Modified Radioimmunoassay for Murine Sarcoma-Leukemia Virus Group-Specific Antigen

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Iodination of disrupted Moloney strain murine sarcoma-leukemia virus resulted in labeled group-specific (gs) protein which was subsequently purified on an isoelectrofocusing column. This iodinated purified gs antigen, prepared from a relatively small quantity of purified virus, was used in a radioimmunoassay. A radioimmunoassay inhibition method was developed so that antibody specific for mammalian C-type gs antigen could be measured in undiluted or low dilutions of test serum without altering the known reagents of the test. The gs antigen isolated from purified Moloney strain murine sarcoma-leukemia virus has an isoelectric point (pH 5.95) which is significantly lower than that reported for other murine leukemia viruses.

A major polypeptide associated with mammalian C-type oncornaviruses with an approximate molecular weight of 30,000 ± 4,000 contains two antigenic determinants: the intraspecies (gs-1) and interspecies (gs-3) reactivities (9, 11, 14, 16, 23, 24). Isoelectric pH values ranging from pH 6.4 to 9.1 have been reported for the major polypeptide (~30,000 molecular weight) derived from C-type virions of different mammalian species (13, 22, 27). The recent development of a highly sensitive radioimmunoassay (RIA) has provided a powerful tool for analyzing the interrelationship of these antigens isolated from purified C-type virions and from avian or mammalian infected and transformed cells (10, 25, 30–33). In this study, preparations of radiolabeled gs antigen derived from small amounts of purified Moloney strain of murine sarcoma-leukemia virus (MSV (M-MuLV)) and the use of a modified RIA inhibition test to detect antibody in undiluted sera are described. Studies with antisera prepared against the purified gs antigen of MSV (M-MuLV) show that the MSV (M-MuLV) gs antigen has an isoelectric point of 5.95, significantly lower than that reported for other murine leukemia viruses.

MATERIALS AND METHODS

Cells. The 78A1 cell line of rat embryo fibroblasts transformed by and chronically infected with MSV (M-MuLV) was grown in Eagle medium with 10% fetal calf serum and 0.075% NaHCO3 (1, 4).

Virus. Preparations of MSV (M-MuLV) virions were purified from supernatant fluids of 78A1 transformed cultures as previously described (3). Briefly, 1 liter of fluid derived from a culture of 78A1 cells was clarified by differential centrifugation, followed by sedimentation on a double-layer cushion of sucrose solution (30 to 60%). The virus band was collected and centrifuged again through a linear 20 to 60% sucrose density gradient. This yielded 3 ml of purified virus with a concentration of 250 to 300 μg of virus protein per ml. Purified preparations of the Rauscher strain of murine leukemia virus (R-MuLV) and woolly monkey virus (SSV-1) (17) containing 1.13 and 2.28 mg of virus protein per ml, respectively, were kindly supplied by J. Gruber, National Cancer Institute, Bethesda, Md.

Purification of gs antigen. The gs protein was prepared from purified virus preparations using either nonidet P-40 (NP-40), Tween-80, or Triton X-100 to disrupt the virions (0.1% NP-40 being the reagent of choice). The virus lysate (crude antigen) was extracted five times with ether to remove the detergent and lipids and then aerated with nitrogen to remove the ether. Ribonuclease (RNase) (100 μg/ml) was added to the crude preparations for 30 min at room temperature to remove virus ribonucleic acid (RNA), followed by centrifugation at 100,000 × g for 60 min and dialysis of the supernatant against 1% glycine. A 1-ml amount of this preparation was fractionated on an LKB isoelectrofocusing column with an ampholyte pH range of 3 to 10 at 4°C. The proteins were electrophoresed for 72 h at a final voltage of 500 V. Fractions (2 ml each) were collected and the gs activity was measured both by agar gel diffusion (AGD) and complement fixation (CF) methods as described below. A yield of 2 to 3 ml of purified gs antigen containing 15 to 30 μg of protein per ml was obtained from 500 μg of purified virus.

Antisera. Antiserum against either crude or purified MSV (M-MuLV) gs antigen was prepared by four inoculations of 3-kg male albino rabbits at 10-day intervals with 50 to 100 μg of protein emulsified with Freund complete adjuvant. Each preparation was
given both by subcutaneous and intramuscular routes. Anti-rabbit IgG serum was produced in goats by immunization with rabbit IgG purified by diethylaminoethyl-cellulose chromatography. Rabbit anti-goat globulin was purchased from Antibodies Incorporated (Davis, Calif.). Rabbit antisera to the gs antigen of R-MuLV or SSV-1 were obtained from W. P. Parks, National Cancer Institute, and a goat anti-MuLV gs antigen serum was supplied by R. V. Gilden, Flow Laboratories (Rockville, Md.). The latter antisera had a titer as measured by RIA of 1:5,000 with SSV-1 gs antigen and approximately 1:50,000 with MuLV gs antigen (R. V. Gilden, personal communication).

Iodination. Both purified and crude antigen preparations were iodinated by the method of Greenwood et al. (15), with slight modifications. Twenty-five to 100 μl of the antigen preparations were incubated with 45 μCi of 125I (1 μl) (Amersham/Searle Corp., Arlington Heights, II.), and 25 to 50 μg of chloramine-T (5 to 10 μl). The reaction was stopped by adding sodium metabisulfite (50 μl) (added in double molar concentration relative to the amount of chloramine-T) and 500 μg of KI (50 μl). Free iodine was removed by gel filtration on a Sephadex G-25 column (1 by 25 cm) pretreated with 100 mg of bovine serum albumin. Exact conditions of labeling different preparations are given in Results.

RIA method. RIA precipitation was carried out as follows. The reaction tubes each contained 0.1 ml of 1% normal rabbit serum (NRS) (to serve as constant equivalent quantity of normal rabbit IgG to react with the precipitating anti-IgG antibody), 0.1 ml of a 1:10 dilution of normal human serum (NHS), 0.1 ml of 125I-purified gs antigen (2,000 counts per min per 0.1 ml), and 0.1 ml of two-fold dilutions of rabbit or goat anti-murine gs antigen serum with a starting dilution of 1:200. The 1:10 diluted NHS was required to prevent sticking of the iodinated protein to the glassware. This reaction mixture was incubated for 2 h at 37°C and for 18 h at 4°C. One-tenth milliliter of a 1:8 dilution of goat anti-rabbit IgG serum was added to each tube followed by a subsequent 18-h incubation at 4°C. In those experiments in which goat anti-gs antigen was tested, 0.1 ml of 0.25% normal goat serum served as carrier, and the immune complexes were precipitated with rabbit anti-goat globulin diluted 1:4. The precipitating anti-IgG sera were standardized by the quantitative precipitin test as described by Kabat and Mayer (19). A 2-ml amount of cold 0.15 M NaCl was then added to each tube followed by centrifugation at 2,500 rpm at 4°C in an HL-8 rotor, RC3 Sorvall centrifuge for 20 min. The supernatants were decanted and the washed precipitates were counted in an automatic gamma counter ( Nuclear Chicago Corp.). The protocol utilized to measure levels of gs antigen is essentially that described previously (18) with minor modifications (Table 1). The protocol devised to measure anti-gs antibody is also illustrated in Table 1.

Other serological procedures. Agar gel diffusion was performed in 1.0% agarose in phosphate-buffered saline (PBS), pH 7.2, on microscope slides (1 by 3 inches) (26). The slides were stained with 1% amido black in 7.5% acetic acid. Radioagar gel diffusion was carried out by mixing equal volumes of 125I-labeled purified gs antigen with unlabeled crude gs antigen. This antigen mixture was precipitated in AGD slides as described above against specific antisera. The slides were subsequently washed with 0.15 M NaCl, dried, stained with 1% amido black, and exposed to X-ray film for 72 h as previously described (8). A micro-CF test (7) was employed by using 2 units of complement and 4 units of either antibody or antigen.

Polyacrylamide gel electrophoresis. Electrophoresis in 10% acrylamide gels in the presence of urea and sodium dodecyl sulfate (SDS) was utilized (6). The radioactivity of 1- to 2-mm gel slices was counted and recorded by an automatic gamma counter. Molecular weight markers (cytochrome-c, chymotrypsinogen a, ovalbumin, and phosphorylase) were used to determine the molecular weights.

Gel filtration chromatography. Sephadex G-100 (Pharmacia, Uppsala, Sweden) equilibrated in PBS was poured to give a column of 1.5 by 30 cm. Bovine serum albumin as well as the known proteins listed above were used to determine the relative molecular weights. Fractions of 1 ml were collected, counted, and tested for their ability to precipitate with 1/1,000 rabbit anti-MSV (M-MuLV) gs antigen serum, by using the RIA technique.

Protein determination. The protein content of samples was determined by the method of Lowry et al. (20) utilizing crystalline bovine serum albumin as standard.

**RESULTS**

Characterization of MSV (M-MuLV) gs antigen. Crude antigen preparations derived from purified MSV (M-MuLV) were fractionated on an LKB electrofocusing apparatus. The antigenic activity, monitored by using a rabbit anti-R-MuLV gs antigen serum, was detected by both AGD and CF in fractions with an isoelectric pH (IE-pH) of 5.95 (±0.1). Since the

| Table 1. Radioimmunoassay inhibition for gs antigen or antibody |
|---------------------------------------------------------------|
| **For antigen**                                              | **For antibody**                |
|---------------------------------------------------------------|
| 1:100 Normal rabbit serum                                    | 1:100 Normal rabbit serum        |
| 1:10 Normal human serum                                      | Competing test antibody         |
| Competing test antigen                                       | Antibody (40 to 45% precipitation), 2 h 37°C* |
| Antibody (110, 2,000 counts/ min)                            | 2 h 37°C                        |
| Antigen (114, 2,000 counts/ min)                             | 2 h 37°C                        |
| 1:8 Goat anti-rabbit IgG, 18 h 4°C                          | 1:8 Goat anti-rabbit IgG, 18 h 4°C |

* Each of the reagents listed was used in 0.1-ml amounts.
* Known anti-gs antigen serum at that dilution which precipitates 40 to 50% of the iodinated gs antigen.
* The goat anti-rabbit IgG was added at equivalence in reference to 1:100 diluted normal rabbit serum.
observed IE-pH of MSV (M-MuLV) gs antigen was considerably lower than that reported for the murine gs antigens of other murine C-type viruses, other detergents (Triton X-100, Tween-80) were used as well as treatment with RNase (100 μg/ml) and/or deoxyribonuclease (100 μg/ml). However, an IE-pH of 5.95 ± 0.1 was obtained with each of the alternate treatments. The validity of this observation was tested further by using similar methods to obtain gs antigens from purified R-MuLV or SSV-1. These preparations yielded antigens with IE-pH of 6.53 and 6.32, respectively, agreeing closely with results reported by others (13, 28).

**Iodination of gs antigen.** Iodination of 100 μl of purified MSV (M-MuLV) gs antigen containing 20 μg of protein per ml resulted in a drastic loss of antigenicity in that only 10 to 20% of the radioactivity was precipitated with anti-MSV (M-MuLV) gs antigen serum. Fractionation of this radiolabeled antigen on a Sephadex G-100 column resulted in elution of approximately 20% of the iodinated protein in a peak corresponding to a molecular weight of 30,000 to 35,000. The remaining 80% was eluted in the void volume. Decreasing the levels of chloramine-T by fivefold (5 μg) again resulted in material that was antigenically altered in that only 30 to 40% was precipitable by specific antiserum.

To circumvent this problem of the drastic loss of antigenic activity, we iodinated crude antigenic preparations (5 to 15 μg of total protein) using 25 μg of chloramine-t. This labeled material was then fractionated by isoelectrofocusing, and antigenic activity was detected by radiomunodiffusion with a 125I-labeled protein peak which had an IE-pH of 5.95 (Fig. 1). Thus, the IE-pH of the gs antigen was not changed by iodination under these conditions. Assuming uniform protein labeling, the specific activity of the gs antigen ranged from 5 to 11 μCi/μg. The material was 80 to 95% precipitable with anti-gs serum. This iodinated purified gs antigen could be kept in the presence of 10% guinea pig serum, 10% Triton X-100, and 1.0 M NaCl at -40 C more than 3 months, and still yield an 80 to 90% specific precipitation.

The purity of the labeled purified gs antigen preparations was analyzed by SDS-urea-PAGE (Fig. 2). One single peak of radioactivity with a molecular weight of 29,000 was detected. The same material was fractionated by gel filtration utilizing G-100 Sephadex and eluted with PBS (Fig. 3). More than 90% of the activity eluted with an estimated molecular weight of 34,000 and a minor peak of labeled material was eluted in the void volume. This purified iodinated gs protein eluted from the gel filtration column was 94% precipitable with specific rabbit anti-MSV (M-MuLV) gs antigen serum.

**RIA test utilizing gs antigen purified from labeled crude antigen.** A rabbit anti-MSV (M-MuLV) gs antigen serum was titrated by

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**Fig. 1.** Isoelectrofocusing of disrupted crude MSV (M-MuLV) antigen labeled with 125I. The pH gradient (pH range of 3 to 10) was electrophoresed at a final voltage of 500 V for 72 h at 4 C. Fractions (1.8 ml each) were collected and counted in an automatic gamma counter (○). Group-specific antigen was measured by radioimmuno-agar gel diffusion (+). The pH was determined at 4 C (O).

**Fig. 2.** Polyacrylamide electrophoretic analysis of 125I gs antigen. Iodinated protein standards (phosphorylase, 95,000; ovalbumin, 45,000; chymotripsinogen, 25,000; cytochrome c, 12,800) were used to approximate the molecular weight. The gels were sliced to 1.5 mm per slice, and were counted in an automatic gamma counter.
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The results of an RIA inhibition test using a crude MSV (M-MuLV) gs antigen are shown in Fig. 5B. As illustrated, as little as 7 ng of virus protein inhibited the reaction of the labeled gs antigen with the rabbit anti-gs serum. Since the gs proteins represent approximately 30% of the total virion proteins (data not shown), it can be calculated that the sensitivity of the RIA inhibition test for specific gs antigen is in the range of 1 to 2 ng.

DISCUSSION

The RIA test as originally developed for virus antigens (12) has been utilized as a sensitive

The inhibitory test devised to detect antibody to gs antigen required that the test serum and a known competing antibody be derived from different species (Table 1). A goat anti-MSV (MuLV) serum, which had an AGD titer of 1:4 and a CF titer of 1:128, was tested by both the RIA precipitation and the RIA inhibition techniques. The precipitation method yielded a titer of 1:51,200 (Fig. 4A). To assay this same goat antiserum by the inhibition method, a rabbit anti-MSV (M-MuLV) gs antigen serum was used as the known competing antibody. The rabbit antisera were added to the reaction at a dilution of 1:15,000 which precipitated approximately 40% of the labeled antigen (Fig. 4). The titer of the goat anti-MSV (M-MuLV) serum, tested with these conditions by the RIA inhibition method, was 1:12,800. Therefore, the comparative titers of the goat antisera as determined by these serologic assays was 1:4 by AGD, 1:128 by CF, 1:51,200 by the direct RIA precipitation test, and 1:12,800 by RIA antibody inhibition method. The inhibition approach allows the testing of unknown sera of supposedly low antibody without adjustment of the quantity of anti-globulin antibody required to precipitate the immune complex.
probe for the detection of numerous virus antigens and their associated antibodies. A prerequisite common to all RIA methods is the availability of purified antigens which can be radiolabeled. Radiolabeling of the purified antigen to high specific activity without loss of antigenicity is important since it has been shown that increased levels of radio-specific activity enhance the sensitivity of the assay (2). Several reports have been published in which the RIA has been used to measure mammalian C-type virus proteins (10, 25, 31-33); however, it is a costly procedure to obtain sufficient amounts of highly purified virus and virus subunits to provide the reagents which are required for the test.

The present study was initiated by iodinating gs protein, which had been isolated from purified MSV (M-MuLV), obtained from 1 liter of supernatant of suspension cultures of 78 A1 cells. Iodination by the chloramine-t method of these purified gs antigens, containing 15 to 30 \( \mu \)g of protein per ml, resulted in a significant loss of antigenic activity. This was not unexpected since iodination of low concentrations of several other proteins utilizing the chloramine-t method have resulted in protein denaturation (21). To circumvent this obstacle, we iodinated crude antigen preparations containing 10-fold higher levels of protein than the purified protein preparations; these labeled crude antigens were subsequently fractionated by isoelectrofocusing. An iodinated protein antigenically identical to the purified gs protein was isolated from the isoelectrofocusing column which was free of any other detectable iodinated virus protein, as shown by analytical SDS-urea-PAGE and by gel filtration chromatography. It should be noted that the RNase added during the preparation of the crude antigens prior to iodination (see Materials and Methods) did not contaminate the final preparation of purified gs antigen. RNase labeled with \(^{125}\)I has an IE-pH of 9.1 and migrates electrophoretically at that pH, as shown in Fig. 1.

This labeled gs antigen was subsequently utilized in an RIA inhibition test which was similar in design to that reported by others with similar levels of sensitivities (25, 30, 31), in that as little as 1.0 to 2.0 ng of specific gs protein could be detected. The labeled gs antigen, which was 80 to 95% precipitable with specific antisera, was obtained by labeling 30 \( \mu \)g of crude virus lysate which yielded sufficient material to perform several thousand assays for antigen or antibody in duplicate. This approach of labeling crude preparations before subunit purification allows preparation of reagents which can be utilized in an RIA for specific subunit antigens of mammalian C-type viruses from relatively small volumes of infected cell supernatants. Since this study has been completed, we have prepared iodinated gs proteins from similar quantities of R-MuLV and SSV-1 which meet the criteria of purity and high levels of antigenic reactivity. Scolnick et al. (30) have mentioned iodination of crude antigen preparations, but no data were presented with their labeled product.

We have devised an inhibition method which can be used to measure specific anti-gs antibody activity. The test is designed so that the test serum must be of a different species of origin than that of the known competing anti-virus antibody. This approach has several advantages when compared to the direct precipitation method of measuring antibody: (i) The test antisera can be screened at undiluted or low dilutions since the precipitating anti-globulin reagent is added in equivalent levels in reference to the known competing antibody. (ii) Increasing dilutions of test antisera can be tested while the other reagents in the assay are held constant. (iii) An index of specificity is incorporated in that any antibody present is competing with a known antibody of predetermined specificity and characteristics. (iv) The immunoglobulin class of the putative antibody in the test serum does not affect the results because the precipitating anti-globulin reagent is directed against IgG of the known competing antibody.

Previous studies have indicated that gs protein derived from murine viruses obtained from AKR mice and R-MuLV virus had identical IE-pH values of 6.6 (13, 22). In this study it was shown that the gs protein obtained from purified MSV (M-MuLV) has a molecular weight of approximately 30,000, similar to that reported for other mammalian C-type gs proteins. However, the MSV (M-MuLV) gs protein had significantly lower IE-pH, namely, 5.95. Thus, the gs antigen of MSV (M-MuLV) appears to be more acidic than that of any previously reported mammalian C-type viral gs proteins.

Whether MSV (M-MuLV) (~30,000 molecular weight) protein contains more acidic amino acid residues than other mammalian C-type virus gs proteins is not known. It may be that small amounts of RNA contaminate the MSV (M-MuLV) gs proteins. However, the configuration of the protein would have to differ from the other known C-type viruses since the MSV (M-MuLV), R-MuLV and SSV-1 crude antigens were all prepared by identical protocols. While this manuscript was being prepared,
Chuat et al. (5) reported on studies relating to the IE-ph of MSV (M-MuLV) gs antigen. Our study has confirmed and extended their observations by showing that the bulk of gs antigen has an IE-ph of 5.9.

Studies are currently in progress to determine if MSV (M-MuLV) carries antigenic determinants which are distinct from those associated with Rauscher virus gs protein. In this regard, Schäfer et al. (29) proposed that mammalian C-viruses contain at least two (a and b) interspecies determinants.

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