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Plasma levels of a viral protein as a diagnostic signal for the presence of tumor: The murine mammary tumor model

(glycoprotein/radioimmune assay/lactation/spontaneous tumor/transplant)

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ABSTRACT **We used the mouse mammary tumor and its associated virus (murine mammary tumor virus) to examine the possibility of using plasma levels of a viral protein (gp52, the glycoprotein of 52,000 molecular weight) as a diagnostic indicator of the presence of a solid tumor. The following features have emerged from our studies: (*a*) tumor-bearing animals show markedly elevated (100–1000 ng/ml) plasma levels of gp52 and the mean concentration increases with tumor size; (*b*) mammary tumor tissues located outside the mammary gland are also detected by the elevated plasma gp52; (*c*) low (2–10 ng/ml) plasma levels of gp52 are found in tumor-free mice, whether they are derived from strains characterized by high or low frequencies of spontaneous mammary tumors; (*d*) tumor-free lactating females exhibit the normally low levels of plasma gp52 despite the fact that their milk contains an average of 20,000 ng/ml of this antigen; (*e*) thus, high levels of plasma gp52 are found only in the presence of tumor and are not induced by either predisposition for the disease or by normal production of the antigen during lactation; (*f*) the circulatory clearance time of gp52 is sufficiently rapid to require continued replenishment to maintain the high levels observed in tumor-bearing animals, a feature implying that the gp52 concentration can be a responsive parameter of disease status.**

The information obtained suggests that plasma gp52 is a potentially useful and specific systemic indicator of the presence and extent of murine mammary neoplasia.

We have chosen the mouse mammary tumor as the experimental model to explore the feasibility of using viral-related proteins in the body fluids as quantitative signals for the presence of solid tumors. Viral antigens have been demonstrated in mammary tumor cell cultures, mammary tumor tissues, and in mouse milk (1–11). Furthermore, the level of viral antigens in the milk correlates with the tumor incidence in the mouse strains examined (5, 11). In similar studies comparing individual mice, the concentrations determined by either immunodiffusion (6) or radioimmune assay (9) indicate that mice with higher levels of the antigens in the milk have a greater tendency to develop tumors. While these studies of milk antigens have been informative, they are not applicable to nonlactating individuals, and it is not evident that the data provide diagnostic information on tumor status.

A more generally useful pathway would look to blood plasma as a source of the antigen and to focus on individual viral proteins rather than on whole particles. To this end we devised (12) a convenient method for the purification in high yield of the two gs antigens (gp52, a viral glycoprotein with a 52,000 molecular weight, and p27) of the murine mammary tumor virus (MuMTV). The availability of the pure proteins made it possible for us to develop (12) radioimmune assays sufficiently sensitive to detect these proteins at a level of 1 ng/ml.

Abbreviations: gp52, viral glycoprotein with a molecular weight of 52,000; MuMTV, murine mammary tumor virus; RIA, radioimmune assay.

The present paper uses these technical advances but confines its attention to gp52. Our aim here is to answer the following questions: (*i*) Can gp52 be detected in the plasma of normal and tumor-bearing mice? (*ii*) Is the level of gp52 diagnostic for the presence of a mammary tumor and indicative of its size? (*iii*) Can the plasma level detect mammary tumor masses located in other parts of the body? (*iv*) How do the plasma gp52 levels compare in lactating and nonlactating tumor-free females? (*v*) Is the plasma gp52 present in particulate or in soluble form? (*vi*) Is any significant proportion of the plasma gp52 complexed to antibody? (*vii*) Is the clearance time of gp52 from the circulatory system adequate to make gp52 a potentially responsive indicator of disease status?

The experiments to be described provide resolutions of these and other related issues. The data obtained encourage the belief that the plasma level of gp52 may be usefully employed as a specific and sensitive signal of the presence and extent of mammary neoplasia.

MATERIALS AND METHODS

Mouse Strains. For the analysis of gp52 plasma concentration, both tumor-positive and negative mice of the following strains were used: C3H/HeJ (Jackson Laboratories, Bar Harbor, Maine), RIII (Columbia University, Institute of Cancer Research), Balb/c MuMTV-infected and $CD8F_1$, a hybrid MuMTV-infected Balb/c and DBA8 (Catholic Medical Center, New York). In addition, normal C57L/J (Jackson Laboratories) and NIH Swiss mice were obtained through the Office of Program Resources and Logistics, National Cancer Institute of the Virus Cancer Program as was the plasma from Balb/c mice infected with murine leukemia virus.

Reproducibility of the Radioimmune Assay (RIA) for gp52 in Plasmas and Absence of Interference with Proteases. The MuMTV gp52, purified by concanavalin A affinity chromatography and iodinated with the ^{125}I-Bolton-Hunter reagent, was used for RIA as previously described (12). The test is a blocking assay in which delayed addition of the labeled antigen is used to maximize sensitivity.

Repeated assays were carried out on plasma aliquots from a mouse bearing a small tumor. The mean value (±1 SD) of 10 replicate determinations was 74.1 ± 6.9 ng/ml which yielded a standard deviation less than 10% of the mean value.

The presence of proteases can yield false positives and spuriously high values for radioimmune assays due to destruction of either the labeled antigen or of the antibody. To check for this possibility, plasma samples from a tumor-bearing mouse were assayed in the presence of increasing amounts of the protease inhibitors Trasylol (up to 1.6×10^3 kallikrein inhibitor units/ml, Mobay Chemical Corporation) and diisopropyl fluorophosphate (up to 4 mg/ml). The observed amount of gp52 (corresponding to 33–34% displacement of the labeled antigen)

was unaffected by the presence of either protease inhibitor at any level tested. Nevertheless, to insure that this type of interference would not unknowingly confuse our assay, Trasylol was always included at a level of 50 units/ml. This amount is sufficient to neutralize the activity of 30 μg of trypsin.

Bleeding Procedure. Mice were bled (approximately 500 μl) from the retro-orbital venus plexus, and the blood was collected in heparin-treated tubes. Plasma was separated from blood by centrifugation (1500 × *g* for 10 min) and rapidly frozen in a dry-ice methanol bath. Plasma samples were then stored at −70° prior to the radioimmunoassay.

Determination of Tumor Weight. Weights were determined by measurement with calipers (two perpendicular measurements). Tumor weights (in mg) were estimated (13) from the empirical relationship, 0.4 (LW^2), where L and W represent, respectively, the length and width of the tumor in millimeters.

Procedure for Determining Blood Clearance Rate of gp52. Purified gp52 was iodinated with ^{125}I-Bolton-Hunter reagent and subjected to Sephadex G-100 chromatography. The purified ^{125}I-gp52 (approximately 0.5 μg), which was more than 90% immune precipitable, was injected into the lateral tail vein of three nontumor and three tumor-bearing $CD8F_1$ females. Blood samples (70 μl) were removed from the ventral tail artery and collected in heparin-treated hematocrit tubes. Samples were collected at 1, 3, 9, 24, 48, and 97 hr. Total ^{125}I-radioactivity in each 70 μl of blood sample was quantitated with a Searle gamma counter. One tumor-bearing mouse died during the course of the experiment and has not been included in the results.

Tumor Transplantation. One spontaneously occurring tumor was obtained from a female $CD8F_1$ mouse and used for all transplants. It should be noted that this tumor is hormone-independent (14), and that consequently males are convenient recipients. A 1 mm square piece of tumor tissue was implanted subcutaneously with a trocar in each of six male $CD8F_1$ hybrid mice. After the development of a moderate-sized tumor in all six, they were bled for analysis of plasma antigen.

RESULTS

The Detection and Quantitation of gp52 in the Plasma of Normal and Tumor-Bearing Mice. Mouse plasma samples obtained from both tumor-bearing and tumor-free mice were assayed as previously described (12) for MuMTV gp52 which employed a blocking radioimmunoassay. Two to four aliquots (10–200 μl) of each bleeding were assayed to determine the concentration of gp52 in the plasma of each mouse. The results obtained under a variety of conditions with six different mouse strains are summarized in Table 1.

If we focus first on comparing normal with tumor-bearing animals, then a striking feature is immediately evident. The first three strains (C3H/HeJ, Paris RIII, and $CD8F_1$-hybrid) all have a high incidence of spontaneous mammary tumors. Nevertheless, it is easy to distinguish the tumor-bearing from the control animals on the basis of the gp52 levels. The next two listed (NIH Swiss and C57 L/J) are characterized by a low spontaneous frequency of breast cancer, and the gp52 levels of the nontumored animals are very much like those observed in comparable individuals in the first three high cancer frequency groups. Thus, elevated plasma levels of gp52 are determined not by the predisposition to the disease but rather by the presence of the tumor.

The last two strains involve Balb/c, which has a low spontaneous frequency of mammary tumors but in which this disease can be readily induced by infection with the mouse mammary tumor virus. It will be noted that infected Balb/c females free of tumors have low values of gp52 (6.3 ng/ml) whereas those with tumors average around 767 ng/ml. Finally, the last strain listed is Balb/c free of breast tumors but made leukemic by infection with Rauscher leukemia virus. It is evident that the presence of this mesenchymal neoplasia introduced no complication in identifying individuals without mammary neoplasias. It would appear that elevated gp52 levels are not associated with malignancy in general but rather with the presence of mammary tumors.

Table 1. gp52 concentration in the plasma of normal and tumor-bearing mice

Group	Mouse strain	Sex and tumor status	No. tested	ng/ml Avg. mouse plasma
1	C3H/HeJ	Male (−)	5	5.6
		Female (−)	10	4.6
		Female (+)	20	570.0
2	Paris RIII	Virgin female (−)	9	9.8
		Pregnant female (−)	9	8.5
		Lactating female (−)	9	13.7
		Female (+)	7	122.0
3	$CD8F_1$ hybrid	Female (−)	10	2.3
		Male (−)	10	2.5
		Female (+)	65	301.0
		Tumor transplant in male (+)	6	407.0
4	NIH Swiss	Male (−)	10	5.5
5	C57 L/J	Female (−)	10	5.6
6	Balb/c MuMTV infected	Female (−)	5	6.3
		Female (+)	11	767.0
7	Balb/c	MuLV (Rauscher) infected mice (10^9–10^{10} particles/ml)	(Plasma pool)	10.4

Radioimmune assays were performed on mouse plasma samples from the strains and numbers indicated. The (+) and (−) indicate the presence and absence of tumors, respectively. Averages were determined for the indicated number of individuals within each category with the exception of group 7 (murine leukemia virus-infected mice) where the average of three determinations of the same plasma pool is recorded.

In summary, in all the strains studied the plasma concentration of gp52 was found to be considerably higher (usually 100-fold or more) in tumor-bearing animals than in tumor-free controls. Even in this rather limited comparison, elevated levels of gp52 appeared to provide an unambiguous indication of the presence of a mammary tumor. Data contained in Table 1 are relevant to questions of tumor localization and other issues that we now discuss separately.

Plasma Levels of gp52 in Animals Carrying Tumor Transplants. It was of obvious interest to determine whether breast tumors located outside the mammary gland could be detected by assays of plasma gp52. For this purpose, $CD8F_1$ hybrid male mice received subcutaneous transplants of tumor tissue derived from a $CD8F_1$ female and all six recipients developed moderate-sized tumors. As may be seen from the results recorded in Table 1 (group 3), the gp52 plasma concentrations of the males with transplanted tumors averaged 200-fold higher than those observed in normal control males and were com-

Table 2. gp52 levels in plasma and milk of lactating $CD8F_1$ mice

Mouse no.	ng/ml of mouse plasma*	ng/ml of milk plasma†
1	9.2	37.50
2	11.8	18.30
3	6.7	3.02
4	9.6	7.26
5	3.4	29.40
6	1.8	22.80
Mean ± SD	7.1 ± 3.5	$19.7 \times 10^3 \pm 11.9 \times 10^3$

* Six mice were bled and milked during a period of lactation.

† "Milk plasma" was made by mixing mouse milk 1:1 with 0.15 M EDTA at pH 7.8 and centrifuging at 10,000 × *g* for 10 min. The "milk plasma" was recovered as the zone between the lipid layer and the pellet of precipitated casein.

parable to the levels found in female mice bearing spontaneous tumors. This outcome demonstrates that mammary tumors localized in sites external to the breast can be detected by plasma gp52 levels. Further, finding elevated gp52 in male mice with breast tumors means that tumor growth and the plasma expression of the specific gp52 are both independent of female hormones.

The Influence of Sex and Lactation on gp52 Levels in Tumor-Free Mice. The tumor-free males and females listed in Table 1 reveal no significant influence of sex on plasma gp52 levels. Of particular interest is the second (Paris RIII) group that compares virgins, pregnant, and lactating females, all free of tumor. There is no evidence that either pregnancy or lactation leads to elevated plasma levels of gp52.

The absence of any effect of lactation on plasma gp52 was particularly surprising since large amounts of virus appear in the milk of the Paris RIII strain, a common and convenient source of the murine mammary tumor virus. It was decided therefore to examine this question directly by comparing gp52 levels in the plasma and the milk of lactating $CD8F_1$ mice, our principal experimental model. From the results summarized in Table 2, it is evident that the very high gp52 concentrations in the milk are not reflected in a corresponding rise in blood plasma levels. The 30,000 to 1 ratio of the concentration of gp52 in milk versus blood plasma would suggest the existence of a highly effective barrier between the mammary gland and the circulatory system, a barrier that is destroyed on tumor development.

Is the Plasma gp52 Particulate or Soluble? Diverse data consistent with the presence of particles in mouse blood have been published (4, 10, 15–17) and the question arises whether any significant portion of the gp52 we were detecting was particle bound. Plasmas from tumor-bearing animals were assayed before and after centrifugation at speeds sufficient to pellet virus particles. From the results recorded in Table 3, it is evident that well over 80% of the plasma gp52 is not particulate by the test applied. Examination of the pellet for gp52 (in the case of the C3H/HeJ) revealed the presence of only 0.96 ng, which is less than 1% of the 12% not recovered.

Table 3. The effect of particle removal from plasma of tumor-bearing mice on gp52 determination

Mouse strain	Mouse plasma (ng/ml)		% of initial level after particle removal
	Before centrifugation	After centrifugation	
$CD8F_1$ hybrid	940	783	83.3
C3H/HeJ	64	56	87.5

Mouse plasma has been assayed for gp52 both before and after centrifugation for 114,000 × *g* for 1 hr. The percent of the initial gp52 activity remaining in the virus-free supernatant following centrifugation is seen in the last column.

Table 4. The effect of immune precipitation of mouse IgG from the plasma of tumor-bearing mice on gp52 determination

Mouse strain	Mouse plasma (ng/ml)		% Recovery in supernatant of immune precipitation*
	Control	Precipitated	
RIII	217	195	90
$CD8F_1$♀	595	551	93
C3H/HeJ	306	278	91
$CD8F_1$♂†	450	419	93

Plasma samples (50 and 100 μl aliquots) of the tumor-bearing mouse strains indicated were subjected to immune precipitation (18 hr at 4°) with goat anti-mouse γ globulin (an amount titered to yield optimal precipitation). An equal amount of plasma was incubated with normal goat serum as a control. Immune complexes were removed by centrifugation (1780 × *g* for 20 min), supernatants were assayed for gp52, and the recovery of gp52 in the supernatants of immune-precipitated samples was compared with the control. The gp52 levels are presented for both control and immune-precipitated samples. The percentage of gp52 recovered in the supernatant after removal of mouse IgG is indicated for each category of tumor mouse tested.

* Recovery is the average recovery for two mice of each category.

† Transplant tumors.

On the Problem of Immune Complexes Involving gp52. The possible presence of antigen-antibody complexes raises questions directly relevant to the quantitative significance of our radioimmune assays for gp52. This issue can be explored by testing the effect on the observed gp52 levels of removing the IgG fraction from the plasma samples being analyzed. This can be achieved by specific immune precipitation of the mouse IgG by the addition of goat anti-mouse γ globulin in amounts determined by prior titration to yield optimal precipitation. The results of such experiments with four different groups of tumor-bearing mice are summarized in Table 4. The recovery of the gp52 after the removal of the immune complexes ranges from 90 to 93% of the concentrations found before precipitation. The results indicate that our radioimmune assay is predominantly measuring free gp52 and is not detectably influenced by the presence, if any, of immune complexes involving gp52.

Clearance of gp52 from the Circulation. The comparative responsiveness of gp52 levels as a parameter for determining disease status is of obvious importance to our ultimate aim. To get some insight into this question, we sought to determine the rate at which gp52 was removed from the circulation, using ^{125}I-gp52 according to the procedure detailed in *Materials and Methods*. The five mice (three tumor-free and two tumor-bearing) used yielded indistinguishable findings and the results are presented as averages at each time point in Fig. 1. Semilogarithmic analysis of the results indicates a rapid clearance (half-life of about 7.5 hr) in the first 2 days followed by a slower (half-life of about 35 hr) removal of the residual 10% in the next 4 days.

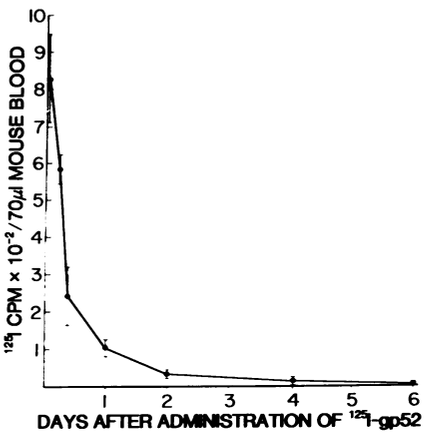

FIG. 1. *In vivo* clearance of ^{125}I-labeled gp52. The ordinate (^{125}I-cpm × 10^{-2}/70 μl of mouse blood) indicates the amount of ^{125}I-label remaining in the blood as a function of time presented on the abscissa. The values indicated at each time point are mean values for five CD8F$_1$ mice. One standard deviation from the mean is indicated at each time point with bars.

The relatively rapid clearance of the gp52 suggests that the maintenance of the elevated plasma levels in tumor-bearing animals requires continued replenishment and augurs well for the usefulness of gp52 plasma concentration as a quantitative monitor of disease status.

Concentration of gp52 as a Function of Tumor Size. Several mice strains were surveyed to determine whether the plasma level of gp52 provided information indicative of tumor size. The most extensive examination was carried out with CD8F$_1$, our principal experimental model, and the results obtained with it are illustrated in Fig. 2 as a semi-logarithmic scatter plot. Table 5 summarizes all the data, including those obtained with C3H/HeJ and Balb/c infected with MuMTV. It is clear from both Fig. 2 and Table 5 that the mean concentration of gp52 in the plasma increases with tumor size in all strains examined. However, the wide range of values within each tumor size class suggests that comparison of individual values with population averages will not be as clinically informative on tumor status as serial assays on the same individual.

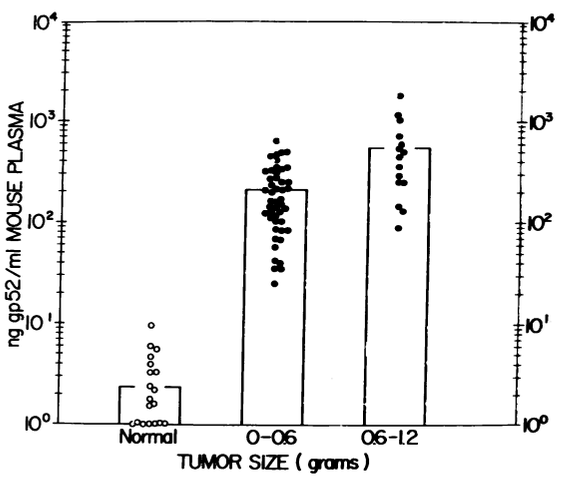

FIG. 2. The gp52 concentration in the plasma of CD8F$_1$ hybrid mice without and with tumors of different size ranges. The ordinate (ng of gp52/ml of plasma) is on a log scale. Open and solid circles indicate the absence and presence of tumors, respectively.

Table 5. gp52 concentration versus tumor size

Mouse strain	Tumor size (g)	No. tested	Mean concentration (ng/ml) Mean	Range
CD8F$_1$	None	20	2.4	(0.0–9.7)
	0.07–0.60	47	210.0	(25–640)
	0.60–1.20	15	555.0	(90–1800)
C3H/HeJ	None	15	4.9	(0.0–0.6)
	0.07–1.00	14	154.0	(6.9–1050)
	1.00–3.00	5	433.0	(82–920)
	3.00–6.00	4	745.0	(192–1500)
MuMTV-infected Balb/c	None	5	6.3	(42.–8.8)
	0.07–2.00	6	235.0	(16–840)
	2.00–7.50	5	1406.0	(320–4000)

Radioimmune assays were carried out on the plasmas of the mouse strains, and with the numbers of animals indicated. The controls without tumors are compared with animals bearing tumors of the size range noted.

DISCUSSION

The experiments described here were designed to examine the possibility of using plasma concentrations of a viral protein to provide information on mammary tumor status. The viral glycoprotein, gp52, is found at markedly elevated levels in the plasmas of animals with mammary tumors and the absolute level is a rough indicator of tumor mass. The fact that subcutaneous transplants also result in increased gp52 concentrations suggests that mammary tumor tissue external to the mammary glands can be detected by this device. It will ultimately be necessary to extend such findings to the clinically more useful situation of natural metastases to other sites. Because, in this instance, the lungs are almost always the first organs involved, it should be readily possible to perform experiments that will yield the desired information.

The fact that lactation does not lead to high plasma levels of gp52 means, of course, that this condition will not generate the confusion of false positives. Although the correlation between gp52 concentration and tumor size is hopeful, it is evident that the wide range of gp52 levels for a given size range means that factors other than tumor mass are playing a role, and these will have to be identified. Experiments involving surgical removal of tumors, and then following tumor regrowth and plasma gp52 levels in individual mice should yield relevant information. In any event, it is clear from the data presented here and other preliminary data that serial assays of individuals will probably provide the most illuminating information on disease status.

The motivations underlying investigations such as those described here are both immediate and long range. The CD8F$_1$ hybrid system has already proved (14) its value as a predictive guide for exploring various therapeutic modalities for the most effective management of human breast cancer. Our immediate goal is to provide a systemic measure of disease status during therapy. Hopefully this will augment the value of the mouse model and in the process catalyze a more rapid accumulation of clinically useful information. In this connection, it is obviously necessary to explore the usefulness of other viral-related proteins as diagnostic signals. Further, if very disparate levels of expression in the plasma are found for different viral proteins, it will be necessary to discover the underlying mechanisms involved.

The knowledge and understanding gained from such studies of the mouse mammary tumor model should help us attain our ultimate objective, the identification of a correspondingly useful

particle-related protein in the corresponding human disease. The fact that we have demonstrated (18–20) the presence in human breast cancers of particles possessing biochemical features very similar to those of the murine mammary tumor virus makes this approach towards a specific diagnostic test sufficiently plausible to warrant further efforts.

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