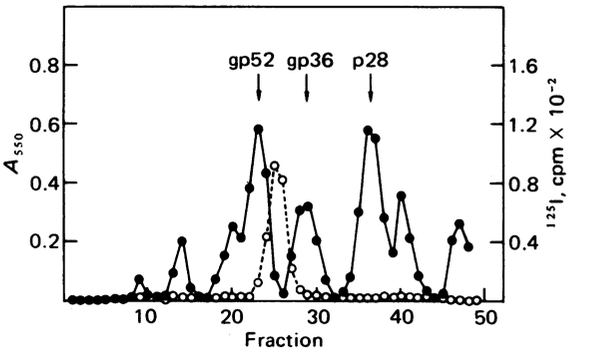

FIG. 4. Analysis of glycosidase-treated MMTV gp52. Purified gp52 was treated with mixed glycosidases, externally labeled with ^{125}I (Bolton and Hunter Reagent), and purified by gel chromatography (Ultrogel AcA 34). The sample was mixed with 100 μg of MMTV and electrophoresed on a $NaDodSO_4$/5% polyacrylamide gel. Prior to analysis of radioactivity, the gel was stained with Coomassie brilliant blue (●). Thereafter, the gel was sliced in 2-mm sections for determination of radioactivity (○).

Preparation of Deglycosylated Glycoproteins. The enzymatic removal of the sugars from the glycoproteins of MMTV and from purified gp52 is illustrated in Fig. 2 by the responses of the relevant bands to the protein (Coomassie blue) and carbohydrate (PAS) detecting stains. Gels A and B show the proteins and glycoproteins of MMTV prior to treatment with the glycosidases. Gel C shows that the deglycosylated purified gp52 moves somewhat faster than its untreated counterpart shown in A and B. That the glycosidase treatment has effectively removed the sugar residues is indicated by the absence of a detectable band with PAS staining in gel D. [^{3}H]Glucosamine-labeled MMTV was employed to provide a more quantitative assessment of the extent of the carbohydrate removal. As shown in Fig. 3, the majority of the label appears in the two peaks corresponding to gp52 and gp36. It is clear from the profile of

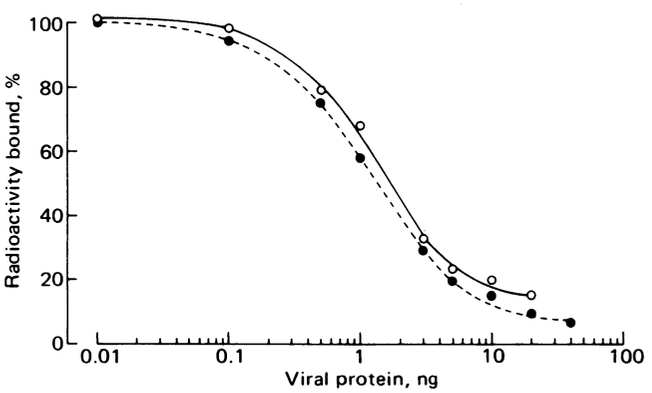

FIG. 5. Competitive radioimmunoassay for purified MMTV gp52 and deglycosylated gp52. Competitive radioimmunoassay for MMTV gp52 was carried out with rabbit anti-gp52 (1:5000 final dilution in the assay) and MMTV ^{125}I-labeled gp52 as described (16). Purified MMTV gp52 (○) and deglycosylated gp52 (●) were competitors.

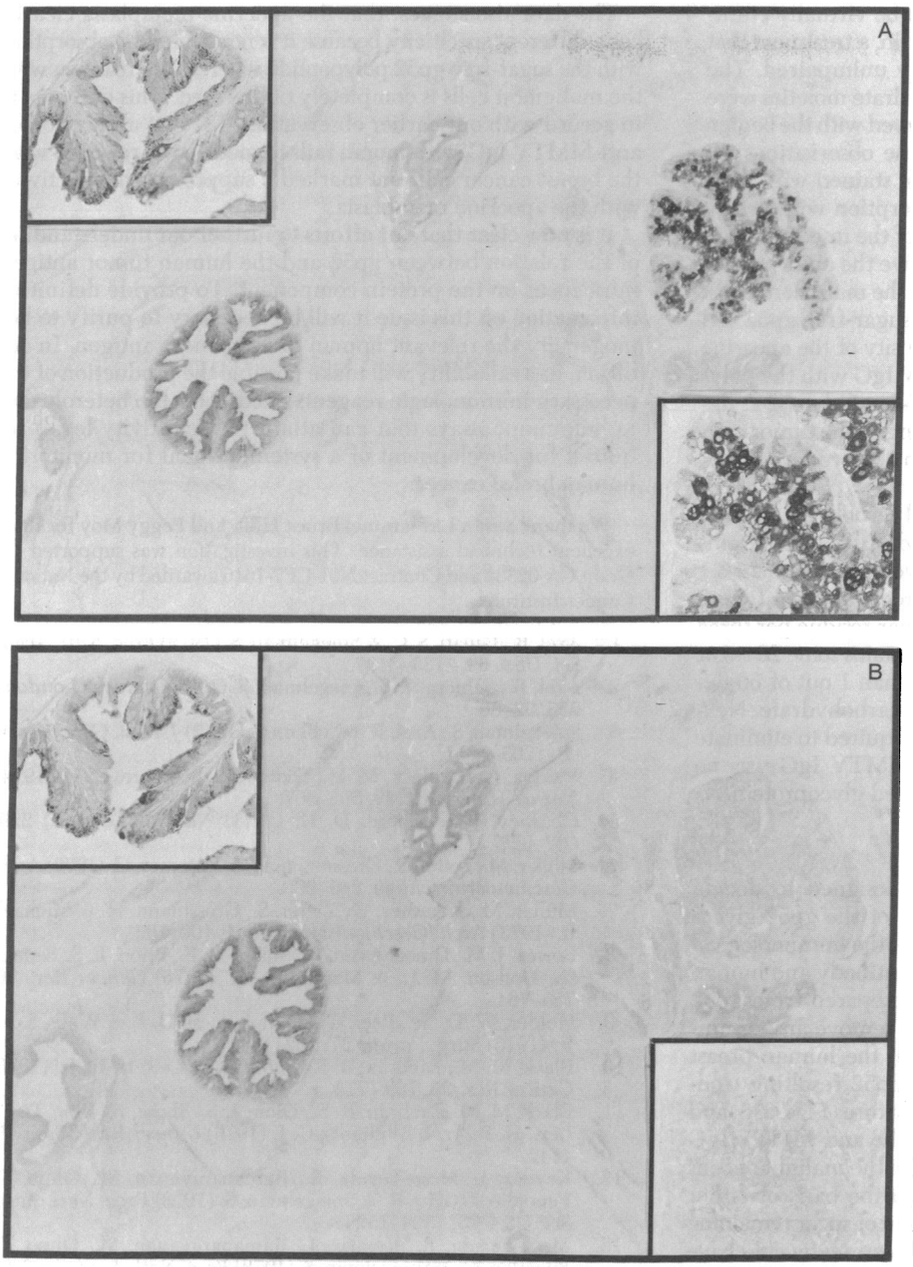

FIG. 6. (*A*) Immunoperoxidase stain with anti-MMTV of cross section of nipple duct from a case of intraductal and invasive carcinoma of the breast showing one duct involved by *in situ* carcinoma (right) and another with apocrine metaplasia (left). (*Insets*) Details of the above, showing considerable intracellular reaction product in the neoplastic cells and characteristic intracellular granular staining in the apocrine epithelium. (Methylene blue counterstain, ×75; *Insets*, ×180.) (*B*) Immunoperoxidase stain of adjacent serial section using anti-MMTV previously absorbed with sugar-free gp52. Note complete absence of reaction product in the neoplastic cells, whereas the intensity of the staining reaction in the apocrine epithelium is essentially the same as in *A*. (Methylene blue counterstain, ×75; *Insets*, ×180.)

the mixed-glycosidase-treated sample that exposure of the labeled viral proteins to mixed glycosidases removes more than 90% of the glucosamine-labeled material from gp52 and gp36.

The position of the deglycosylated band in gel C of Fig. 2 indicates that the removal of the sugars from gp52 results in a protein that moves somewhat faster than intact gp52. To obtain a more accurate estimate of molecular weight, deglycosylated gp52 was iodinated with ^{125}I and coelectrophoresed with the total proteins of MMTV. From its relative position to the other proteins (Fig. 4), the iodinated deglycosylated gp52 appears to migrate as a protein of about 48,000 daltons.

Immunologic Competence of Deglycosylated gp52 as Measured by Radioimmunoassay. The immunologic reactivity of the deglycosylated gp52 was first examined in a standard radioimmunoassay (16) in which it was challenged to compete with ^{125}I-labeled intact gp52. The titration curve exhibited in Fig. 5 shows that removal of the sugar residues from gp52 does not detectably alter its capacity to react with the relevant antibody. The reverse titration with ^{125}I-labeled sugar-free gp52 competing with unlabeled intact gp52 gave similar results (data not shown).

Ability of Deglycosylated gp52 to Eliminate the Immunohistochemical Staining of Human Breast Cancer Sections by Anti-MMTV IgG. We now come to the key issue of the present investigation, which centers on the question of whether removal of the sugar residues from gp52 interferes in any way with its ability to block the specific antibody from anti-MMTV IgG responsible for the immunohistochemical staining of human breast cancer sections (13). Four clearly positive tumors were selected for testing and the results with all were satisfyingly clear-cut. The deglycosylated gp52 was as effective in removing the immunohistochemical reaction as was absorption with untreated gp52. The similar outcomes are exemplified in Fig. 6 by a uniquely informative instance. We have here a section from a case with intraductal and invasive breast carcinoma which shows a nipple duct involved with lobular carcinoma *in situ* and a nearby duct exhibiting apocrine metaplasia, a benign microscopic component of cystic disease that we have earlier reported (13) to stain with anti-MMTV IgG. This combination of malignant and benign lesions in the same section was of particular interest to us. We found (13) that the staining

reaction of the apocrine metaplasia could be virtually eliminated by absorbing the anti-serum with mucin, a treatment that left the tumor cell reaction comparatively unimpaired. The results suggested that antibodies to carbohydrate moieties were principally responsible for the staining observed with the benign lesion, a suggestion fully confirmed by the observations we describe here. Fig. 6 compares the section stained with anti-MMTV IgG before (*A*) and after (*B*) absorption with deglycosylated gp52. The higher magnification of the insets provides an even better appreciation of how effective the absorption is in eliminating the staining reactions from the malignant cells. Note, however, that absorption with the sugar-free gp52 did not appreciably decrease the staining intensity of the apocrine metaplasia. Absorption of the anti-MMTV IgG with the polysaccharide isolated from the proteinase K-treated gp52 as described in Fig. 1 failed to affect the staining of the tumor cells but decreased the intensity of the reaction observed with apocrine metaplasia.

The numerology of the possible residual carbohydrate in the deglycosylated gp52 may be briefly noted. Fifty micrograms of the glycosidase-treated gp52 was assayed and less than 0.1 μg of sugar, the lower limit of the assay procedure, was found. This would correspond to less than one sugar residue per three polypeptide chains. Because intact gp52 contains some 20 sugar residues per polypeptide chain (23), less than 1 out of 60 deglycosylated chains could have the entire carbohydrate. Nevertheless, the amount of sugar-free gp52 required to eliminate the tumor-specific antibody from anti-MMTV IgG was no different from that observed with untreated glycoprotein.

DISCUSSION

The experiments described here were designed to decide whether the sugar or the protein moiety of the gp52 glycoprotein is responsible for its ability to block the immunological reaction observed between anti-MMTV antibody and human breast cancer cells. The polysaccharide prepared from gp52 with proteinase K (Fig. 1) was unable to remove any immunohistochemical reactivity observed with the human breast cancer cells. In contrast, the sugar-free gp52 resulting from treatment with the glycosidase enzyme mixture (Figs. 2, 3, and 4) did absorb out those antibodies from the anti-MMTV IgG that were responsible for the reaction with the malignant cells in sections of human breast cancers. On the basis of either chemical or radioactive analysis, the amount of sugar remaining per polypeptide strand after glycosidase treatment would have corresponded to less than one sugar molecule for every three protein molecules. Consequently, less than 1 out of 60 polypeptide strands could have had a complete carbohydrate polysaccharide complex. Nevertheless on a weight basis, the sugar-free protein was fully as effective in removing the relevant antibodies as the original intact glycoprotein (Fig. 6). The radioimmunoassay titration curve of Fig. 5 also demonstrates that removal of the sugars from gp52 does not influence its ability to compete with intact gp52 for the relevant antibodies.

The fact that it is the polypeptide rather than the polysaccharide portion of the gp52 that is responsible for the immunological reactivity between the human antigen and antibodies against mouse gp52 adds additional weight to the biological implications of the similarities that exist between the human and murine mammary neoplasias. At the very least, these results indicate that the immunological interrelationship between the human tumor antigen and gp52 is more than a chance correspondence of polysaccharide complexes.

The data also suggest that the apocrine metaplasia clearly has a different specificity because it is not altered by absorption with the sugar-free gp52 polypeptide whereas the reaction with the malignant cells is completely obliterated. This outcome is in accord with our earlier observation (13) that absorption of anti-MMTV IgG with mucin fails to modify the reaction with the breast cancer cells but markedly suppresses the reactivity with the apocrine metaplasia.

It is now clear that our efforts to further our understanding of the relation between gp52 and the human tumor antigen must focus on the protein component. To provide definitive information on this issue it will be necessary to purify to homogeneity the relevant human breast cancer antigen. In addition, its availability will make possible the production of the necessary immunologic reagents needed for the heterologous radioimmunoassays that can attain the sensitivity levels required for development of a systemic signal for monitoring human breast cancer.

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