Quantitation of Viral Antigens Released into Plasma and Culture Fluids by Murine Mammary Tumor Cells

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ABSTRACT

Radioimmunoassays capable of measuring mouse mammary tumor virus (MMTV) 52,000 M.W. envelope glycoprotein (gp52) and 27,000 M.W. protein (p27) have been used to quantitatively compare plasma concentrations of these viral antigens in mice bearing spontaneous mammary tumors. Although gp52 was detected in the plasma of all tumor-bearing mice tested, p27 was detected in only a portion of tumor-bearing animals. In p27-positive animals, gp52 was detected in higher concentrations than p27. These findings demonstrate that gp52 has preferential utility as a plasma marker for the presence of mammary tumors in MMTV-infected hybrid (BALB/c x DBA/8 F1), Paris Rill, and C3H/HeJ mice. In addition, cultures of MMTV-producing cells [GR-MMTV and MMTV(C3H)Fel I] were used as models to study the release of viral antigens in the absence of serum antibody or additional host factors. Comparisons of extracellular soluble and particulate antigen concentrations demonstrated that gp52 and p27 were present in substantially higher concentrations as soluble than virion-associated antigens. The mean ratio of non-virion-associated gp52 to virion-associated gp52 was 12.5:1 for GR-MMTV and MMTV(C3H)Fel I cells and 37.3:1 for MMTV(C3H)Fel I cells. The marked stability of MMTV in culture fluids suggested that virion breakdown was not responsible for the accumulation of soluble viral antigens in culture. The information obtained suggests that abundant virus-free antigens may be of greater use than virion-associated antigens as a source of viral antigen to evaluate mammary tumor status.

INTRODUCTION

The mouse mammary tumor and its associated virus MMTV\(^3\) have been used as a model to study the relationship between viral antigens in body fluids and the onset of mammary tumors. Concentrations of viral antigens in milk have correlated with the tumor incidence of mouse strains examined, and mice with higher levels of viral antigens in their milk have had a greater tendency to develop tumors (4, 7, 8, 10); however, milk antigens cannot be utilized to detect the presence of tumor in nonlactating individuals. For this reason, gp52 was studied in plasma of tumor-bearing and tumor-free mice to determine if gp52 levels of plasma reflected the presence of mammary tumors. Results of studies utilizing gp52-specific RIA's revealed that gp52 levels were elevated in plasma samples obtained from tumor-bearing animals (1, 15, 20). Subsequent investigation indicated a 10- to 100-fold decrease in the level of gp52 within 9 days of tumor removal, thus demonstrating that the tumor was the principal source of plasma gp52 (16). Fractionation of plasma samples by centrifugation revealed that gp52 was detected as a soluble antigen in supernatants rather than as a virion-associated antigen in viral pellets (15). Measurement of gp52 levels in plasma of tumor-bearing mice has also been used to monitor changes in tumor status during chemotherapy. This approach has provided a measure of therapeutic effect for combination 5-fluorouracil:thymidine treatment (14, 17).

Although gp52 has proven to be useful as a systemic marker for mammary disease, our knowledge of the utility of other MMTV structural proteins to serve as markers for disease is still incomplete. p10 has been detected in serum of tumor-bearing mice (2). In addition, the recent results of Osterrieth et al. (9) have suggested that the 27,000 M.W. protein (p27) is generally not found in the serum of MMTV-infected tumor-bearing Swiss albino mice; however, only a small number of a single strain of mice were studied and mean gp52 levels of serum were also relatively low.

The present study used gp52 and p27 RIA's to quantitate these antigens in the plasma of tumor-bearing mice to determine the comparative utility of these 2 viral antigens as systemic markers for murine mammary tumors and utilized cell culture models to compare the concentrations of extracellular MMTV antigens released in the absence of potentially lytic serum factors (1, 19).

In the present study, comparison of gp52 and p27 levels in the plasma of 3 different strains of tumor-bearing mice has revealed that gp52 was detected with greater frequency and abundance than p27. In addition, the analysis of viral antigen ratios in culture fluids has demonstrated that gp52 was relatively more abundant than p27 as a soluble antigen and that both proteins were detected predominantly as soluble rather than virion-associated antigens. The marked stability of MMTV particles in culture fluids has suggested that virion degradation is an unlikely cause of the high concentrations of soluble antigens detected in virus-free supernatants.

The parallel observations that gp52 is present principally as a soluble non-virion-associated antigen in plasma of tumor-bearing animals and extracellular culture fluids of murine mammary tumor cells suggest that mammary tumor cells release an abundance of soluble viral antigen and therefore provide a useful model system for studying factors that influence the release and accumulation of soluble viral antigens.

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MATERIALS AND METHODS

Cells. The GR-MMTV cell line, which was derived from a murine mammary adenocarcinoma of a GR mouse, was acquired from Dr. David K. Howard, Meloy Laboratories (12). A feline cell line, designated MMTV(C3H)Fel I, was obtained from the same source. MMTV(C3H)Fel I is a C3H MMTV-infected feline embryonic kidney cell line (6). The Mm5mt/c1 C3H mammary tumor cell line was obtained from Dr. Fine (5). Cells were maintained on DMEM containing 10% heat-inactivated fetal calf serum, insulin (250 IU/liter), tyloxolene (60 μg/ml; Amersham/ Searle Corp.) at a concentration of 10 μCi/ml. Purified MMTV gp52 and p27 were iodinated with 125I-labeled Bolton-Hunter reagent. The iodination and chromatography procedures have been described previously (13); however, for p27, the procedure was modified by substituting Sephadex G-75 to separate iodinated protein from low-molecular-weight reaction products. Iodinated MMTV gp52 was greater than 90% immune precipitable with anti-gp52 serum and iodinated MMTV p27 was greater than 90% immune precipitable with anti-p27 serum.

RIA for gp52 and p27. Purified iodinated MMTV gp52 was used in a blocking RIA in which delayed addition of the labeled antigen was used to maximize the sensitivity of the measurement. The RIA procedure, its reproducibility, application, and lack of interference from proteases have been reported (15, 16). The RIA buffer was composed of 0.1 M potassium phosphate buffer, pH 7.4, and solubilized with 0.1% Triton X-100 and 0.1 M KCl for 30 min at 37°. Viral antigen concentrations in pellets and soluble antigen fractions were standardized to cell number and have been reported as ng/10^6 cells.

RESULTS

Quantitation of gp52 and p27 in the Plasma of Tumor-bearing Mice. Previous studies have demonstrated the utility of gp52 as a systemic marker in mice bearing spontaneous mammary tumors (15, 16). The purpose of the present study is to quantitate both gp52 and p27 in plasma samples, thus comparing the relative frequency of detection and abundance of these viral antigens in tumor-bearing mice.

Plasma samples from RII, C3H/HeJ, and CD8F1 mammary tumor-bearing mice were obtained for comparative measurement in gp52 and p27 RIA's, and small numbers of tumor-free animals were used as controls. Viral antigen levels were compared in mice bearing a range of different tumor burdens. The majority of RII tumors was in excess of 2.0 g and the majority of CD8F1 and C3H/HeJ tumors was in excess of 1.0 g. The distribution of plasma (10 to 200 μl) from the same bleeding of each normal and tumor-bearing mouse were assayed in the presence of Trasylol, a protease inhibitor, for gp52 and p27 as described previously (13).

The gp52 levels of plasma from tumor-free controls were low (≤10 ng/ml) and p27 was not detected. The results obtained with tumor-bearing mice indicated that gp52 was detected in plasma from mice with tumors of the mammary gland. The RIA's for gp52 and p27 similarly demonstrated a sensitivity of <10 ng/ml. Cross-reactivity testing with gp52, p27, and Rauscher murine leukemia virus antigen from JLSV9 cells has demonstrated that each assay was specific for its homologous protein. Repeated assays were performed on plasma sample in both gp52 and p27 RIA's, and the mean value (± S.D.) for 10 replicate determinations was 74.1 ±6.9 (± S.D.) for 10 replicate determinations was 74.1 ±6.9, (15, 16). The RIA's for gp52 and p27 similarly demonstrated a sensitivity of <10 ng/ml.
Release of MMTV Antigens

**Chart 1.** Comparison of gp52 and p27 concentrations in plasma samples of CD8F, tumor-bearing mice. The concentrations of gp52 and p27 have been determined for each plasma sample by parallel determinations in their respective RIA's. The ordinate (antigen concentration) is on a log scale. ○, concentration of p27; ●, concentration of gp52.

**Chart 2.** Comparison of gp52 to p27 concentration is illustrated as a series of histograms for individual tumor-bearing mice.

**Table 1**

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>No. of mice tested</th>
<th>Concentration of gp52 (ng/ml)</th>
<th>Concentration of p27 (ng/ml)</th>
<th>gp52:p27</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8F</td>
<td>14</td>
<td>446±150–840</td>
<td>24</td>
<td>6–54</td>
</tr>
<tr>
<td>Rill</td>
<td>25</td>
<td>169±7–740</td>
<td>14</td>
<td>1–73</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>4</td>
<td>170±39–424</td>
<td>11</td>
<td>8–12</td>
</tr>
</tbody>
</table>

Indicates that gp52 is present at higher concentrations than p27 in CD8F, Rill, and C3H/HeJ mice. The difference between mean concentrations of gp52 and p27 has been evaluated for significance in a 2-tailed Student's t test. The difference in means is statistically significant for CD8F, mice (p < 0.001) and Rill mice (p < 0.01). Although C3H/HeJ mice demonstrate the same trend, the number of p27-positive mice was too small to demonstrate a statistical difference (p > 0.1).

**Accumulation of Non-Virion-associated gp52 in Extracellular Culture Fluids.** Analysis of gp52 levels in plasma samples of tumor-bearing mice revealed that gp52 was detected predominantly as a soluble rather than a virion-associated antigen (15). To further our understanding of MMTV antigen release from murine mammary tumor cells, GR-MMTV cells have been utilized as a model system to study the release of non-virion-associated gp52.

As a first step in our study of viral antigen release in culture, the accumulation of non-virion-associated gp52 was examined as a function of time by gp52 blocking RIA. Culture medium was harvested from growing GR-MMTV cells cultures at various times from 1 to 120 hr after medium change. At each time...
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point, medium was clarified by low-speed centrifugation, centrifuged as indicated in "Materials and Methods" to obtain a virus-free non-virion-associated antigen fraction, and quick frozen for subsequent RIA analysis. Virus-free medium samples (volumes of 4 to 160 μl) from each time point were analyzed by blocking assay.

The blocking curves obtained for time intervals from 0 to 120 hr after medium change are presented in Chart 3. All samples (except the zero time point) produced a reduction in gp52 precipitation with the addition of increasing sample volumes, thereby demonstrating the presence of gp52 determinants in each virus-free supernatant. Further analysis of these blocking curves indicates that both the initial and maximum inhibition achieved increase with time after medium change. The results clearly demonstrate continued gp52 release and accumulation as non-virion-associated antigen for the entire time interval studied (0 to 120 hr). The extent of blocking detected indicated that a 24-hr interval would provide a sufficient amount of gp52 accumulation in culture to quantitatively compare gp52 levels in virion-associated and non-virion-associated antigen fractions.

Quantitation of Virion-associated and Non-virion-associated Extracellular Viral Antigens. Murine mammary tumor cells of GR mice (GR-MMTV cells) and cat kidney cells infected with C3H MMTV [MMTV(C3H)Fel I cells] have been used as an in vitro model to determine if gp52 and p27 antigens, released by MMTV-producing cells, are detected predominantly as virion-associated or non-virion-associated antigens. The ability to measure both gp52 and p27 by RIA has provided a direct quantitative comparison of the amount of each antigen detected in viral pellets (virion-associated antigen fractions) and virus-free supernatants (non-virion-associated antigen fractions).

Medium samples were obtained from growing dexamethasone-treated GR-MMTV and MMTV(C3H)Fel I cells, and samples were separated into soluble and particulate antigen fractions as described in "Materials and Methods." Pooled medium samples from 3 cell cultures were used to reduce variation among cultures, and 4 separate experiments were performed for each cell line. Viral pellets were solubilized (0.1% Triton X-100 and 0.1 M KCl) for 30 min at 37° prior to assay, and both antigen fractions were assayed simultaneously for comparison. Relatively mild solubilization conditions were used to permit direct assay of samples without assay interference, a possible consequence of high concentrations of salt or detergent. In each experiment, the concentration of gp52 detected in virion-associated and non-virion-associated fractions has been normalized to cell number, and the data obtained from 4 experiments with each cell line are presented in Table 2. The values obtained for both GR-MMTV cells and MMTV(C3H)Fel I cells demonstrate that gp52 is substantially more abundant as a soluble non-virion-associated antigen than as a virion-associated particulate. The difference between gp52 concentration means in these 2 antigen fractions is statistically significant for each cell line (p < 0.01), and markedly higher levels of soluble gp52 were detected in each experiment. The ratios of soluble to particulate antigen for GR-MMTV and MMTV(C3H)Fel I cells indicate that soluble gp52 is, respectively, 12.5- and 37.3-fold more abundant than virion-associated gp52. Although this comparison was conducted with dexamethasone-treated cells, soluble gp52 was also substantially more abundant in untreated cultures.*

In addition to gp52 quantitation, extracellular soluble and particulate antigen fractions were compared by p27 assay. Again, 24-hr medium harvests of growing GR-MMTV cells were obtained. The results of p27 assay for 3 separate experiments demonstrated a higher concentration of soluble compared to particulate antigen, and the ratio of soluble to particulate p27 was 2.3 ± 0.5. This ratio is substantially smaller than that obtained for gp52 (2.3 versus 12.5) and demonstrates that gp52 is relatively more abundant as a soluble antigen.

Stability of MMTV in Medium of Mammary Cell Cultures. The possibility that non-virion-associated gp52 might arise through a process of virion breakdown has been investigated using [3H]leucine-labeled MMTV purified by equilibrium gradient centrifugation. The procedures used to determine virion stability are detailed in "Materials and Methods." Labeled virus (either C3H MMTV or GR MMTV) was added to flasks containing each of the following: (a) medium and mammary cell cultures producing the same strain of MMTV; (b) conditioned medium alone, and (c) DMEM with additives routinely used for cell growth. Medium samples were removed at different time points and separated into soluble and particulate antigen fractions, and the recovery of trichloroacetic acid-precipitable [3H]leucine-labeled MMTV was determined for the particulate antigen fraction. The recovery of C3H MMTV from Mm5mt/c1 cell cultures and medium controls is presented for each time point in Table 3. These results indicated that MMTV recovery as a particulate antigen ranged from 83 to 96% and the recovery of MMTV did not decrease with increasing time of incubation for medium samples from cell cultures or controls. The high recoveries of MMTV obtained after 24 hr in culture medium and the failure to see a decline in MMTV recovery with increasing time of incubation demonstrate that the incubated MMTV was relatively stable in culture fluids. The recovery of [3H]leucine-labeled GR MMTV from GR-MMTV cultures was performed in an identical manner, and GR MMTV recoveries ranged from 91 to 96% in culture fluids and medium controls.

* B. A. Smith and E. M. Ritzi, unpublished observations.

Chart 3. Accumulation of non-virion-associated gp52 in culture fluids of GR-MMTV cells. Soluble antigen fractions (virus-free supernatants) were obtained from culture medium as indicated in "Materials and Methods" at various times from 0 to 120 hr after medium change. Increasing volumes of each soluble antigen fraction were used as blocking antigen in a gp52 assay. The percentage of total [3H]gp52 precipitated is illustrated for triplicate volumes of sample; point, mean; bars, S.D. The blocking curves obtained for each soluble antigen fraction are identified on the right by the time of collection (hr after medium change).
Release of MMTV Antigens

Table 2
Distribution of gp52 in extracellular medium fractions

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Concentration of MMTV gp52* (ng/10^6 cells)</th>
<th>B:A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virion-associated antigen fraction (A)</td>
<td>Non-virion-associated antigen fraction (B)</td>
</tr>
<tr>
<td>GR-MMTV</td>
<td>15.1^2 Mean 9.2-14.1</td>
<td>7.1 Mean 6.8-7.3</td>
</tr>
<tr>
<td>MMTV(C3H)Fel I</td>
<td>178 Mean 126-226</td>
<td>309 Mean 227-404</td>
</tr>
</tbody>
</table>

*In each experiment, a 24-hr medium collection was fractionated by centrifugation (as indicated in “Materials and Methods”) into a virion-associated antigen fraction (viral pellet) and a non-virion-associated antigen fraction (virus-free supernatant). Viral pellets were solubilized with 0.1% Triton X-100 and 0.1 M KCl prior to assay. Extracellular antigen fractions (virus-associated and non-virion-associated) were quantitated in the same RIA (either gp52 or p27).

Table 3
Stability of C3H virus in extracellular culture fluids of Mm5mt/cl cells

<table>
<thead>
<tr>
<th>Time of incubation (hr)</th>
<th>% of recovery of C3H MMTV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture A</td>
<td>Culture B</td>
</tr>
<tr>
<td>1</td>
<td>90.4</td>
</tr>
<tr>
<td>4</td>
<td>93.0</td>
</tr>
<tr>
<td>8</td>
<td>91.5</td>
</tr>
<tr>
<td>12</td>
<td>94.5</td>
</tr>
<tr>
<td>24</td>
<td>95.9</td>
</tr>
</tbody>
</table>

*Purified ³H-labeled C3H MMTV (2 x 10^6 cpm) has been added to medium in each of 4 flasks. Two flasks (A and B) contain monolayer cultures of Mm5mt/cl cells while the remaining flasks contained only medium (standard DMEM plus additives or conditioned medium). Medium samples (2.0 ml) were removed at each of the specific times and were fractionated by centrifugation as indicated in “Materials and Methods” into high-speed viral pellets and virus-free supernatants. Fractions were trichloroacetic acid precipitated, and the percent recovery of C3H MMTV was quantitated in the same RIA (either gp52 or p27). Two cell lines were used. The GR-MMTV cell line is derived from GR mouse mammary tumor cells whereas the MMTV(C3H)Fel I line is a feline embryonic kidney cell infected with C3H MMTV.

These results strongly suggest that virion degradation is not a principal pathway for the release of MMTV antigens into the circulation. Even in p27-positive animals, very little similarity in the gp52:p27 ratio was noted when individuals were compared. These differences in antigen concentration ratios may not only reflect differences in the release of these 2 antigens but may also reflect secondary differences in immune response, blood clearance rates, or the presence of serum lytic activity.

The comparative measurement of gp52 and p10 in tumor extracts and mouse sera (2) resulted in ratios of gp52 to p10 ranging from 1.8 to 5.1; however, the average ratio of gp52 to p27 in the present study of CD8F, mice was 27. In both studies, gp52 was more abundant, but the comparatively low ratio of gp52 to p10 may indicate that p10, like gp52, may serve as a more useful marker than p27. Although lower concentrations of p27 were detected, further investigation of the physical state of p27 determinants in plasma is warranted to determine if any alterations in the p27 of plasma might render it more difficult to detect by RIA.

The extension of plasma antigen studies to an in vitro cell culture model has permitted a quantitative assessment of viral antigen release and accumulation in the absence of secondary in vivo considerations. Although the mechanisms of in vivo and in vitro viral antigen release may in part differ, the cell culture model has permitted viral antigen release to be studied in the absence of serum antibodies and potential viral lytic activity.

The levels of 2 MMTV structural proteins have been examined in the plasma of tumor-bearing animals to compare the utility of these proteins as systemic markers for the presence of mammary tumors and to understand more about the processes involved in the release of MMTV antigens from tumor cells. The envelope glycoprotein gp52 has been detected in plasma of tumor-bearing animals both in greater frequency and abundance than the nucleoid protein p27. Although these results confirm the previous report that gp52 levels rise with the onset of tumor (5, 8), they do not consistently demonstrate a similar relationship for p27.

If viral antigen concentrations of plasma were solely the result of budding and degradation of mature virions, the stoichiometric ratio of these 2 proteins in the virion [approximately 1:1 (9, 18)] should be reflected in the concentrations detected in plasma. The marked differences in antigen concentrations, when gp52 and p27 levels were compared, suggests that the production of mature virions may not be a principal pathway for the release of MMTV antigens into the circulation. Even in p27-positive animals, very little similarity in the gp52:p27 ratio was noted when individuals were compared. These differences in antigen concentration ratios may not only reflect differences in the release of these 2 antigens but may also reflect secondary differences in immune response, blood clearance rates, or the presence of serum lytic activity.

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These results strongly suggest that virion degradation is not a principal pathway responsible for the accumulation of non-virion-associated extracellular gp52.

DISCUSSION

The high levels of non-virion-associated gp52 cannot be attributed to virus breakdown during the fractionation of particulate and soluble antigens since this procedure with ³H-labeled virus achieved recoveries in excess of 90%. This finding suggests that the majority of virus-free gp52 detected in culture and plasma samples of tumor-bearing animals does not arise as a result of virion degradation and favors alternative pathways of antigen release such as cell surface shedding. Although experiments with purified ³H-labeled virus demonstrate that
breakdown was minimal, the possibility that a second population of less stable virions are continually released and rapidly degraded is not eliminated by the present experiments.

Quantitation of p27 in culture fluids revealed a lower ratio of soluble to particulate antigen than detected for gp52 (2:1 versus 12:1). Since virion solubilization conditions (0.1 M KCl and 0.1% Triton X-100) were relatively mild to avoid interference in assay, p27 levels obtained in assay represent minimum estimates of virion-associated p27; despite this fact, ratios demonstrated that gp52 was more abundant than p27 as a soluble antigen.

The results obtained herein argue that non-virion-associated viral antigens are present in higher concentrations and, therefore, offer greater practical utility than virion-associated antigens as markers for mammary disease. The use of mammary cell cultures to study viral antigen release should permit a more complete understanding of the factors that regulate the release and accumulation of non-virion-associated antigens. Such studies of soluble MMTV antigens can be extended to include mammary tumor cells that are cloned virus nonproducers. The amount of soluble viral antigen released by nonproducer cells should help to determine if virus production by tumor cells significantly contributes to the concentrations of soluble antigens released in culture and detected in plasma of tumor-bearing animals.

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