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Detection and Characterization of Mouse Mammary Tumor Virus Cell Surface Antigens: Estimation of Antigen Abundance by Protein A Assay

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Antisera against the following mouse mammary tumor virus (MMTV) structural proteins were used to detect MMTV cell surface antigens: (i) the 27,000-dalton nucleoid protein, p27; (ii) the 36,000-dalton envelope glycoprotein, gp36; and (iii) the 52,000-dalton exterior envelope glycoprotein, gp52. We report here the development of an adherent-cell isotopic staphylococcal protein A (SPA) test (ISPAT) for MMTV structural proteins which allows for the detection of an MMTV membrane-associated antigen as well as an estimate of its relative abundance on the cell surface. This test demonstrated that the gp52 was the predominant MMTV cell surface antigen detected on both C3H and GR mouse mammary tumor cells. In a comparative study with anti-gp52 and anti-gp36 sera, SPA-specific binding with anti-gp36 serum was found to be only 5 to 6% of that obtained for the external virion glycoprotein, gp52. Both direct and indirect ISPAT indicated the presence of a low but detectable number of gp36 determinants on GR-MMTV cells; however, these gp36 determinants, unlike gp52 determinants, appeared to be exposed by the fixation procedure used. Only 0.9 to 1.1% of the gp52-specific binding was detected when anti-gp36 serum was allowed to react with viable cells. The binding of [^{125}I]SPA achieved with anti-p27 serum was even less than that detected with gp36-directed reagents, indicating that p27 is not a cell surface antigen. The use of fluoresceinated SPA further demonstrated that p27 and gp36 reactivity was only associated with a small number of cells in each of the mammary cultures tested. When *N*-[4-(5-nitro-2-furyl)-2-thiazoly]-formamide-induced C3H bladder tumor cells were subjected to a gp52-directed ISPAT, the failure to detect gp52-specific binding demonstrated the specificity of this assay for MMTV gp52-expressing cells. In addition to detecting and characterizing MMTV cell surface antigens, the newly developed adherent cell assay could measure changes in the abundance of cell surface gp52. When dexamethasone-treated and untreated GR cells were compared, measurements of gp52-specific SPA binding indicated that dexamethasone stimulation leads to a 12.2-fold increase in the amount of cell surface gp52 detected.

The mouse mammary tumor and its associated virus, mouse mammary tumor virus (MMTV), have recently been studied as a model system to test the feasibility of using plasma or serum levels of a viral protein as a systemic marker for the presence of mammary tumors. These studies comparing tumor-free and tumor-bearing mice have revealed that the level of the 52,000-dalton envelope glycoprotein (gp52) of MMTV is elevated in the plasma of tumor-bearing mice (1, 19, 20, 28). The results of Ritzi and co-workers further demonstrate that the plasma level of gp52 can be used to provide both diagnostic and prognostic information with respect to tumor status of surgically treated mice (20).

The present study attempts to further elucidate the relationship between plasma viral antigens and the presence of tumor by further characterizing MMTV cell surface antigens (CSAs). This goal is approached through the development of an adherent-cell isotopic staphylococcal protein A (SPA) test (ISPAT). This test, when performed with saturating or near-saturating levels of a viral structural protein-specific serum, provides data that allow one to further characterize MMTV CSA expression not only through the detection of specific MMTV CSAs, but also by providing relative estimates of the abundance of each antigen detected. This approach should ultimately allow one to evaluate whether a relationship exists between the relative abundance of a specific MMTV antigen

on the tumor cell surface and its corresponding abundance or detectability in the sera of tumor-bearing animals.

Although initial studies using immunofluorescence, immunoperoxidase, immunoelectron microscopy, and cytotoxicity assays have provided evidence for the association of MMTV antigens with cells of murine mammary tumors and lymphoid tissues (3, 4, 7, 9, 10, 12, 24–26), many of these investigations used polyvalent antisera and procedures which detected intracellular viral antigen as well as CSA. More recent studies designed to characterize MMTV CSAs have focused more directly upon the cell surface and used monospecific antisera directed against MMTV structural proteins. The use of immunoelectron microscopy by Holder et al. (11) has indicated the presence of gp52 on C3H mammary carcinoma cells.

A second major approach to the study of MMTV CSA has involved the use of cell surface-labeling procedures. Lactoperoxidase radioiodination of CSAs followed by specific immune precipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis has demonstrated the presence of MMTV gp52 on virus-producing murine mammary tumor cells (21, 23, 27). The use of antisera of different specificity (anti-MMTV, anti-gp36, anti-p27, and anti-p10) did not demonstrate the presence of any additional MMTV CSAs (21, 23). Interestingly, gp52 was also the only MMTV antigen detected by this procedure on B9 cells, a cloned C3H mammary nonproducer cell line (23). This procedure has also been used to demonstrate that the precursor for gp52, gPr75-MMTV*env*, is not found on the cell surface (21, 23). Despite the inability to surface iodinate the 36,000-dalton MMTV glycoprotein (gp36), this antigen has been labeled by the galactose oxidase method and immunoprecipitated (23). This observation demonstrates that at least the carbohydrate regions of gp36 must be exposed on the cell surface. Although the use of cell surface labeling has provided considerable insight into the characterization of MMTV CSAs, this approach has the inherent weakness that one antigen may be preferentially labeled with respect to another. Thus, the detectability of an antigen may depend upon the procedure used. Complement-dependent antibody-mediated cytotoxicity using monospecific viral protein-directed sera has also been used as an alternative means of analysis. Cytotoxicity tests have demonstrated that only anti-gp52 serum was cytotoxic, indicating the presence of gp52 on the cell surface (21, 22).

In this study, an adherent-cell ISPAT was developed and used to detect and estimate the relative abundance of MMTV gp52, gp36, and p27 determinants on the surface of C3H and GR mammary tumor cells. The abundance of each antigen detected was analyzed with respect to total MMTV CSA (that detected with a polyvalent serum). The cellular specificity of antigen expression was demonstrated by comparing the abundance of gp52 CSA detected on murine mammary tumor cells with that detected on C3H *N*-[4-(5-nitro-2-furyl)-2-thiazoly]formamide (FANFT)-induced bladder tumor cells.

MATERIALS AND METHODS

Cells. The C3H mouse mammary tumor cell line (Mm5mt/cl), a continuous cell line producing C3H MMTV (15), was obtained from D. Fine and grown according to the procedure of Fine et al. (5). A second GR MMTV-producing cell line (17), designated GR-MMTV, was kindly supplied by J. Schlom. This cell line was maintained on Dulbecco modified Eagle minimal essential medium-high glucose containing 10% heat-inactivated fetal calf serum, insulin (250 IU/liter), tylocine (60 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml). A FANFT-induced C3H epithelial bladder tumor cell line was kindly supplied by C. O'Toole and was maintained under the same culture conditions as used for GR-MMTV cells.

Purification of viral antigens and production of immune sera. Antisera against RIII gp52, p27, and solubilized MMTV were produced as previously described (18) with the exception that equal portions of the purified antigens, gp52 and p27, were iodinated after concanavalin A affinity chromatography and used as trace markers for further antigen purification. Each concanavalin A antigen fraction was purified before immunization by gel filtration on G-100 or G-75 Sephadex.

Radioimmunoassay (RIA) of antiserum specificity. The purified external virion glycoprotein, gp52, was iodinated as previously described (18). To iodinate lower-molecular-weight MMTV proteins, a protein fraction containing MMTV p27, p14, and p10 was iodinated with Bolton-Hunter reagent (2), and the iodinated antigens were separated on G-75 Sephadex. All four iodinated proteins (gp52, p27, p14, and p10) were assessed for purity by SDS-PAGE analysis. No cross-contamination of the iodinated protein fractions was detectable by SDS-PAGE analysis. The four purified iodinated MMTV proteins were then each tested under RIA conditions (18) with a series of serum dilutions to analyze the reactivity of each immune serum tested. This test is designated a radioimmune titration (RIT).

Immunoprecipitation of MMTV proteins. Approximately 100,000 cpm of [^{3}H]leucine-labeled lysed MMTV was diluted 1:2 with RIA buffer (0.1 M potassium phosphate buffer, pH 7.2) containing 0.1% bovine serum albumin (BSA) and 0.001 M EDTA. A rabbit anti-MMTV or anti-MMTV structural protein-directed serum was added to achieve a final dilution of 1:25 in a volume of 500 μl. This mixture was then

incubated for 1 h at 37°C. To each incubation mixture, 4.0 U of goat anti-rabbit immunoglobulin G (IgG) (Calbiochem) was added, and incubation at 4°C was continued overnight. The precipitate was collected by centrifugation at 2,500 × *g* for 30 min, washed twice with RIA buffer, and suspended in electrophoresis sample buffer (0.06 M Tris-hydrochloride, pH 6.8, 12.5% glycerol, 1.3% [wt/vol] SDS, and 1.3% β-mercaptoethanol). To ensure complete solubilization of the pellet, each sample was incubated at 80°C for 1 h and dialyzed overnight against electrophoresis sample buffer.

PAGE. The procedure used was based upon the system of Laemmli (14). Dialyzed samples obtained from immunoprecipitation were adjusted to 210 μl with sample buffer and heated at 100°C for 3.5 min. Samples were applied to polyacrylamide gels composed of a 3% polyacrylamide stacker and a 13% polyacrylamide running gel. Electrophoresis through the stacker was performed at a constant current of 1.0 mA/gel for 1 h. A constant current of 2.0 mA/gel was then applied for 4 h. The following proteins were routinely used as molecular weight standards: phosphorylase B (94,000), BSA (68,000), ovalbumin (43,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (21,000), and lysozyme (14,300).

Iodination of SPA. SPA obtained from Pharmacia Fine Chemicals Inc. was iodinated by a modification of the method of Hunter and Greenwood (13).

Affinity chromatography of [^{125}I]SPA. The affinity column used for further purification of iodination reaction products was composed of a human gamma globulin-Sepharose 4B matrix. This bed material was prepared by coupling 40 mg of human gamma globulin (Cappel Laboratories) to 2.0 g of cyanogen bromide-activated Sepharose 4B (Pharmacia). The human gamma globulin-Sepharose was used to pack a column 1.3 cm in diameter by 2.7 cm high. The mixture containing SPA iodination reaction products was adsorbed onto the column and then washed with 0.1 M sodium phosphate buffer, pH 7.4. The [^{125}I]SPA which remained bound to the affinity matrix was eluted with 3.5 M potassium thiocyanate, using a flow rate of 10.2 ml/h. The specific activity of the [^{125}I]SPA preparation, assuming a labeling efficiency of 40%, similar to that of Qualtiere and Pearson (16), was 7.0×10^6 cpm per μg of SPA.

Direct method for adherent-cell ISPAT. Multiwell tissue culture plates (Falcon 3008) were prepared for cell growth by aseptically placing round glass cover slips of 1.77-cm^2 surface area (15-mm diameter) in each growth chamber. Cells were trypsinized with a solution of 0.2% trypsin containing 5 mM EDTA and then seeded into a volume of 1.0 ml of growth medium at a density of 1.5×10^5 to 2.0×10^5 cells per well. After 48 h of growth at 37°C (5.0% CO_2), cells became confluent and were assayed.

Medium was first aspirated from each growth chamber, and adherent confluent cultures which remained in the multiwell plates were washed twice in Dulbecco phosphate-buffered saline, pH 7.4 (PBS). At this point, standard procedure involved a fixation step (1.0% paraformaldehyde) for 20 min at 4°C. Alternatively, in certain specified assays, fixation of cells was delayed until after the specific antiserum incubation had been completed. After fixation, cells were washed twice with PBS and once with PBS containing 0.1% BSA (PBS-BSA). The washed fixed cells were then incubated in growth chambers with 240 μl of each dilution of virus-directed antiserum for 2 h at 4°C. Each antiserum dilution was tested in triplicate with three culture wells. Free antibody was removed with three PBS-BSA washes. Each confluent culture was further incubated with 240 μl of ^{125}I-labeled, affinity-purified SPA (150,000 to 200,000 cpm/well) in a solution of PBS-BSA for 1 h at 37°C. The level of [^{125}I]SPA used was chosen to provide the highest percentage of input counts bound without marked increases in the statistical variation of triplicates. Free [^{125}I]SPA was subsequently removed with three PBS-BSA washes, and glass cover slips were removed from growth chambers and counted for 1 min in a Beckman 300 gamma counter. The mean and standard error of the mean were determined for triplicate samples of each antiserum dilution tested. Differences in SPA binding were evaluated for significance by a two-tailed Student *t*-test.

Indirect method for adherent-cell ISPAT. Cells were prepared for assay, washed, fixed, and incubated with a virus-directed antiserum in a similar manner to that used for a direct ISPAT. After three washes with PBS-BSA, a second immunoglobulin fraction of swine anti-rabbit serum (Bio-Rad Laboratories) was added at 1:200 dilution, and incubation was continued for 1 h at 37°C. Cells were again washed three times with PBS-BSA to remove free swine antibody, and [^{125}I]-SPA was added (100,000 cpm/well). Incubation was continued for 1 h at 37°C, and the assay was completed with three PBS-BSA washes as indicated in the direct method.

Preparation of fluorescein-conjugated SPA. The reaction of fluorescein isothiocyanate with SPA (Pharmacia) was performed according to the method of Garvey et al. (6). A ratio of 50 μg of fluorescein isothiocyanate per mg of SPA was used for conjugation. The conjugate obtained was purified on a human gamma globulin-Sepharose 4B affinity column before use.

RESULTS

Specificity of viral and viral protein-directed sera. The first step in developing an ISPAT capable of analyzing MMTV CSAs was to assess the specificity of sera produced in response to solubilized MMTV, purified envelope glycoprotein (gp52), and purified core protein (p27). Three different procedures were used to evaluate the specificity of each serum. As an initial test, each serum was assessed in an RIT against each of the following purified iodinated MMTV proteins: gp52, p27, p14, and p10. Each of these iodinated proteins produced a single peak of the appropriate molecular weight upon SDS-PAGE. This assured their purity before assay. The reactivity of these sera in a double-antibody RIT is presented for each of the MMTV structural proteins tested in Fig. 1. Antiserum produced against solubilized MMTV re-

acted with all four MMTV proteins but demonstrated a higher titer and greater immunoprecipitability with gp52 and p27 than with p14 or p10 (Fig. 1a). The lower level of immunoprecipitability for p14 and p10 may in part be a consequence of antigen alteration due to iodination with Bolton-Hunter reagent. The serum produced against purified gp52 demonstrated reactivity only with iodinated gp52 and not with p27, p14, or p10 (Fig. 1b). The results in Fig. 1c further demonstrate the specificity of anti-p27 serum for iodinated p27. Again, no reactivity was detected with the other MMTV proteins tested. The results demonstrate both high titer and specificity for the viral protein-directed sera (anti-gp52 and anti-p27).

To further assess the specificity of each serum before use in assay, an immunoprecipitation SDS-PAGE analysis was performed. For this evaluation, [^{3}H]leucine-labeled MMTV produced by Mm5mt/cl C3H mammary tumor cell cultures was solubilized and allowed to react with each of the sera to be tested. The immune precipitates obtained after repeated washes were subjected to SDS-PAGE, and the gel profiles of ^{3}H-labeled proteins were evaluated (Fig. 2). The proteins precipitated by anti-solubilized MMTV serum were gp52, gp36, p27, and two lower-molecular-weight MMTV proteins. The presence of precipitable counts in the low-molecular-weight region of the gel further indicates that some reactivity with p14 and p10 is characteristic of this serum. This profile obtained with polyspecific anti-MMTV serum, when compared with the profile obtained for virus which was not precipitated, differed only in the size of lower-molecular-weight protein peaks. This means of analysis demonstrates that polyspecific anti-MMTV serum is not only reactive with the four MMTV proteins tested in RIT, but is also reactive with a fifth protein, gp36. In Fig. 2b, tritium-labeled protein was only detected in the gp52 region of the gel, whereas in Fig. 2c, counts were predominantly found in the p27 region. Although some protein was detected in the region of the stacker gel, counts were not detected in the position of a second MMTV structural protein. Precipitable MMTV proteins were also not demonstrated when a preimmune serum was used for comparison. These results further support the contention that anti-solubilized MMTV serum is polyspecific for MMTV proteins, whereas anti-gp52 and anti-p27 are monospecific sera. Ouchterlony analysis with these three sera (data not presented) also produced results supporting this conclusion. As additional reagents for use in ISPAT, monospecific goat anti-gp36 serum was obtained from J. Schlom, of the National Cancer Institute, and a rabbit anti-gp36

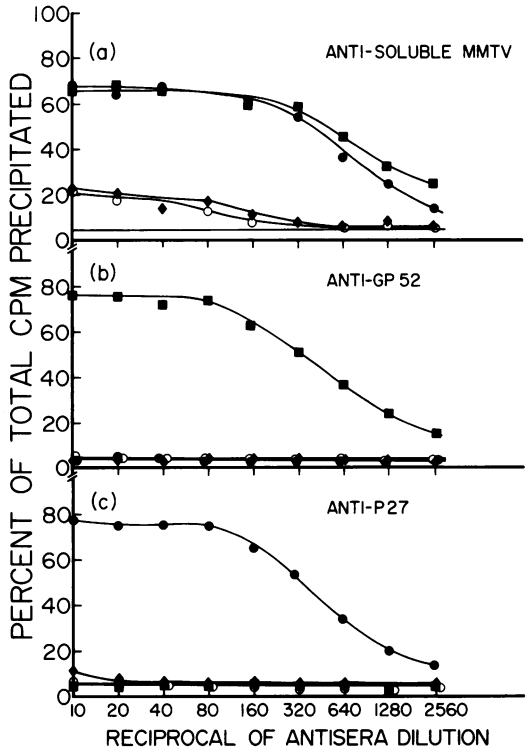

FIG. 1. *RIT of antisera produced with solubilized MMTV and purified MMTV structural proteins. Each antiserum indicated was titrated to determine their specificity against ^{125}I-labeled MMTV gp52, p27, p14, and p10. The maximum level of precipitation obtained with NRS and any of the iodinated proteins is indicated by a solid line in (a). Symbols: ■, gp52; ●, p27; ◆, p14; ○, p10.*

serum with slight cross-reactivity for p27 was obtained through R. Massey, of the Frederick Cancer Research Center. This combination of polyspecific and monospecific sera provided the basic immune reagents for an MMTV ISPAT. All of the sera used for this test were absorbed with acetone mouse liver powder before use to reduce or eliminate reactivity with normal murine cellular antigens.

Preparation of affinity-purified ^{125}I-labeled SPA. To acquire a preparation of high purity and suitable specific activity, SPA was iodinated by the chloramine T method (13), adsorbed to a Sepharose 4B-human gamma globulin affinity column, washed, and then eluted. The purified products obtained demonstrated greater than 90% binding when allowed to react a second time with the human gamma globulin affinity matrix and had a specific activity of 7.0×10^6 cpm/μg. Immune sera were first titrated in an assay to determine saturating levels, and the input of [^{125}I]SPA was varied to determine optimal conditions for assay. When

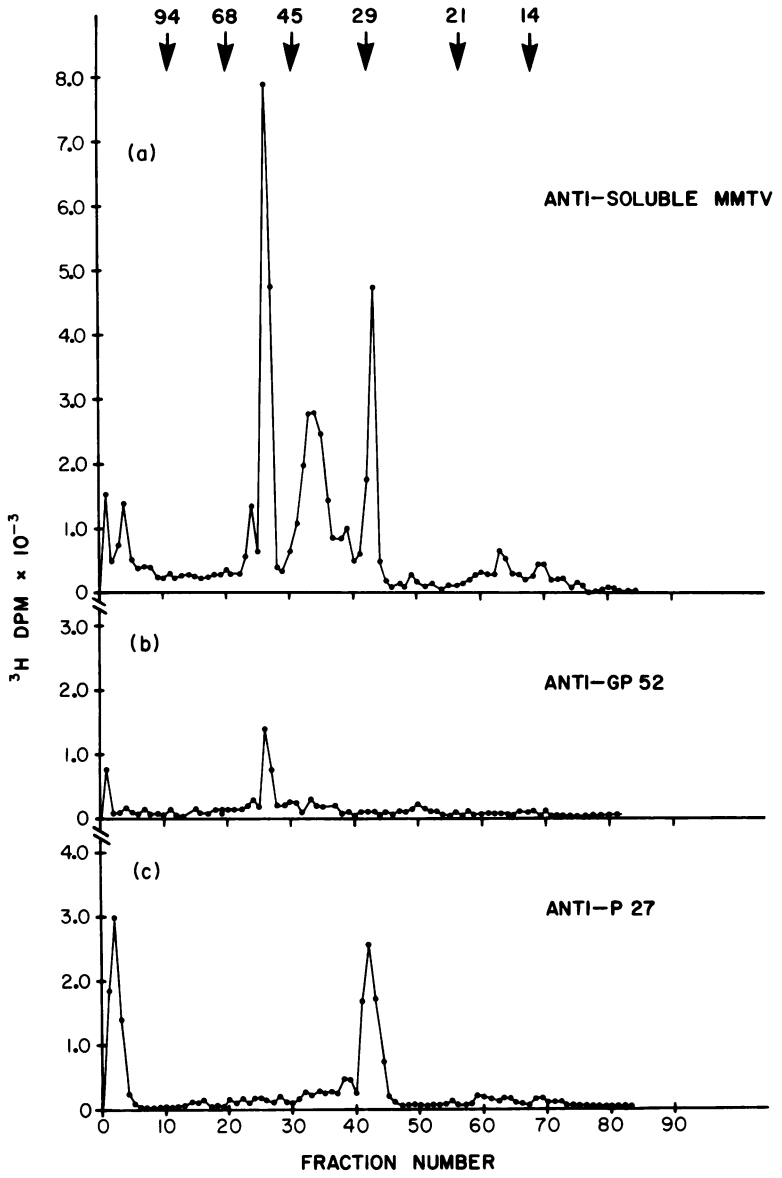

FIG. 2. *Immunoprecipitation SDS-PAGE analysis with solubilized [^{3}H]leucine-labeled C3H MMTV. Each antiserum indicated was permitted to react with solubilized [^{3}H]leucine-labeled MMTV proteins. Each immune precipitate was analyzed on 13% SDS-polyacrylamide gels. A gel profile of [^{3}H]leucine-labeled proteins is presented for each of the sera tested. Tritium-labeled proteins were not detected when NRS was used.*

the amount of [^{125}I]SPA was increased from 1.0 $\times$ 10^5 to 4.4 $\times$ 10^5 cpm per confluent cover slip culture, the number of counts bound continued to increase for both normal rabbit serum (NRS) and immune sera. Analysis of these results revealed that, for each serum tested, the highest levels of binding with acceptable statistical variation occurred at approximately 1.75 $\times$ 10^5 cpm per confluent cover slip culture. Increases in the amount of [^{125}I]SPA added above this level produced both higher levels of nonspecific binding to cells and a marked increase in the statistical variation of triplicate samples.

Direct versus indirect ISPAT. Before these reagents were used in an attempt to detect and provide relative estimates of the abundance of individual MMTV structural proteins on the surface of murine mammary tumor cells, a com-

parison of direct and indirect ISPAT (one using swine anti-rabbit IgG in addition to immune serum) was conducted. Swine anti-rabbit IgG, which binds protein A exceptionally well (8), was chosen as the second antibody. A comparison of direct and indirect methods indicated that [^{125}I]MMTV-specific binding was greater in the indirect test (8,500 cpm) than the direct test (2,125 cpm). Since binding of the preimmune serum control (500 cpm) increased by only a factor of 2, the indirect test allowed one to detect a greater fold increase above control than did the direct test.

Thus, the indirect test enhanced low levels of binding and provided greater sensitivity in the detection of CSAs; however, the direct test was chosen to evaluate different MMTV antisera since the binding in this test should be more directly proportional to the relative abundance of each MMTV antigen detected on the cell surface.

Effect of serum fractionation and absorption. GR-MMTV cells were used in ISPAT to evaluate whether SPA binding differed markedly when an immunoglobulin fraction (33% NH_4SO_4 precipitate) or mouse liver powder-absorbed serum was used. Fractionated or absorbed sera were tested against whole serum, and the extent of [^{125}I]SPA binding in each instance was compared. The results obtained with ammonium sulfate-fractionated anti-gp52 serum were nearly identical to those obtained with the whole serum (32,106 versus 31,450 cpm bound). This result indicates that relative binding levels for whole or fractionated serum are similar. In addition, the reactivity of each serum tested was found not to be significantly altered by multiple absorptions with acetone mouse liver powder.

Use of the ISPAT to detect and estimate the abundance of MMTV CSAs. The two mammary tumor cell lines chosen for study were producers of C3H MMTV (Mm5mt/c1 cells) and GR MMTV (GR-MMTV cells). Both lines were grown in the presence of dexamethasone (1.2×10^{-5} M) for 48 h to stimulate virus production. The cell lines were then used to titrate each of the following sera: anti-solubilized MMTV, anti-gp52, anti-p27, and a preimmune serum. A mild 1% paraformaldehyde treatment was used in these assays. The sera were tested at different dilutions to determine whether antiserum levels were saturating and to evaluate whether [^{125}I]SPA binding decreased upon antiserum titration. This experiment was designed to detect and compare the relative abundance of gp52, p27, and the total complement of MMTV antigens exposed on the cell surface. The levels of binding achieved with each antiserum are presented in Fig. 3 for Mm5mt/c1 cells. Triplicate cultures were used for each antiserum dilution, and brackets are used to indicate the

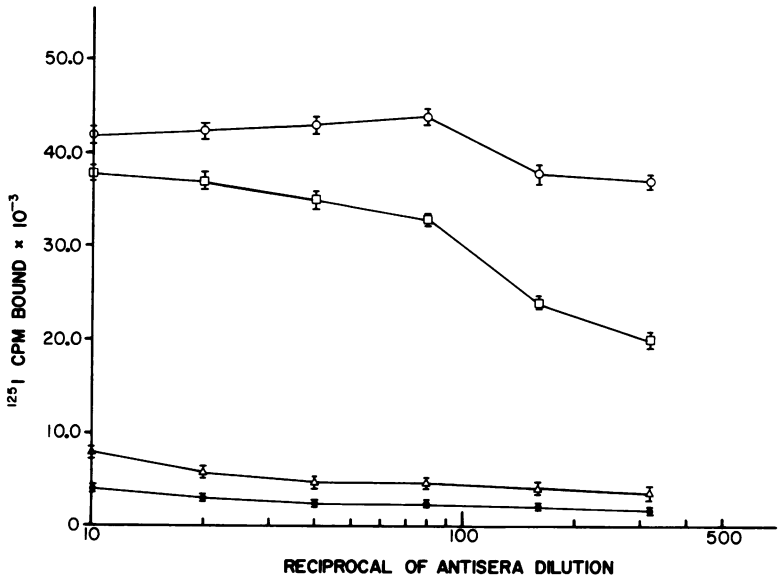

FIG. 3. *SPA binding of immune and preimmune sera with C3H mammary tumor cells. Four sera were titrated on 48-h cultures of Mm5mt/c1 cells, using a direct ISPAT. [^{125}I]SPA binding can be assessed with respect to both the serum tested and the dilution of serum used. The following sera are compared: ○, anti-soluble MMTV serum; □, anti-gp52 serum; △, anti-p27 serum; and ●, a preimmune serum. Bars indicate the standard error of the mean for triplicate samples.*

standard error of the mean for determinations. The results clearly demonstrate that SPA binding for each of the three immune sera tested was greater than binding with a preimmune serum or with [^{125}I]SPA alone (not shown). With increasing concentrations of both anti-MMTV and anti-gp52 serum, [^{125}I]SPA binding increased and relative levels of antisera saturation were obtained. In the case of anti-p27 serum, binding was low but consistently above that obtained with more than one normal rabbit serum. The relative mean levels of binding for saturating or near-saturating antiserum dilutions (1:40 dilutions) were: anti-soluble MMTV, 42,983 cpm; anti-gp52, 35,490 cpm; anti-p27, 5,178 cpm; and a preimmune serum, 2,715 cpm. Since the level of binding achieved with anti-gp52 serum closely approached that obtained with polyspecific anti-MMTV serum, gp52 must be the predominant MMTV CSA detected. The difference in binding between polyspecific serum and monospecific anti-gp52 serum indicates that the polyspecific antiserum recognizes additional antigenic determinants on the Mm5mt/c1 cells. However, this result neither suggests nor rules out the presence of additional MMTV CSAs since reactivity of polyspecific anti-MMTV may be directed against other non-MMTV CSAs despite the serum absorption procedures used. Although the low but detectable binding obtained with anti-p27 serum would tend to argue for the presence of p27 or *gag* precursor protein on the cell surface, two additional studies in progress strongly suggest that p27 cell surface determinants are not responsible for the binding detected. First, the use of fluoresceinated SPA has demonstrated that, unlike anti-gp52 reactivity, anti-p27 reactivity is directed against only a small number of cells in the cultures tested. The results obtained for C3H Mm5mt/c1 cells when fluoresceinated SPA was substituted for iodinated SPA in an assay for p27 antigen are presented in Fig. 4. Comparison of the fluorescence detected with anti-p27 serum with that detected with a preimmune serum shows that only a few positive cells (indicated by arrows) were detected with anti-p27 serum. Moreover, the fluorescence detected appeared to be more cytoplasmic in nature than membrane associated. Second, this anti-p27 serum was further tested and shown to have the same low level of reactivity with murine cells producing Moloney leukemia virus (M-MuLV) (A. H. Callis and E. M. Ritzi, unpublished data). These data suggest that the low level of binding detected could result from serum reactivity with normal murine cellular antigens or viral C-type antigens. The anti-gp52 serum used was not reactive with the same murine cells producing M-MuLV.

The results of a similar ISPAT using the same antisera and GR-MMTV cells are presented in Fig. 5. The extent of SPA binding obtained for each serum with these GR mammary tumor cells was quite similar to that found with the C3H cells. The mean relative binding levels obtained for saturating or near-saturating dilutions (1:40) were: anti-soluble MMTV, 33,300 cpm; anti-gp52, 24,950 cpm; anti-p27, 4,950 cpm; and a preimmune serum, 1,980 cpm. Again, gp52 was a more abundant CSA than p27. The similarities detected in the extent of SPA binding for GR and C3H cells with anti-gp52 serum and polyspecific anti-MMTV serum must reflect a considerable extent of shared MMTV antigenic determinants on the cell surface, since these RIII-directed sera demonstrated the predominance of gp52 on both GR and C3H tumor cells.

Effect of fixation on the abundance of

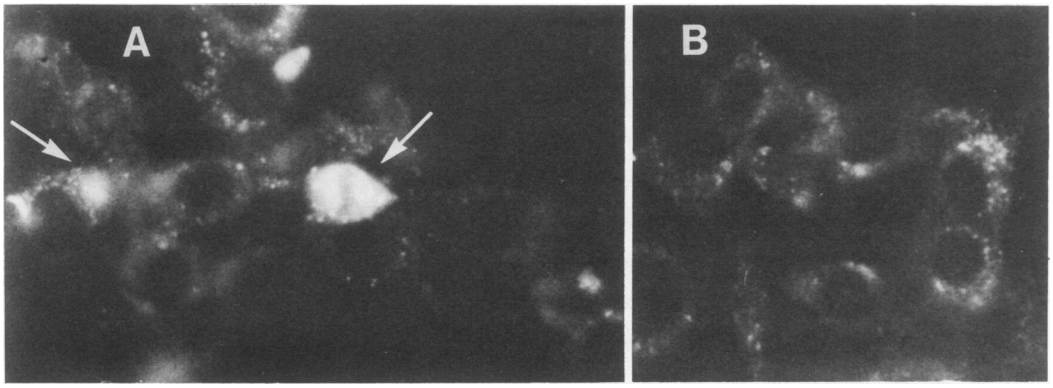

FIG. 4. *Indirect immunofluorescence staining of C3H mouse mammary tumor cells, using fluorescein-labeled SPA. The reactivity of a preimmune serum is compared with reactivity of anti-p27 serum after assay with fluoresceinated SPA. The few positive cells detected with anti-p27 serum are indicated with arrows. (A) Mm5mt/c1 C3H cells incubated with anti-p27 serum. (B) Cells incubated with a preimmune serum.*

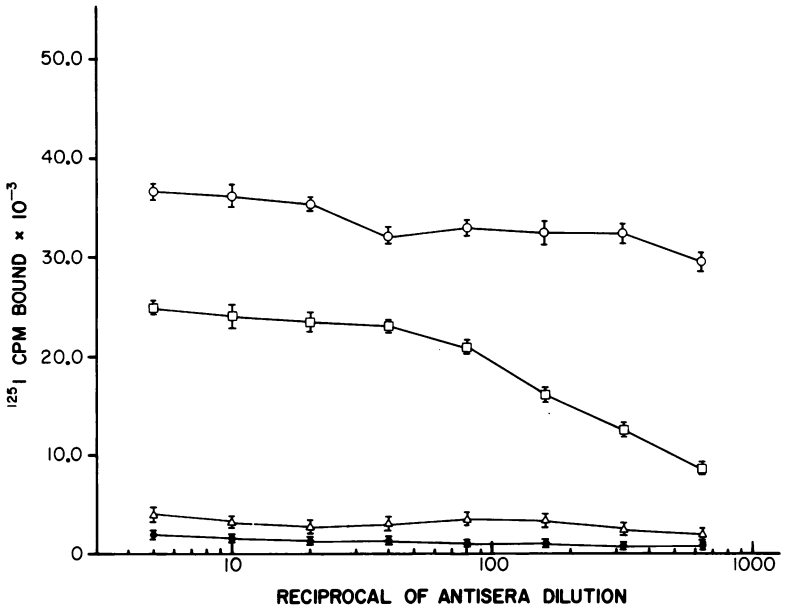

FIG. 5. *SPA binding of immune and preimmune sera with GR-MMTV mammary tumor cells. Four sera were titrated on 48-h cultures of GR-MMTV cells, using a direct ISPAT. [^{125}I]SPA binding can be assessed with respect to both the serum tested and the dilution of serum used. The following sera are compared: ○, anti-soluble MMTV serum; □, anti-gp52 serum; △, anti-p27 serum; and ●, a preimmune serum. Bars indicate the standard error of the mean for triplicate samples.*

viral CSA detected by ISPAT. The results of ISPAT were compared by using a fixation procedure (1% paraformaldehyde) either before the addition of protein-specific immune sera or after incubation of antisera to determine whether fixation exposed antigen which was not on the cell surface or led to a noticeable reduction in immune reactivity by altering CSA. This comparison was conducted with GR-MMTV cells grown for 48 h in the presence of 1.2×10^{-5} M dexamethasone. The extent of [^{125}I]SPA binding for serial dilutions of anti-gp52 serum, anti-p27 serum, and a preimmune serum is presented in Fig. 6 as a histogram for cells fixed both before and after antibody incubation. SPA binding was slightly enhanced when anti-gp52 serum was incubated with viable cells as compared with previously fixed cells. This was true for all dilutions of anti-gp52 serum tested. These data indicate a slight loss in CSA reactivity upon fixation, but suggests that fixation is not exposing additional cytoplasmic gp52. This analysis is important in demonstrating that even with fixation, the adherent-cell ISPAT detects CSA. In addition, results with fluoresceinated SPA, anti-gp52 serum, and similarly fixed cells have demonstrated fluorescence that is membrane associated (Callis and Ritzi, unpublished data). This finding further indicates the cell surface nature of the antigen detected. Results differed with anti-p27 serum and a preimmune serum, suggesting that fixed cells demonstrated enhanced SPA binding. This effect may contribute in part to the low level of SPA binding detected with the rabbit anti-p27 serum, but the reactivity of this serum with the viable cell surface is still in excess of that detected with a preimmune serum.

Use of the ISPAT with FANFT-induced C3H bladder tumor cells. To clearly determine that the detection of gp52 determinants by ISPAT was specifically an indication of gp52 expression on the surface of murine mammary tumor cells, C3H bladder tumor cells which do not produce MMTV were analyzed by ISPAT. These cells were grown for 48 h and assayed for the presence of gp52. The results obtained for preimmune and immune serum are presented in Table 1. For each dilution of anti-gp52 serum tested, no significant difference between SPA binding with immune and preimmune sera was evident. Failure to detect gp52-specific binding with these control cells demonstrates the specificity of the ISPAT for gp52. Furthermore, the use of M-MuLV-producing HPME cells has indicated that anti-gp52 serum does not react with M-MuLV-producing cells (Callis and Ritzi, unpublished data).

Use of the ISPAT to detect MMTV gp36. A further comparison of the abundance of MMTV proteins, including the virion envelope

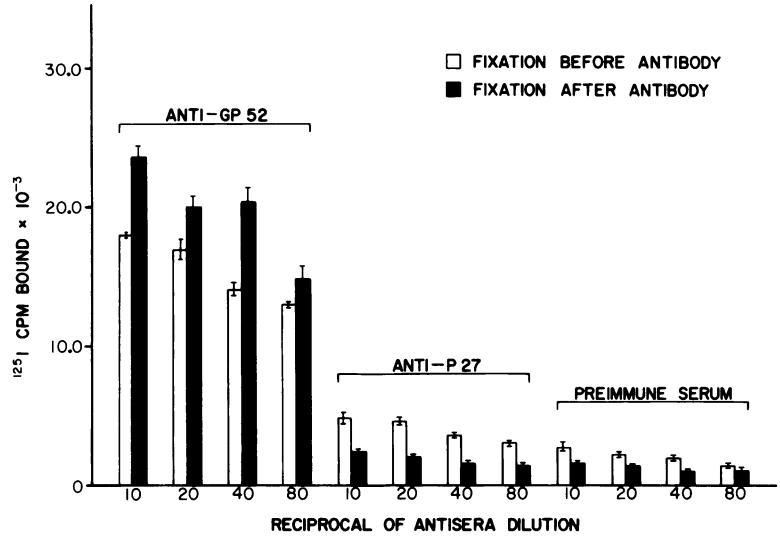

FIG. 6. *Effect of fixation upon [^{125}I]SPA binding. Each of the immune sera indicated was assessed for comparative [^{125}I]SPA binding in direct assays which used 1.0% paraformaldehyde fixation either before or after incubation with a specific serum. Fixation after antibody incubation indicates the level of SPA binding when antibody reacts with viable cells. The mean ^{125}I counts bound with each of the two assay conditions is represented as a histogram for each serum dilution tested. Bars indicate the standard error of the mean for triplicate samples.*

TABLE 1. *Lack of gp52-specific binding with FANFT-induced C3H bladder tumor cells*

Antiserum dilution	^{125}I cpm bound[a]	
	Preimmune serum	Anti-gp52 serum
1:10	2,249 ± 119	2,161 ± 91
1:20	1,924 ± 68	1,908 ± 174
1:40	1,466 ± 55	1,514 ± 40
1:80	1,155 ± 111	1,131 ± 45

[a] Mean and standard error of the mean for triplicate determinations.

glycoprotein gp36, was made possible through the aid of L. Arthur and Y. Teramoto, who supplied rabbit and goat anti-gp36 sera. Immunoprecipitation SDS-PAGE analysis by Dr. Arthur demonstrated that MMTV gp36 was the predominant protein precipitated by the rabbit serum. Although this serum demonstrated a low level of reactivity with MMTV p27, no reactivity with MMTV gp52 could be detected (Callis and Ritzi, unpublished data). This anti-gp36 serum was first used in a direct assay with GR-MMTV cells, using standard fixation procedures to detect and assess the abundance of cell surface gp36. This serum and those structural protein-directed sera used in the previous studies were titrated in the adherent-cell assay against 48-h cover slip cultures of GR-MMTV cells grown in 1.2×10^{-5} M dexamethasone. The level of [^{125}I]SPA binding achieved is compared for each serum tested in Table 2. If the binding in excess of that achieved with NRS is evaluated, it is evident that gp52-specific SPA binding was far greater than either p27 or gp36 at each of the serum dilutions tested. The binding detected with anti-gp36 serum was only 5 to 6% of the gp52-related binding. However, the low level of binding detected with anti-gp36 serum was consistently above that obtained with NRS or anti-p27 serum. This result argues for the detection of a small quantity of gp36 determinants.

In a further attempt to detect and verify the presence of gp36 cell surface determinants, a monospecific high-titer anti-gp36 goat serum was obtained from Y. Teramoto of Meloy Laboratories. This antiserum was used with 48-h GR-MMTV cultures, using standard fixation procedures. The test used was an indirect ISPAT which used rabbit anti-goat IgG as a second antibody. The [^{125}I]SPA binding levels achieved with this serum and normal goat serum (NGS) are presented for comparison in Table 3. Although the nonspecific binding attributable to NGS was higher than that obtained with direct assays using NRS, a detectable binding in excess of NGS was noted even at serum dilutions from 1:160 to 1:1,280. Considering the amplified sensitivity of this indirect assay, the level of specific SPA binding obtained still only demonstrates the detection of a low level of gp36 determinants. The detection of specific gp36-related binding

TABLE 2. *Comparative SPA binding with sera directed against MMTV structural proteins*

Antiserum	Binding at given antiserum dilution[a]					
	1:40			1:80		
	^{125}I cpm bound	^{125}I cpm (in excess of NRS)[b]	% of anti-gp52 serum binding	^{125}I cpm bound	^{125}I cpm (in excess of NRS)	% of anti-gp52 serum binding
NRS	3,993 ± 36			2,480 ± 87		
Anti-p27	4,500 ± 141	507	2.0	3,211 ± 228	731	4.0
Anti-gp36[c]	5,069 ± 821	1,076	5.0	3,623 ± 81	1,143	6.0
Anti-gp52	26,059 ± 513	22,066	100.00	20,607 ± 488	18,127	100.00

[a] Mean and standard error of the mean for triplicate samples of GR-MMTV cells.
[b] Mean number of counts bound with NRS has been subtracted from each immune serum mean.
[c] This serum also has a low level of reactivity with MMTV p27.

TABLE 3. *Indirect ISPAT for the detection of MMTV gp36*

Antiserum dilution[a]	^{125}I cpm bound[b]		
	NGS	Goat anti-gp36 serum	gp36-specific binding in excess of NGS[c]
1:160	11,631 ± 797	16,521 ± 543	4,890
1:320	8,070 ± 270	12,002 ± 671	3,932
1:640	5,943 ± 180	10,062 ± 473	4,119
1:1,280	4,007 ± 242	7,012 ± 141	3,005

[a] Dilution of first antibody. The second rabbit anti-goat IgG was used at a 1:3,200 dilution.
[b] Mean and standard error of the mean for triplicate samples of GR-MMTV cells.
[c] Mean number of counts bound with NGS at each antiserum dilution has been subtracted from the mean binding with anti-gp36 serum.

with two different hyperimmune sera, one rabbit and one goat, suggested that a limited number of gp36 determinants may be exposed on at least a portion of the cells in each culture tested.

To further test this assumption and the possibility that gp36 might be revealed by the fixation procedure used, an assay was performed with GR-MMTV cells in which the fixation step was delayed to allow rabbit anti-gp52 serum to react with viable (unfixed) mammary cells. A comparison of SPA binding with anti-gp52 serum, anti-gp36 serum, and NRS revealed that at a 1:20 dilution, binding by anti-gp36 serum in excess of NRS was only 248 cpm, whereas binding by anti-gp52 serum was 27,774 cpm. A very similar result was noted at a 1:40 dilution, demonstrating that the binding attributed to the presence of gp36 on viable cells was only 0.9 to 1.1% of that attributed to gp52. This level of anti-gp36 serum binding is substantially lower than that detected with fixed cells (5.0 to 6.0%), and the number of counts bound is sufficiently similar to that obtained for NRS that the difference cannot be considered statistically significant when analyzed in a two-tailed *t*-test. This result indicates that little if any gp36 is exposed on the viable cell surface but that the very mild fixation procedures used in the two previous tests must have exposed this viral antigen. An immunofluorescence analysis was also performed with rabbit anti-gp36 serum and fluoresceinated SPA. Both fixed and viable fluorescences were assessed with GR-MMTV cells. The results of fluorescence microscopy with anti-gp36 serum, using both fixed and viable cells, were virtually identical to those depicted in Fig. 4 when anti-p27 serum was used. In both cases, isotopic binding was enhanced by prior fixation, and only a few cells in each mammary culture demonstrated positive fluorescence.

Use of the ISPAT to detect dexamethasone-mediated changes in the abundance of cell surface gp52. GR-MMTV cells were grown in the presence and absence of 10^{-5} M dexamethasone to determine whether the adherent-cell ISPAT could be used to specifically detect a change in the cell surface abundance of gp52 as a consequence of dexamethasone stimulation. Cells were grown for 48 h as control or dexamethasone-treated cultures and then subjected to ISPAT with preimmune and anti-gp52 serum. The [^{125}I]SPA binding achieved and the fold stimulation detected with immune serum are presented in Table 4. Whereas gp52 was detectable without dexamethasone treatment (692 cpm in excess of NRS), SPA binding was markedly enhanced by dexamethasone stimulation. Comparison of the counts bound in excess of preimmune serum binding for control and treated cultures demonstrates that dexamethasone treatment led to a 12.2-fold increase in the amount of cell surface gp52 detected in treated cultures. These results indicate that the ISPAT can be used as a responsive indicator of changes

TABLE 4. *ISPAT analysis of gp52 abundance in the presence and absence of dexamethasone*

Treatment	Serum	^{125}I cpm bound[a]	^{125}I cpm bound in excess of preimmune serum binding[b]	Fold stimulation by dexamethasone
Control	Preimmune	2,386 ± 81		
	Anti-gp52	3,078 ± 58	692	
Dexamethasone[c]	Preimmune	2,384 ± 121		
	Anti-gp52	11,501 ± 258	9,117	12.2

[a] Mean and standard error of the mean for triplicate determinations.

[b] Mean counts bound with preimmune serum has been subtracted from the mean obtained with anti-gp52 serum.

[c] Concentration of dexamethasone used was 10^{-5} M. GR-MMTV cells were grown for 48 h before assay.

in the abundance of gp52 cell surface determinants.

DISCUSSION

Comparison of SPA binding with anti-gp52 serum and a polyspecific anti-MMTV serum clearly indicates that MMTV gp52 accounts for the majority of MMTV CSA detected on both GR and C3H murine mammary tumor cells. This result agrees with previous studies concluding that gp52 is present on the cell surface (11, 21, 23) and further explains the observation of Schochetman et al. (22) which indicated that gp52 was the only MMTV structural protein capable of serving as the target for a complement-dependent antibody-mediated cytotoxic reaction. Additional studies still in progress using fluoresceinated SPA provide further assurance that gp52 detected under the conditions used for assay is plasma membrane associated. The marked abundance of MMTV gp52 on the tumor cell surface may also help to explain the abundance of gp52 detected in the plasma of tumor-bearing mice; however, the exact process(es) by which viral antigens are produced in tumor cells and ultimately appear in the circulatory system remains unclear. The results further demonstrate that the cell surface determinants of the external virion glycoprotein, gp52, are responsible for approximately 20-fold-greater SPA binding than the determinants of the major envelope glycoprotein, gp36, when fixation procedures are used in the adherent-cell assay. The lack of statistically significant gp36-related SPA binding with viable cells suggests that little if any gp36 is exposed on the viable mammary cell surface. This result would support the findings of previous cell surface-labeling studies (21, 23) which have argued for the presence of only gp36 carbohydrate moieties. The low level of SPA binding achieved with anti-gp36 sera further suggests that sufficient determinants are not exposed to provide a good target for complement-dependent antibody-mediated cytotoxicity. This observation is consistent with the finding that anti-gp36 serum is not cytotoxic with C3H mammary tumor cells; however, treatments which expose additional viral cell surface determinants (trypsin or EDTA) render the cell surface susceptible to cytotoxic attack by anti-gp36 serum (21). The ability to expose gp36 determinants with trypsin or EDTA may suggest that fixation with 1.0% paraformaldehyde similarly produced an alteration in the cell surface which exposed additional gp36 determinants but did not enhance gp52 detection. Furthermore, the presence of p27 on the cell surface seems unlikely if the following results are considered: (i) binding by anti-p27 serum was very similar to that obtained with NRS; (ii) this binding could be attributed, in large part, to the effect of fixation; (iii) only a small number of cells were found to be positive in p27 immunofluorescence studies; and (iv) the same low level of p27-related binding was detected when M-MuLV-producing cells were analyzed.

In addition to detecting and characterizing the relative abundance of MMTV CSAs, the results obtained with dexamethasone-stimulated cells demonstrate that the newly developed adherent-cell assay can detect and measure changes in the abundance of cell surface gp52. Although the specificity of each antiserum used in assay may affect both the detection of specific determinants and the extent of SPA binding achieved, the ability to measure changes or detect differences in the cell surface abundance of a particular set of viral antigenic determinants, such as MMTV gp52 determinants, should permit future studies to answer the following questions. How does the cell surface abundance of MMTV determinants compare with the abundance of a set of MuLV determinants when mammary cells of the same and differing mouse strains are compared? How does the detectability and abundance of gp52 differ when MMTV producer and nonproducer mammary tumor cells are compared by ISPAT? Can gp52 cross-reactive determinants be detected on cell lines of human mammary origin, and, if detected, how does the abundance of these determinants compare with MMTV gp52 determinants detected on murine mammary tumor cell lines?

In addition to answering these questions, the ability to measure changes in an MMTV CSA should provide an opportunity to further investigate the relationship between expression of a

cell surface viral antigen and the process(es) by which it is released from the cell as either an MMTV virion or a soluble antigen.

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