Procedures for Radioimmunoassay of the Mouse Mammary Tumor Virus

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SUMMARY

A procedure for radioimmunoassay of the major glycoprotein antigen derived from murine mammary tumor virus is described. The assay is sensitive to 0.05 ng of antigen and is highly reproducible. The antigen, gp55, has been found to be group specific and will detect viruses in 13 separate mouse strains, as well as from continuous cell lines. Factors affecting the assay have been examined.

INTRODUCTION

For studies of the distribution and synthesis of the mouse MuMTV, we have found it necessary to detect small quantities of virus-related materials in a large background of unrelated materials. There have been several reports of radioimmunoassays for MuMTV, based either on whole (1) or disrupted (19) virus and purified components (11, 12). In our studies of these assay techniques, we have attempted to characterize the reaction in detail and to define the parameters for optimum sensitivity. We have concentrated on a single antigen assay, using a highly purified MuMTV glycoprotein, gp55. We have also investigated possible sources of errors in the assay and have obtained information that will, we feel, be of value to others who are developing such systems.

MATERIALS AND METHODS

The virus used in this study was obtained from the milk of females of various mouse strains at 3rd or greater parity and purified by a combination of rate zonal and isopycnic centrifugation (14). The purity of the virus was monitored by electron microscopy. One ml of milk yielded, on the average, 0.25 mg of virus protein. Specific saccharides were provided by L. Warren of the Wistar Institute, Philadelphia, Pa. The saccharides used were: a-methyl-D-galactoside; a-methyl-O-glucoside; methyl-\(\beta\)-D-xylopyranoside; methyl-\(\alpha\)-D-glucopyranoside; O-nitrophenyl-\(\beta\)-D-thiogalactoside; p-amino-nophenyl-\(\beta\)-D-thiogalactoside; and a-methyl-D-mannoside.

Purification of gp55 from RIII-MuMTV. The technique for the purification of gp55 has been described in detail (13). Briefly, density-banded virions were disrupted (3 mg viral protein/ml) in 0.2 M sodium phosphate buffer containing 0.01 M EDTA, 0.2% (v/v) \(\beta\)-mercaptoethanol, and 1.0% (v/v) Nonidet P-40. Before chromatography, the solubilized virus was diluted 10-fold with DEAE-cellulose buffer (0.22 M Tris-HCl, pH 7.2, 0.2% \(\beta\)-mercaptoethanol, 0.2% Nonidet P-40, and 30% glycerol) and applied to a DEAE-cellulose column equilibrated with the same buffer. Under these conditions, gp55 is not adsorbed to the column and is recovered in the application and wash fractions. For final purification of the highly enriched gp55 resulting from this step, the application and wash fractions were pooled, dialyzed overnight at 4° against 0.1 M ammonium acetate, concentrated by dialysis in a vacuum in collodion bags (No. 100; Carl Schleicher and Schuell, Keene, N. H.), and applied to a Sephadex G-100 column (2 x 34 cm) equilibrated with 0.1 M ammonium acetate. Effluent fractions were monitored with an LKB Uvicord (280 nm), and individual fractions were monitored by SDS-polyacrylamide gel electrophoresis. Finally, fractions containing only gp55 were pooled, lyophilized, and resuspended in sterile water.

Labeling Procedures. Purified MuMTV component gp55 was labeled with \(^{125}\)I by the Chloramine-T method (6) with some modifications. Initially, 5 \(\mu\)g of protein in 20 \(\mu\)l of 0.25 M phosphate buffer (pH 7.5) were mixed with 10 to 30 \(\mu\)l of \(^{125}\)I in NaOH at 0.1 mCi/\(\mu\)l. Ten \(\mu\)l of Chloramine-T (250 \(\mu\)g/ml) were then added and allowed to react with gentle mixing for 1 min, at which time 10 \(\mu\)l of sodium metabisulfite (750 \(\mu\)g/ml) and 50 \(\mu\)l of 0.1 M potassium iodide were added to terminate the reaction. The total reaction volume was diluted to 1.0 ml with 0.05 M phosphate buffer, pH 7.5, containing 0.25% gelatin and 0.5% Nonidet P-40 (radioimmune buffer), and dialyzed against several changes of a 10° excess of buffer. Upon removal from the dialysis bag, the activity was determined, and the material was diluted and separated into aliquots for storage at ~70°. Labeled material was dialyzed every 2 weeks to limit the effects of degradation. Purity and integrity of the protein preparations were monitored by SDS-polyacrylamide gel electrophoresis and radioautography. We generally obtained specific activity of 1 to 3 \(\times\) \(10^6\) cpm/ng.

Antisera. For most of the experiments, we used polyclonal antisera generated against isopycnically banded MuMTV extracted from the milk of RIII mice after their 3rd

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\[^{3}\] The abbreviations used are: MuMTV, murine mammary tumor virus; SDS, sodium dodecyl sulfate.

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litter or later. The sera were made by repeated i.m. inoculations of whole or ether-extracted RII virus emulsified in Freund’s complete adjuvant, into rabbits. Since these polyspecific sera reacted with C57 virus-free milk, absorption was necessary. Two sequential protocols were tested. For in vitro absorption, 1 volume of serum was mixed with 2 volumes of skimmed C57 milk (5 min at 250 × g) and incubated at 37° for 0.5 hr, after which it was centrifuged for 0.5 hr at 100,000 × g. One-half ml of in vitro absorbed serum was inoculated i.p. into C57 female mice, and the mice were exsanguinated after 18 hr. The resulting in vivo and in vitro absorbed serum, although diluted by passage through the mouse, still bears reactivity against MuMTV.

Immunological Procedures. The system used was a 2-step precipitation assay (15). An antibody dilution series was first performed with labeled antigens by combining 30,000 cpm of antigen in 100 μl of radioimmune buffer, 100 μl of serially diluted anti-MuMTV, and 100 μl of radioimmune buffer. The reaction mixture was agitated by inversion and incubated at 37° for 2 hr, after which 100 μl of appropriately diluted goat anti-rabbit immunoglobulin (Miles Laboratories, Elkhart, Ind.) were added to each tube, mixed, and incubated at 4° overnight. The immune precipitate was then centrifuged at 1000 × g in an International Model PR-6 centrifuge for 15 min, and 120 μl of the supernatant were withdrawn and counted. After determination of the 50% binding antibody dilution (with our current serum, 1:1350), competition inhibition assays were performed, substituting the competing antigen for the 100 μl radioimmune buffer. We compared direct competition experiments, in which labeled and test antigen were added simultaneously, with a binding site occupation assay, in which the test antigen was added to the antibody and incubated for 2 hr at 37°, after which labeled antigen was added for another 2 hr before adding the goat anti-rabbit sera. All antigen dilution series were performed in radioimmune buffer and were run in parallel with standardized amounts of unlabeled protein. Normal rabbit serum controls were run, as well as nonantigenic samples.

A major complication in developing this assay was the difficulty of pipetting constant volumes of the reagent solutions. The main reason for this was the presence of detergent, which caused the solution to wet the tips of most of the microliter pipetting devices, leading to significant variations. In order to obtain values which were accurate to 1%, it was necessary to use positive displacement pipetting systems. We found the Kimble Model D-73 dispenser satisfactory for dispensing reagents and the Scientific Manufacturing Industries (Emeryville, Calif.) Micro/pettor an excellent unit for sampling. Any system that used the displacement of a column of air was unsatisfactory.

Electrophoresis. During this study, several electrophoresis conditions were used. Acrylamide gel slabs (7.5%) were prepared using 0.1 M phosphate buffer (pH 7.8), 0.1% SDS, and 0.1% 2-mercaptoethanol, and run as described previously (16). Samples were dissociated in 0.01 M phosphate, pH 7.8, 1% SDS, and 1% 2-mercaptoethanol at 60° for 30 min. Gels were stained with Coomassie Blue G-250, as described previously.

Acrylamide gel slabs (10%) were prepared in an E-C vertical gel apparatus (EC Apparatus Corp., St. Petersburg, Fla.), using a modification of the methods described by Laemmli (9). The 10% separation gel buffer contained 0.375 M Tris (pH 8.8), 0.06 M HCl, and 0.1% SDS. The 3% stacking gel buffer contained 0.06 M Tris (pH 6.8), 0.06 M HCl, and 0.1% SDS. Samples were dissociated in 0.06 M Tris (pH 6.8), 0.06 M HCl, 2% SDS, and 5% 2-mercaptoethanol at 60° for 30 min, followed by the addition of bromphenol blue in 100% glycerol, to a final glycerol concentration of 10%. The electrode buffer contained 0.025 M Tris (pH 8.3), 0.192 M glycine, and 0.1% SDS. Gels were run at 200 V until the bromphenol blue had migrated 9 to 10 cm through the separation gel.

Slabs were fixed overnight in 7% acetic acid-5% methanol. Gels containing 125I-labeled protein were then rinsed in distilled water, dried in vacuo with heat onto Whatman No. 1 filter paper, and pressed with Kodak RP Royal-X-Omat film and exposed at −70° for an appropriate length of time.

Densitometry was performed with a Model EC910 densitometer (EC Apparatus Co.) with filters at 650 nm for Coomassie Blue-stained gels and radioautograms. Sensitivity settings were identical for matched gels.

RESULTS

Chart 1b shows a radioautogram of the iodinated gp55 after SDS-polyacrylamide gel electrophoresis compared with a Coomassie Blue-stained gel of the starting material (Chart 1a). Only a single labeled component is visible. An antibody precipitation curve is presented in Chart 2. The antiserum is able to precipitate greater than 80% of gp55. Immune precipitation of purified iodinated gp55 is not affected by either of the absorption protocols used.

Kinetics of Binding to Antibody. The time curve of association of antigen with antibody was determined by adding the goat anti-rabbit serum at various times after the start of the reaction. The curve is presented in Chart 3. It can be seen that 30% of the antigen is bound within 2 hr at a 1:4000 dilution, and 50% is bound at an antibody dilution of 1:800. This information was used in determining conditions for a binding site occupation assay in which the test antigen was added to the antibody and incubated before the addition of labeled antigen. For practical reasons, we allowed the test antigen to interact with the antibody for 2 hr before adding the labeled antigen for the same time period. Under these conditions, 50% of the labeled antigen would be bound to the antibody in the absence of competition. The results of such an assay, compared with a direct competition assay, are given in Chart 4. The binding site occupation assay is more sensitive than the other by a factor of 4.

Inhibition Curves. The inhibition of the precipitation of iodinated gp55 by disrupted virus and by purified gp55 can be seen in Chart 5. In this case, the curve appears simple with a linear region from 0.1 to 1.5 ng of gp55 and 0.5 to 10 ng of RII virus. It is significant that the amount of gp55 detected in the disrupted virion is less than predicted by Sarkar and Dion (13) who indicated that gp55 represents 27% of the total protein. In repeated experiments, a 10-fold excess of viral protein is required to displace gp55, indicating that gp55 is 10% of the virus. Murine Rauscher leukemia
Factors Affecting the Assay. The influence of KCl concentration on the immune precipitation reaction was determined. As shown in Chart 6, surprisingly low amounts of KCl caused significant displacement of antigen from the immune complex. This occurs with both antigen systems. In order for us to obtain meaningful results, we have found it necessary to maintain the salt concentration at or below 0.10 M. Otherwise, the salt-induced displacement would be interpreted as a false positive.
Glycerol, which is used as a stabilizing factor in virus protein purification procedures (4), also has a similar effect on the immune reaction (Chart 7). Presumably, this is due to additional viscosity which interferes either with successful centrifugation of the precipitate or reduces the collision frequency of the reactants.

Proteolytic release of counts from the labeled antigen is a possible source of false readings. This possibility remains even though the reaction mixture contains 2.5 mg of gelatin per ml which should overwhelm such activity. We have adopted 2 controls for proteolytic activity. First, the immune reaction mixture is resuspended and allowed to incubate for an additional 24 hr, and the supernatant is examined for additional release of material. Second, an aliquot of the supernatant is precipitated with 5% trichloroacetic acid to detect any soluble counts. With these tests we have not yet encountered any evidence of such activity in our samples.

Studies on the Nature of the Reactive Sites of gp55. In a recent publication (18), we discussed the sensitivity of iodinated gp55 to trypsin and demonstrated that up to 30% of the immune reactivity was sensitive to this protease, while the remaining 70% consisted of several antigenic fragments of molecular weights from 36,000 to 12,000 daltons. We have attempted further characterization of the reactivity of this antigen.

Attempts to inhibit the precipitation of iodinated gp55 with the saccharides listed under "Materials and Methods" in concentrations of up to 10 μg/ml were unsuccessful. Similarly, treatment of the antigen with β-galactosidase or galactose oxidase had no effect; neither did periodate treatment.

Cross-Reaction between Inbred Mouse Strains. We have compared the displacement of gp55 by MuMTV from several sources and observe essentially the same degree of displacement for all virus samples tested. However, the relative content of gp55 varies extensively, as can be seen in Chart 8. The data are summarized in Table 1. It appears from these data that gp55 is a group-specific antigen, confirming the observations of others (8, 10, 15), but that conversion from antigen content to virus content may not be strictly linear. In fact, the specific gp55 content of various "purified" virus samples may vary over a 10-fold range. This may be due to a combination of factors. Electron microscope observations in this laboratory have demonstrated a considerable variation in the virion:nonvirion material ratio in preparations of virus from different sources. Studies are now in progress to determine whether the degree of impurity as determined by

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Table 1

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Protein recovered (mg/ml milk)</th>
<th>μg gp55/mg protein</th>
<th>Total gp55 recovered (μg/ml milk)</th>
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<tr>
<td>A</td>
<td>0.39</td>
<td>77</td>
<td>30.0</td>
</tr>
<tr>
<td>GR</td>
<td>0.46</td>
<td>180</td>
<td>82.8</td>
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<td>DBA/2</td>
<td>0.39</td>
<td>11</td>
<td>4.3</td>
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<tr>
<td>BALB/cCF3H</td>
<td>0.51</td>
<td>33</td>
<td>16.8</td>
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<tr>
<td>C3H</td>
<td>0.18</td>
<td>80</td>
<td>14.4</td>
</tr>
<tr>
<td>RII</td>
<td>0.24</td>
<td>100</td>
<td>24.0</td>
</tr>
<tr>
<td>C3H/C57</td>
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<td>35</td>
<td>3.8</td>
</tr>
<tr>
<td>A/C57</td>
<td>0.15</td>
<td>3</td>
<td>0.5</td>
</tr>
<tr>
<td>DD</td>
<td>0.09</td>
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electron microscopy correlates with that calculated from the radioimmunoassay. An additional source of variation may be that the virions have different specific contents of gp55, as indicated by Sarkar and Dion (13), virus from strain A mice has a larger specific content of the major glycoprotein. Also, as discussed below, gp55 is readily lost from the surface of virions and remains soluble. It is possible that the envelopes of the different strains of MuMTV have different affinities for gp55 and so retain different amounts through the purification.

Application of the Assay. The gp55 assay has, because of its antigenic simplicity and high sensitivity, been used for our routine assaying of virus production and antigenic content. Because of the cross-reaction between gp55 (spike protein) of virus from different sources, it has been possible to use the assay for the detection and quantitation of virus antigen in the milk of various mouse strains and for the routine screening of mouse milk for evidence of infection. The radioimmunoassay has been compared to the immunodiffusion assay, which is in extensive use in this laboratory (2), and has been found to be in essentially complete agreement, but with the added advantage of quantitation. We have observed that the virus content of infected mouse milk is not a strict function of the original inoculating dose, nor does it represent a 1-step increase from a low to a high value. Our results indicate a broad distribution of antigen content, as indicated in Chart 9.

The assay has also been used to analyze virus antigen released from continuous cell lines in vitro (J. B. Sheffield, T. M. Daly, R. M. McCaffrey, and N. Taraschi, studies in progress). In these studies, we have noticed a considerable amount of soluble antigen similar to that described by Kimball et al. (5, 7, 8). We have been able to reproducibly separate the soluble antigen from the virus particles by filtration through a 0.1 μm nucleopore filter. Chart 10 shows the progressive release of soluble gp55 antigen upon incubation of RIll MuMTV in 0.01 M phosphate, pH 4–0.15 M NaCl at 37°. The initial virus was obtained by the standard procedure of centrifugal purification and presumably had no soluble antigen after the final centrifugation. Nevertheless, during the overnight resuspension at 4°, 7% of the viral antigen became soluble. Incubation at 37° accelerated the process so that 25% of the antigen was soluble within 4 hr. It is not yet clear whether this is due to selective solubilization of gp55 or to the gradual destruction of the virions. Moore and Sarkar (10) showed several years ago that virus incubated at 37° was rapidly degraded morphologically. These studies have emphasized the need to define our assay carefully, since soluble antigen is thus not necessarily an indication of particulate virus.

DISCUSSION

We have described the features of a radioimmunoassay for MuMTV. We have found that the use of detergent (Nonidet P-40) in the reaction mixture confers greater specificity by reducing nonspecific trapping and also ensures the solubility of the reacting materials. The difficulties we have encountered with pipetting have been overcome by using positive displacement systems. The use of a high concentration of nonspecific protein (gelatin) reduces nonspecific adsorption of labeled antigen and minimizes the effects of proteases. By measuring the supernatant after immune precipitation, we can eliminate the problems of successive washing of the pellet.

The gp55 assay is highly specific for a single protein. In its optimum form, it is able to detect as little as 50 pg of purified gp55. We have not encountered the conformation shift problems mentioned by Parks et al. (12). Since the gp55 we use as our test antigen cross-reacts with virus from all sources tested, we have used the assay to monitor virus production by cells derived from tumors of BALB/c, C3H, GR, A, and RIll strains.

Our attempts to characterize the antibody-reactive sites of
the antigen suggest that the particular serum we use reacts with protein portions of the molecule, since reagents that react with polysaccharides do not have any effect on the immune precipitation (17). The failure to inhibit precipitation with various sugars is suggestive, but it is inappropriate to draw absolute conclusions on the basis of a limited number of saccharides. The cross-reaction of gp55 from different mouse strains, however, is further evidence that the protein moiety contains the pertinent antigenic sites, since the protein appears to be conserved among strains while variation in glycosylation has been postulated (3). Other sera may react more strongly with the sugar portion of the molecule, but we have not thus far encountered this.

Our experience with unexpected sources of error such as salt concentration and the observation of a significant degree of solubilization of antigen during incubation emphasize a need to define and carefully control the conditions of the assay. Once the conditions are understood, however, we feel that such assay procedures can give reproducible and accurate results.

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