Radioimmunoassay for Protein p28 of Murine Mammary Tumor Virus in Organs and Serum of Mice and Search for Related Antigens in Human Sera and Breast Cancer Extracts

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ABSTRACT

The main protein of the core of murine mammary tumor virus, with a molecular weight of 28,000 (p28), was solubilized by deoxycholate treatment of the virus and purified by Ultrogel ACA-54 filtration and hydroxyapatite chromatography. This protein was used as labeled antigen in a highly specific and reproducible radioimmunoassay. Organ extracts of uninfected C57BL mice did not contain p28, but organ extracts of infected RIII mice did contain the antigen. Despite the high content in the mammary gland, the level of p28 in the other organs was identical in male and female mice. Sera of uninfected mice and the majority of the sera of infected mice did not contain the antigen. The investigation included 338 human sera (50, normal; 157, breast cancer; 77, polycystic disease; 32, benign mastopathy; 12, fibroadenoma; 10, at risk of developing breast cancer). None contained an antigen related to p28. Eight of 24 extracts of human breast cancer gave results that appeared weakly positive, possibly as a result of proteolysis. Extracts of healthy breast tissue and the serum from the breast arterial and venous blood of corresponding patients were negative.

INTRODUCTION

We have described previously a radioimmunoassay for gp47, the main envelope glycoprotein of MuMTV (2, 4), and have confirmed this antigen to be a valuable marker of viral expression (15). To gain additional information on the biology of the virus in the mouse, we have now worked out a radioimmunoassay for p28, the main core protein of MuMTV (2, 4), in a attempt to detect the latter polypeptide in organ extracts and serum from mice.

The observations that a protein with a molecular weight of 27,000 was described in "virus cores" prepared from the gland, the level of p28 in the other organs was identical in male and female mice. Sera of uninfected mice and the majority of the sera of infected mice did not contain the antigen. The investigation included 338 human sera (50, normal; 157, breast cancer; 77, polycystic disease; 32, benign mastopathy; 12, fibroadenoma; 10, at risk of developing breast cancer). None contained an antigen related to p28. Eight of 24 extracts of human breast cancer gave results that appeared weakly positive, possibly as a result of proteolysis. Extracts of healthy breast tissue and the serum from the breast arterial and venous blood of corresponding patients were negative.

MATERIALS AND METHODS

Animal and Virus

RIII, Swiss albino, and C57BL mice and Fischer rats were used (15). MuMTV was purified from the milk of Swiss albino mice (4).

MuMTV Antigens

p28: purified MuMTV [12 ml; protein (2 mg/ml) in 0.02 M ammonium acetate] was diluted 4 times with Tris-maleate buffer (0.025 M Tris:0.025 M maleic acid adjusted to pH 7.4 with sodium hydroxide) containing sodium deoxycholate (0.5 mg/ml). The resulting suspension was left for 15 min at room temperature and then centrifuged (Spinco SW 27 rotor; 26,000 rpm; 1 hr; 20°). The supernatant, concentrated in a dialysis bag with Aquacid, was submitted to gel filtration on a 1.5- x 33-cm column of Ultrogel ACA-54 (LKB, Bromma, Sweden). The filtration was carried out at 0.3 ml/hr with 25 mM P¡, pH 7.4 (Sörensen's), containing sodium deoxycholate (0.01 mg/ml), sodium azide (0.1 mg/ml), and 0.5 mM dithiothreitol. Fractions (0.9 ml each) containing p28 (after monitoring by SDS-PGE) were pooled and diluted in 2 volumes of distilled water. The pool was adsorbed onto a 0.6- x 10-cm hydroxyapatite column and then eluted with 100 ml of a 0.02 M Sörensen's phosphate buffer gradient, pH 7.4, containing 0.5 mM dithiothreitol at 30 ml/hr. Fractions (0.8 ml each) containing p28 (after monitoring by SDS-PGE) were pooled, concentrated in dialysis tubing with Aquacid, dialyzed for 1 hr against distilled water, and stored at -70°. The preparation of the other peptides was described previously (15).

Protein Analysis

SDS-PGE was carried out in 12.5% acrylamide (4). The calibration proteins were bovine serum albumin (M.W.
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68,000; Boehringer Mannheim, Mannheim, Germany), phosphocreatine kinase (M.W. 40,000; Sigma Chemical Co., St. Louis, Mo.), and soybean trypsin inhibitor (M.W. 21,500; Boehringer Mannheim).

Protein concentration was determined according to the method of Lowry et al. (6).

Rabbit Antisera

Anti-p28 Antisera. Polypeptide p28 (120 μg) was diluted in 1 ml of 0.12 M sodium chloride and emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Inc., Detroit, Mich.). Sixty μg were injected intradermally into rabbits on the 1st and 45th days of the immunization. The animals were bled 2 weeks after the second injection (14). These antisera will be referred to as Anti-p28 Antisera 2 and 3.

Anti-gp47 Antisera 2 and 3, Anti-p10 Antiserum and Anti-p8 Antiserum. These were obtained by the same technique except for the last one, in which case the rabbit was given only 1 injection.

Other antisera were described earlier (15).

Other Sera

Human Sera. Human sera were collected from 50 normal individuals and from 245 women in the following groups: 32 with various benign mastopathy; 10 considered to be at risk of developing breast cancer; 12 with fibroadenoma; 77 with polycystic disease; and 114 with breast cancer. The venous blood was also collected from 21 breast cancers, and the arterial blood from 22 breasts was collected during breast cancer surgical removal.

Mouse Sera. Mouse sera were collected from Rill, Swiss albino, and C57BL mice.

Organ Extracts

Organ Extracts from Mice and Rats. They were the same as those used for the detection of gp47 (15).

Human Breast Cancer Extracts. The ground tissues were extracted with 3 M potassium chloride as described for mouse organ extracts (15).

Amniotic Fluids. Amniotic fluids were collected from mice that were 19 days pregnant.

Radioimmunoassay

Iodination procedure and detection techniques were carried out as previously described for gp47 (15).

RESULTS

Preparation of Antigen p28. When carried out as described in "Materials and Methods," treatment of MuMTV by sodium deoxycholate solubilized mainly 4 polypeptides: glycoprotein with a molecular weight of 72,000, gp47, p28, and a protein with a molecular weight of 8,000 together with a small amount of a glycoprotein with a molecular weight of 31,000 (Chart 1). Filtration-chromatography on Ultrogel ACA-54 resolved the proteins in 3 large peaks (Chart 2A). All fractions of Peak 2 containing p28 eventually contaminated with some amounts of gp47 (Chart 3A) were chromatographed on hydroxyapatite. The elution pattern showed 3 peaks (Chart 2B). The first did not contain protein; the second, eluted around 85 mM P1, contained gp47; and the third, eluted around 300 mM P1, contained p28. Fractions containing p28 were pooled, concentrated, and dialyzed, resulting in a preparation of 99% pure p28 as judged by SDS-PGE (Chart 3B). Reproducibility of the technique was excellent, and 4 batches of p28 (0.5 mg) were pooled for radioimmunoassays.

Preparation of Labeled p28. Iodination was carried out with chloramine-T (15). The protein-specific activity was 50 μCi/μg. The fraction of the eluate corresponding to the maximum radioactivity was filtered on Sephadex G-100, and the main peak thus obtained was used in immunological reactions.

Assay of Different Antisera (Chart 4). Fixation of p28 by
the different antisera varied from one antiserum to another. Anti-p28 Antiserum 2 and 3 fixed p28 following a dilution curve such that 50% fixation was obtained at dilutions of 1:3,000 and 1:50,000, respectively; when they were diluted 1:500 they fixed, respectively, 74 and 81% of the labeled antigen. Anti-gp47 Antiserum 2 and 3 diluted 1:100 fixed 59 and 54% of the antigen, respectively. Anti-protein with a molecular weight of 8,000 antiserum diluted 1:500 fixed 13.5% of the label. Anti-p10 antiserum did not fix p28 (Chart 4A).

Anti-p28 Antiserum 1 fixed 50% of the labeled p28 when diluted 1:500. Anti-crude virus Antiserum 2 fixed p28 following a dilution curve such that 50% fixation was obtained with a dilution of 1:250. At the same dilution of 1:100, anti-crude virus Antiserum 1 fixed 37%, anti-gp47 Antiserum 1 fixed 21%, and anti-glycoprotein with a molecular weight of 160,000 [also designated as gp160(12)] Antiserum fixed 11% of the labeled antigen. Anti-MFGM antiserum did not fix p28 (Chart 4B).

**Standardization of the Radioimmunoassay (Chart 5).** Anti-p28 Antiserum 3 was chosen for the assay. It was used diluted 1:150,000 so as to obtain 25% fixation of the labeled antigen. The nonspecific fixation of p28 to the (rabbit antibodies plus anti-rabbit IgG sheep antibodies) complexes in the absence of anti-p28 antibodies amounted to 2%. Chart 5 shows a typical standard curve as obtained under our experimental conditions. The sensitivity of the assay calculated on the basis of 2 S.D. of B0 varied between 0.3 and 0.7 ng/ml. Amounts of antigen smaller than 0.8 ng/ml were considered too low for significant detection. The S.E. calculated for each value of the unlabeled p28 utilized in the reference curve ranged from 1 to 10%. The interassay coefficient of variation was between 10 and 15%.

**Specificity of the Radioimmunoassay (Chart 5).** Five other proteins isolated from purified MuMTV did not inhibit the reaction between labeled p28 and anti-p28 Antiserum 3.

Inhibition curves were obtained with a series of dilutions of p28-containing tissue extracts, 1 extract from a mammary tumor of a Swiss mouse, and 1 extract of the mammary gland from a pregnant Swiss mouse. These curves were parallel to that obtained with purified p28, indicating the presence of the same antigen.

**Detection of p28 in the Organs of Animals.** Organ extracts of Fischer rats and C57BL mice did not contain detectable amounts of p28 (Table 1). By contrast, organ extracts of RIII mice contained significant quantities of p28. The relative content in different organs varied widely. The higher values were observed in the mammary glands and MFGM and next, although roughly 10 times less, in brain and spleen. Kidneys, lungs, and heart contained the lowest quantities of antigen. The results indicate that both sexes are infected.

Large amounts of p28 were found in mammary tumors of Swiss mice (Table 2) but differed widely from one extract to another.

None of the 10 amniotic fluids tested contained p28. Detection of p28 in the Serum of Mice. The presence of p28 was investigated in sera from tumor-bearing and normal female mice and from males of various strains (Tables 2 and 3). Only 3 sera contained p28. One was the serum of a mouse with necrotic and purulent tumor; the 2 others were collected from pregnant females.

Search for p28 in Samples from Humans. None of the 50 sera from normal individuals contained detectable amounts of p28 or related antigens, and neither did the 245 sera from women with various breast diseases.

The 15 cyst fluids and the 12 milks (skimmed by low-
Radioimmunoassays with MuMTV p28

None of the other proteins of MuMTV thus far tested inhibited the reaction between labeled p28 and anti-p28 at dilutions that gave clear-cut reaction when comparable anti-p28 antiserum was used.

It might be inferred from the results obtained with antiviral virus Antiserum 2 that the virus preparation used to immunize the rabbit contained immunogenic amounts of p28. Nevertheless, the response obtained with the virus core antigen was much lower than that obtained with gp47 from the virus envelope (15); the titers corresponding to a 50% fixation of the antigens were 1:250 and 1:4000, respectively. Conversely, MFGM preparations used to immunize the rabbit did not contain immunogenic amounts of p28.

Antigen p28 appeared as serologically unrelated to glycoprotein with a molecular weight of 160,000, protein with a molecular weight of 56,000, and p10. It is probably not related to gp47, although anti-gp47 Antisera 2 and 3 at high concentrations reacted slightly with the labeled antigen. Immunoelectrophoresis (unpublished data) showed that this could be due to the presence of small amounts of gp47.

**Table 1**

<table>
<thead>
<tr>
<th>Organs</th>
<th>Concentration of p28 (ng/mg protein)</th>
<th>RIII mouse</th>
<th>C57BL mice (both sexes)</th>
<th>Fischer rats (both sexes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>ND b</td>
<td>0.4</td>
<td>BD</td>
<td>BD</td>
</tr>
<tr>
<td>Heart</td>
<td>ND</td>
<td>2.1</td>
<td>BD</td>
<td>BD</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.8</td>
<td>0.5</td>
<td>BD</td>
<td>BD</td>
</tr>
<tr>
<td>Spleen</td>
<td>5.1</td>
<td>20.1</td>
<td>BD</td>
<td>BD</td>
</tr>
<tr>
<td>Brain</td>
<td>ND</td>
<td>11.1</td>
<td>BD</td>
<td>BD</td>
</tr>
<tr>
<td>Seminal vesicles</td>
<td>BD</td>
<td>M</td>
<td>BD</td>
<td>M</td>
</tr>
<tr>
<td>Mammary glands</td>
<td>ND</td>
<td>ND</td>
<td>F</td>
<td>BD</td>
</tr>
<tr>
<td>Lactating mammary glands</td>
<td>861.0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MFGM</td>
<td>250.0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Pool of organs of 5 mice of each sex or of 2 rats.
b ND, not done; BD, below detection level.

speed centrifugation) investigated were devoid of p28.

Eight of 24 breast cancer extracts were weakly positive, but further analysis indicated that proteolysis might be responsible for the majority of these results. None of the 16 extracts available from noncancerous breast tissue obtained from corresponding patients contained any p28-related antigen. The 21 venous blood samples from the tumors and the 22 arterial blood samples collected from the breast during the surgical removal of the cancer were all devoid of detectable p28-related antigen.

All these human samples were tested for gp47 as described in a previous paper (15); none was positive.

**DISCUSSION**

We prepared the main core protein of MuMTV by the simple technique of rapid solubilization of p28 from purified virus followed by gel filtration and final purification on hydroxypatite. The p28 prepared by this procedure is pure as revealed by PGE analysis; moreover the radioimmunoassay and inhibition curves subsequently showed that the protein obtained is antigenically undenatured.

The first set of experiments carried out with labeled p28 demonstrated the specificity of the radioimmunoassay.

**Table 2**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Tumor extract (ng/mg protein)</th>
<th>Serum (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21.5</td>
<td>BD a</td>
</tr>
<tr>
<td>2</td>
<td>176.1</td>
<td>BD</td>
</tr>
<tr>
<td>3</td>
<td>124.4</td>
<td>BD</td>
</tr>
<tr>
<td>4</td>
<td>12.8</td>
<td>6.4</td>
</tr>
<tr>
<td>5</td>
<td>532.0</td>
<td>BD</td>
</tr>
<tr>
<td>6</td>
<td>33.8</td>
<td>BD</td>
</tr>
<tr>
<td>7</td>
<td>66.2</td>
<td>BD</td>
</tr>
<tr>
<td>8</td>
<td>11.9</td>
<td>BD</td>
</tr>
</tbody>
</table>

a BD, below detection level.
b Mean ± S.E.

**Table 3**

<table>
<thead>
<tr>
<th>Mice</th>
<th>No. tested</th>
<th>Concentration of p28 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swiss</td>
<td>15-29 days</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>15-29 days</td>
<td>F</td>
</tr>
<tr>
<td>First pregnancy</td>
<td>8-12 mos.</td>
<td>F</td>
</tr>
<tr>
<td>Females with mammary tumor</td>
<td>7 (0)</td>
<td>BD</td>
</tr>
<tr>
<td>RIII</td>
<td>F</td>
<td>3 (0)</td>
</tr>
<tr>
<td>Females with mammary tumor</td>
<td></td>
<td>F</td>
</tr>
<tr>
<td>C57BL Lactating</td>
<td>6 (0)</td>
<td>BD</td>
</tr>
</tbody>
</table>

a Numbers in parentheses, number of positives.
b BD, below detection level.
in some batches of p28. The latter technique also revealed that anti-gp47 antiserum did not react with p28, nor did anti-p28 antiserum react with gp47. All anti-p28 antiserum precipitated the labeled p28 much more efficiently than other sera did. Thus the apparent cross-reactions that were observed with concentrated sera are most probably due to nonspecific precipitation.

The fixation curves obtained with organ and tumor extracts showed that purified MuMTV p28, as far as antigenicity is concerned, was not significantly different from fresh p28 present in the extracts.

In brief, the assay is highly specific, its reproducibility is good, and this sensitivity compares well with that of the tests described for gp47 (10, 12, 15).

As far as we know, there has been only 1 report on the use of p28 in radioimmunoassays (9). It was claimed that this assay lacked sensitivity and that the antigen was unstable. Our results show the assay to be satisfactory and valuable for detection of virus expression. In our experience, p28 is fairly stable at -25°C but is somewhat less stable than is gp47; it is much more sensitive to proteolytic enzymes than is gp47.

Organ extracts of Fischer rats and C57BL mice, animals free of milk-transmitted MuMTV, do not contain p28. Conversely, RII male and female mice display detectable amounts of p28 in their organs. These data confirm our earlier results obtained with gp47 radioimmunoassay (15). Inasmuch as MuMTV is synthesized in the mammary gland and excreted in the milk, it is therefore not surprising to find large amounts of p28 in the lactating mammary gland; however, the finding of p28 in the MFGM was less expected. This might be due to a contamination of the MFGM preparation with some MuMTV particles as p28:gp47 is 1:40 in MFGM compared to 1:3 in the mammary gland (15). This latter ratio is closer to that calculated for the purified virus (4).

Most other organs that contained gp47 (15) in small amounts also contained detectable amounts of p28.

The limited data presented here show p28 to be present in mouse mammary tumor extracts, in 1 case at a concentration almost equal to that observed in lactating mammary gland of RII mice. This finding is not surprising inasmuch as the production of virus by mammary tumor cells is a well-known phenomenon (8).

Even in mice with mammary tumors, p28 could not be detected in the majority of the sera, a fact that is at variance with the results obtained with gp47 (15). This might reflect a cytoplasmic localization of p28 as opposed to the location of gp47 at the mammary cell membrane (2) and/or may result from a slower clearance by the liver of glycoproteins as opposed to proteins (1). In the 338 human sera tested, none was significantly positive, an expected result since the sera from the infected mice do not contain the antigen.

Preliminary studies with human mammary cyst fluid and excreted in the milk, it is therefore not surprising to detectable amounts in these tumors. Healthy breast tissue as well as afferent and efferent blood serum were devoid of activity. Nonetheless, further investigations are being carried out with human and animal samples as antibodies to MuMTV core were described in breast cancer patients (7) and as a protein with a molecular weight of 27,000 was isolated from "virus cores" in human milk (5). If similar viruses are responsible for mammary cancer in various species, one might find interspecies determinants common to the major core protein of all these agents. This can be sought since we now possess a sensitive radioimmunoassay.

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