Therapeutic Effect Reflected by Plasma Levels of a Viral Protein during Combination Chemotherapeutic Treatment of Mammary Tumor-bearing Mice

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ABSTRACT

Plasma concentrations of gp52, a M, 52,000 glycoprotein of the mouse mammary tumor virus, have been measured during cyclophosphamide, doxorubicin (adriamycin) and 5-fluorouracil (CAF) treatment of mammary tumor-bearing CD8F, mice. The value of plasma concentrations of gp52 as an indicator of CAF-mediated changes in tumor status was supported by each of the following findings: (a) CAF treatment did not interfere with the detection of elevated viral antigen levels in the plasma of tumor-bearing mice; (b) at 9-11 days after initiation of treatment, a significantly lower mean gp52 level, observed in the group of CAF-treated mice, provided definitive evidence of therapeutic effect; and (c) serial determinations of plasma gp52 levels in individual mice before, during, and after treatment provided a relative measure of therapeutic effect for each individual that was a reflection of corresponding changes in tumor size. Changes in viral antigen levels (i.e., decrease or increase) reflected inhibited tumor growth, as well as tumor regression. These findings demonstrate that plasma concentrations of gp52 can be utilized to provide an alternative measure of therapeutic effect in CAF-treated mice bearing significant tumor loads.

INTRODUCTION

Studies with the murine mammary tumor and its associated virus MMTV4 have demonstrated that elevated plasma levels of viral glycoprotein (gp52) mark the presence of mammary tumors (1, 2). Moreover, surgical excision of mammary tumors resulted in a sharp drop (10-100-fold) in the gp52 concentration of plasma. This finding indicated that the mammary tumor was the principal source of plasma gp52 (3). When viral antigen levels were monitored during a post-operative period of tumor recurrence, regrowths were detected, in some instances prior to palpation, by increases in plasma levels of gp52, and subsequent tumor growth was coupled with further increases in gp52 concentrations (3).

This ability to detect changes in tumor status suggested that gp52 levels during chemotherapy might provide a rapid measure of therapeutic effect. The quantitation of viral antigen in plasma would then provide a means for determining the relative effectiveness of alternative treatments. The present study has incorporated CAF treatment to determine if combination chemotherapy interfered with the use of plasma gp52 concentrations as systemic markers for changing tumor status. If interference was not encountered, plasma viral antigen levels could be evaluated to determine if they provided a measure of success or failure for each animal treated. In addition, monitoring plasma viral antigen levels of individual mice provided an opportunity to determine the length of time required for altered levels of gp52 to become reliable indicators of therapeutic effect.

The results obtained herein have demonstrated that plasma levels of gp52 can be utilized effectively during CAF treatment to provide an accurate measure of therapeutic effect for individual animals bearing significant tumor loads.

MATERIALS AND METHODS

Spontaneous Mammary Tumors. MMTV-infected hybrid (BALB/c x DBA/2)F, mice, designated CAF, were obtained and used as described previously (2-5) as a convenient source of spontaneous mammary tumors. CAF, females with a single spontaneous mammary tumor (100-1000 mg) were utilized for combination chemotherapy studies.

Combination Chemotherapy. Forty female mice with spontaneous mammary tumors (100-1000 mg) were selected and distributed into a control and experimental group so that mice with approximately equal tumor sizes were represented in each group of 20 mice. The experimental group received four i.p. injections of cyclophosphamide (50 mg/kg), doxorubicin (2 mg/kg), and 5-fluorouracil (50 mg/kg), subsequently abbreviated CAF. Note that an A for Adriamycin (doxorubicin) is used in the acronym for this drug combination. Following the initial drug treatment (day 0), drugs were administered once/week for 3 weeks. Control animals received the same course of saline i.p. injections. Treated and control animals were bled on day 0 to obtain a pretreatment antigen level. Following the initiation of CAF treatment, mice were bled in control and treated groups on days 1 and 3 to obtain an early treatment antigen level, on days 9 and 11 to obtain an intermediate treatment antigen level, and on days 24 and 37 to obtain late treatment antigen levels. Therapeutic effect was assessed at various times during the course of treatment by comparing measurements of both tumor mass and plasma viral antigen (gp52) level in treated and control populations.

The levels of gp52 in coded plasma samples collected before, during, and after CAF treatment were determined by gp52 radioimmunoassay in one virology laboratory, whereas chemotherapeutic treatment and the evaluation of tumor status were performed by measurement of tumor size in a second separate chemotherapy laboratory. Therefore, data were collected in a double-blind fashion to provide an unbiased picture of the relationship between plasma levels of gp52 and CAF-mediated changes in tumor status.

Blood Samples. Approximately 500 μl of blood were removed from the retro-orbital venous plexus using heparinized tubes. Blood samples were collected at varied intervals from day 0 to day 37 after initiation of treatment. Plasma was separated from cells by low speed centrifugation (1780 x g for 15 min). The plasma samples obtained were frozen rapidly in a dry ice methanol bath and then stored at −70°C for radioimmunoassay measurement.

Radioimmunoassay of MMTV gp52. Viral antigen (gp52) was purified by concanavalin-A affinity chromatography, iodinated with 125I-Botton Hunter reagent, and used for radioimmunoassay as described previously (6). A blocking radioimmunoassay was utilized to maximize the sensitivity through suppression of nonspecific binding and to determine the existence of an antibody response to gp52.

This work was supported by Grant VC-283 of the American Cancer Society, the Willie Mae Darwin Memorial Grant for Cancer Research, USPHS Grant CA 28305-01, Grant 1 P01 CA 25842, Grant CA 38335-01, and Contract GP71016.

Received 7/1/81; revised 8/1/85; accepted 8/9/85.

The abbreviations used are MMTV, mouse mammary tumor virus; gp52, M, 52,000 envelope glycoprotein; CAP, cyclophosphamide, doxorubicin (Adriamycin), and 5-fluorouracil (6). A blocking radioimmunoassay was utilized to maximize the sensitivity...
of the measurement. This assay was performed in the presence of the protease inhibitor Trasylol (500 Kallikrein Inhibitor Units/ml). Use of Trasylol and diisopropyl fluorophosphate demonstrated that proteases did not interfere with the assay (2). Assay variation was assessed for triplicate assays of 39 plasma samples from all experimental and control animals at an intermediate (9-11-day) point in therapy. The standard error of the mean for 92% of these different mouse plasmas was less than 10%. The maximum SE, detected for only a single sample, was 14%.

Statistical Methods. To obtain the least bias in statistical assumptions and generate valid conclusions about the data distributions of treated and control animals, a non-parametric test, the Mann-Whitney U-test, has been applied to determine if control and treated populations differ significantly (7). Viral antigen levels and tumor mass measurements will be held statistically significant in a two-tailed Mann-Whitney U-test at P ≤ 0.05.

Determination of Tumor Weight. Tumors were measured at each palpation with calipers (two perpendicular measurements). The weights were then estimated using the formula \( mg = 0.5 (L \times W)^2 \), where \( L \) and \( W \) represent, respectively, the length and width of the tumor in mm (6). Thirteen individual assessments of tumor mass were made for each surviving animal. Determinations on bleeding days (0,1,3,9,11,24, and 37) were used for comparison with plasma gp52 concentrations.

RESULTS

Effect of CAF Treatment on Plasma gp52 Levels in Mice Bearing Spontaneous Breast Tumors. Combination CAF therapy was selected for this study primarily because it is a proven and tested regimen for treating both murine and human mammary tumors (9-11). This combination chemotherapy protocol was derived by selecting 3 individual drugs having differing mechanisms of action which each singly demonstrated antitumor activity. Cyclophosphamide, a form of nitrogen mustard, causes alkylation primarily at guanine residues and induces DNA strand breaks. Doxorubicin (formerly Adriamycin) is an anti-cancer antibiotic which intercalates between DNA bases and binds tightly to DNA. The third drug in this combination, 5-fluorouracil, is an anti-metabolite and base analogue of uracil which inhibits DNA synthesis and is incorporated into cellular RNAs as well. Combination CAF therapy was selected over single agent therapy to minimize the number of drug-resistant cells during treatment and to maximize anti-tumor activity against a heterogeneous population of spontaneous mammary tumor cells that demonstrate different sensitivities to single agents.

This effective cyto-reductive therapy and the ability to quantitate MMTV gp52 in the plasma of tumor-bearing animals permitted this study to focus on the following objectives: (a) to determine if combination chemotherapy would interfere with the detection of gp52 in the plasmas of tumor-bearing mice, and (b) if interference was not encountered, to determine if changing gp52 levels of plasma would provide a measure of therapeutic effect.

To study the relationship between plasma levels of gp52 and the effectiveness of CAF treatment, 40 female CDBF mice with spontaneous breast tumors (100-1000 mg) were selected and distributed into two groups so that mice with approximately equal size tumors were represented in each group. Twenty mice received saline (saline controls), and 20 mice were treated (i.p.) with CAF at day 0, 7, 14, and 21. As an initial step following division of mice into a control and experimental group, plasma gp52 levels of these pretreatment tumor-bearing animals were determined. This comparison of tumor-bearing controls and tumor-bearing experimental animals provided the opportunity to determine whether this division of mice bearing spontaneous mammary tumors would produce similar or statistically different distributions of plasma gp52 levels. Since a notably dissimilar distribution of viral antigen levels in pretreatment controls, as compared to CAF-treated animals, might mask subsequent CAF effects on gp52 levels, this initial comparison of viral gp52 levels was important in establishing the similarity of control and experimental groups prior to treatment. The results of these gp52 determinations are presented in Chart 1 as a semi-logarithmic scatter plot of gp52 levels for both the control and CAF-treatment group. The results presented in Chart 1 clearly demonstrate an elevated level of gp52 in both control and treated animals that was consistent with the presence of tumor. Levels in both groups can be compared with previous determinations for tumor-free female mice of this strain which have an average of only 7 ng of gp52/per ml of plasma (2). Comparison of gp52 levels from the control group (mean, 77 ng/ml) and the CAF-treatment group (mean, 102 ng/ml) indicates that these two groups have similar antigen ranges, and further analysis by Mann-Whitney U-test indicates that statistically significant differences in pretreatment gp52 levels or tumor masses are not present.

The similarity in pretreatment antigen levels permitted further analysis of CAF-mediated effects on viral antigen levels. Posttreatment plasma antigen levels of individual control and treated animals measured early (1 or 3 days after initiation of treatment), at an intermediate time (9 or 11 days after initiation of treatment), or late (24 days after initiation of treatment) are presented in Chart 2 as a semi-logarithmic scatter plot. Mean gp52 levels of plasma at each time point have been indicated with horizontal lines to provide a comparative measurement of viral antigen concentration for control and CAF-treated mice. Early mean gp52 determinations demonstrated a drop in viral antigen levels when
Plasma levels of gp52 for both treated and control animals were determined on day 1 and day 3, intermediate in treatment (day 9 and 11), and at a late time, 3 days after terminating therapy on day 24. Plasma levels of gp52 for both treated and control animals have been presented as a semi-logarithmic scatter plot of gp52 concentrations versus time after the initiation of CAF treatment. The mean gp52 levels are represented by horizontal lines (control, solid line and CAF-treated, zigzag line). While no statistically significant difference in control and treated groups was demonstrated by Mann-Whitney U-test at the early time point, statistically significant differences in gp52 levels were noted at the intermediate point ($P < 0.02$) and the late time point ($P < 0.02$). Note that 1 intermediate CAF sample was lost, 4 deaths occurred in the control population, and 1 death occurred in the experimental population by day 24.

Comparison of late gp52 mean levels on day 24 (three days after termination of treatment) revealed a substantial increase in antigen levels of controls and treated animals. However, CAF-treated animals still maintained a lower mean gp52 level (289 ng/ml) than did control animals (780 ng/ml), and the difference between these groups proved to be significant ($P < 0.02$) at this late time point. Furthermore, the only mice at this late time point to retain gp52 levels less than 150 ng/ml were all CAF-treated individuals.

Delay in Re-Establishment of Initial Plasma Levels of Viral Antigen After Pretreatment Bleeding. As indicated above, the pretreatment bleeding resulted in a diminished level of viral antigen in the plasma of both control and treated animals as determined on day 1 and day 3. This decrease in plasma levels of gp52 provided the opportunity to compare the time required for control and CAF-treated animals to re-establish initial gp52 levels. Therefore, the extent of delay detected in CAF-treated animals could be utilized as a measure of CAF effect on viral antigen levels. The number of days required for each control and CAF-treated animal to re-establish initial pretreatment gp52 levels is presented by group as scatter plots in Chart 3. Controls re-established gp52 levels by 3.9 days, which was notably shorter than the 13.2 days required in CAF-treated animals ($P < 0.002$, Mann Whitney U-test). These results further demonstrate compared to pretreatment levels. This decrease was observed in both control (76 ng/ml to 53 ng/ml) and CAF-treated animals (102 ng/ml to 51 ng/ml) and appeared to be a consequence of the short interval between the pretreatment and early bleedings rather than a consequence of the treatment per se. This short bleeding interval affected only the early time point. At this time point in treatment, the mean gp52 level of controls (53 ng/ml) was nearly identical to the mean of CAF-treated animals (51 ng/ml) (Mann Whitney U-test failed to demonstrate any significant difference in gp52 levels between the control and treated groups at this early time). However, with the continuation of therapy, the effect of CAF treatment on gp52 levels could be detected easily. At intermediate times in therapy (day 9 and day 11) gp52 levels in plasma from control animals were rising to a new and substantially higher mean level (240 ng/ml), whereas CAF-treated mice retained a mean antigen level (66 ng/ml) which was lower than pretreatment levels of either the control or experimental group. The difference in antigen levels between groups was held to be significant ($P < 0.02$) at this intermediate point in therapy. Comparison of late gp52 mean levels on day 24 (three days after termination of treatment) revealed a substantial increase in antigen levels of controls and treated animals. However, CAF-treated animals still maintained a lower mean gp52 level (289 ng/ml) than did control animals (780 ng/ml), and the difference between these groups proved to be significant ($P < 0.02$) at this late time point. Furthermore, the only mice at this late time point to retain gp52 levels less than 150 ng/ml were all CAF-treated individuals.

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Chart 2: Plasma levels of gp52 during CAF treatment. CDBF, female mice with spontaneous mammary tumors received CAF injections (CAF-treated) or saline injections (controls) on day 0 and at 7-day intervals for 3 weeks. Mice in each group were bled and assayed for gp52 early in treatment (day 1 and 3), intermediate in treatment (day 9 and 11), and at a late time, 3 days after terminating therapy on day 24. Plasma levels of gp52 for both treated and control animals have been presented as a semi-logarithmic scatter plot of gp52 concentrations versus time after the initiation of CAF treatment. •, control animals; O, treated animals. The mean gp52 levels are represented by horizontal lines (control, solid line and CAF-treated, zigzag line). While no statistically significant difference in control and treated groups was demonstrated by Mann-Whitney U-test at the early time point, statistically significant differences in gp52 levels were noted at the intermediate point ($P < 0.02$) and the late time point ($P < 0.02$). Note that 1 intermediate CAF sample was lost, 4 deaths occurred in the control population, and 1 death occurred in the experimental population by day 24.

Chart 3: Time required to re-establish initial plasma levels of viral antigen. Mice bearing spontaneous mammary tumors were bled for gp52 determination just prior to CAF treatment and at short intervals (1 and 3 days) after initiation of treatment. The majority of animals exhibited a decrease in the concentration of viral antigen in plasma as a result of blood loss at the initial bleeding. Control and CAF-treated animals have been evaluated individually and as groups to determine the number of days required to re-establish initial plasma levels of gp52. While individual delays are presented as scatter plots for control and treated animals, the mean number of days required to re-establish initial gp52 levels for the control group and CAF-treated group is depicted by histogram. The difference between groups in time required to re-establish initial gp52 levels was demonstrated to be significant by Mann-Whitney U-test ($P < 0.002$).
that CAF treatment delays the expected rise in gp52 level that accompanies continued tumor growth (2,3).

Concentration of gp52 as a Function of CAF-mediated Changes in Tumor Mass. To ascertain whether or not the influence of CAF on gp52 levels was a reflection of its cytoprotective effect on tumor mass, both tumor mass and antigen level were compared during the course of CAF treatment. Both average antigen level (solid line) and average tumor mass (interrupted line) have been plotted as a function of time after treatment for both control and CAF-treated mice in Chart 4. CAF treatment resulted in a profound inhibition of tumor growth which was found to be significantly different from that of the control group (P ≤ 0.002) at both intermediate and late times. Importantly, the pattern of changing viral antigen level during the course of chemotherapy was found to reflect the changing tumor mass in each group and therefore provided an accurate measurement of the therapeutic effect.

Assessment of gp52 Levels and Tumor Status in Individual Mice. Evaluation of tumor sizes and gp52 levels for groups of control and CAF-treated mice has supported the hypothesis that chemotherapy induced an inhibition of tumor growth and a relative reduction in tumor mass which was reflected in diminished levels of gp52 in plasma. More insight into this relationship can be gained by an analysis of individual animals. Although tumor growth rate in the group of mice treated with CAF chemotherapy was significantly inhibited in comparison to saline-treated controls, the magnitude of the antitumor effect in individual mice was variable in this group of heterogeneous spontaneous tumors, with some tumors showing considerable increase in size during treatment, while others showed little increase in size, and still others showed a decrease in size compared to tumor measurements taken just prior to the initiation of treatment. Measurement of both tumor mass and plasma gp52 levels in individual mice demonstrated that all control animals and the majority of CAF-treated animals were characterized at each time of bleeding by coordinate increases or decreases in both tumor mass and the gp52 concentration of plasma. Results from 3 representative CAF-treated mice are shown in Chart 5A. Tumors in these mice increased in size during therapy at different rates with one increasing slightly (<200 mg), one increasing a moderate amount (about 400 mg), and one showing a substantial increase (almost 2000 mg). Note that the plasma gp52 level in each mouse shows a coordinate increase that was proportional to the increase in tumor mass in that particular mouse at each of the 4 observation points. Results from another CAF-treated mouse are shown in Chart 5B. The tumor in this mouse decreased in size during chemotherapy. Again this change in tumor mass was accurately reflected in decreasing plasma gp52 concentration. These results demonstrate that gp52 levels reflected changes in tumor status during inhibited tumor growth, as well as tumor regression.

The ability of serial gp52 determinations to accurately reflect both increases and decreases in tumor size for the entire population of CAF-treated animals has demonstrated that for this treatment regimen, viral antigen levels can successfully be utilized as an alternative measure of therapeutic effect.

DISCUSSION

This study has addressed the possibility of utilizing plasma concentrations of a viral protein to monitor the effectiveness of
combination chemotherapy for a solid tumor. The CAF protocol tested has already demonstrated its value as an effective treatment in the management of human breast cancer (9, 10) and of murine breast cancer (11); therefore, this treatment provided a good starting point to determine if plasma levels of gp52 could be utilized to provide a relative measure of therapeutic effect. The ability to successfully utilize plasma concentrations of gp52 for monitoring disease status and evaluating the effectiveness of CAF-treatment is attested to by the following observations: (a) During treatment with the chemotherapeutic agents CAF, tumors that grew despite CAF treatment could be identified by concomitantly rising plasma levels of gp52, which indicates that chemotherapy did not interfere with the synthesis and release of this viral antigen (see Chart 5); (b) A delayed elevation in the plasma concentration of gp52 during therapy provided an indication that CAF-treatment would result in a therapeutic effect; (c) By intermediate times in therapy (days 9 and 11), the difference between plasma gp52 levels of control and treated mice definitively demonstrated the magnitude of the therapeutic effectiveness of CAF treatment; and (d) Coordinate changes in tumor mass and viral antigen levels permitted gp52 levels to be used as a means of monitoring changes in tumor status, providing an individualized measure of therapeutic effect.

During the course of this study, 93 separate, simultaneous measurements of tumor size and plasma gp52 level in individual mice were performed. Eighty-eight of these measurements (95%) showed a coordinated rise or fall in both parameters in each mouse at each time point. In 5 samples from CAF-treated animals, which were serially monitored for plasma gp52 levels before, during, and after therapy, a single observation in time was noted which demonstrated either an increase or decrease in viral antigen level which was not immediately reflected at that time point in measurements of tumor mass. These exceptional transient observations of antigen change may be the result of a sudden and sufficient change in tumor cell death which results in the release of excess cellular gp52 into the bloodstream more rapidly than one could detect by immediate change in tumor mass. However, in each case, all subsequent or prior gp52 determinations in plasma from that animal provided an accurate measure of tumor status which was substantiated by tumor mass measurement and which was indicative of the extent of disease progression.

The ability to utilize plasma concentrations of gp52 in assessing the heterogeneity of chemotherapeutic effect (inhibited tumor growth, as well as tumor regression) has demonstrated that gp52 is an accurate responsive indicator of CAF chemotherapeutic-induced changes in tumor status. The present results have been obtained for and conclusions can only be drawn directly for one chemotherapeutic protocol, namely CAF, and one therapeutic setting, mice bearing significant tumor loads; however, by examining different chemotherapeutic protocols and by monitoring mice during surgical adjuvant therapy, future experiments should help to determine if gp52 plasma levels have broader utility as an alternative measure of chemotherapeutic effect.

Previous data obtained following surgical removal of tumor demonstrated that plasma gp52 levels could be used to monitor disease status in mice suffering mammary tumor regrowths (3). The results of the present study parallel and reaffirm this ability to use plasma gp52 levels to assess mammary tumor status in mice, while the sensitivity of gp52 levels to changes in tumor mass and the rapidity with which results can be obtained suggest that such measurements might in addition provide an efficient way to monitor the comparative therapeutic efficacy of different chemotherapeutic drugs or drug combinations. Furthermore, such measurements could provide an indication of chemotherapeutic activity against metastatic lesions following surgical removal of primary tumors which, at present, can be measured only by examining lungs at sacrifice in this murine tumor model.

It is hoped that these studies with murine breast tumors will provide a model for testing the feasibility of clinically using plasma concentrations of a systemic marker protein to monitor chemotherapy effect. The identification of similarly useful marker proteins in human tumor systems would then permit one to test this approach to disease management with human cancers. In any event, it is anticipated that future studies with MMTV gp52 in the murine tumor model should facilitate the search for more effective chemotherapy.

ACKNOWLEDGMENTS

We thank the Office of Program Resources of the National Cancer Institute for supplying MMTV and express our appreciation to L. Stolfi, H. Hue, and V. D’ella for excellent technical assistance.

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