AD_____

Award Number: DAMD17-94-J-4459

TITLE: A New Immunologic Method for Detection of Occult Breast Cancer

PRINCIPAL INVESTIGATOR: Rishab K. Gupta, Ph.D.

CONTRACTING ORGANIZATION: John Wayne Institute for Cancer Treatment and Research
Santa Monica, California 90404

REPORT DATE: September 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSPECTED 4

20001010 073

PII Redacted

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1999	Final (1 Oct 94 - 31 Aug 99)

4. TITLE AND SUBTITLE
A New Immunologic Method for Detection of Occult Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4459

6. AUTHOR(S)
Rishab K. Gupta, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
John Wayne Institute for Cancer Treatment and Research
Santa Monica, California 90404

E-MAIL:
guptar@jwci.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

These investigations were undertaken to develop an immunologic assay using a 90kD glycoprotein antigen (TA-90) expressed by breast cancer cells and a murine monoclonal antibody to TA-90, which is immunogenic in cancer patients. This novel immunologic assay can detect TA-90 in serum of breast cancer patients even when present as immune complexes. This was confirmed by generating TA-90 specific immune complexes (IC) by reacting purified TA-90 with anti-TA-90 IgG antibodies and subjecting to the immunologic assay (TA-90-IC). Analysis of pre- and post-operative serum samples of breast cancer patients by this assay and correlation of results with clinical follow-up revealed that the TA-90-IC assay is useful in identifying those breast cancer patients who harbor occult micro-metastases, or those patients who are at high risk of developing recurrent disease after surgical resection of their tumor. The assay was also compared with mammography as a screening tool for early breast cancer. Of the 138 patients studied, 109 had an abnormal mammogram, and 29 had a normal mammogram. There was a significant difference in TA-90-IC values between the 82 patients with benign lesions and the 42 patients with invasive carcinoma ($p<0.0005$). Therefore, serum TA90-IC assay appears to be a useful adjunct to mammography. Comparison of the TA-90-IC results in breast cancer patients with evidence of disease with results of CEA and CA15-3 revealed that the incidence of abnormal values was increased to 91% when a sample was considered positive for either of the markers. Thus, use of the TA-90-IC marker in conjunction with CEA and/or CA15-3 may prove to be more sensistive than when used alone for immunodiagnosis and immunoprognosis.

14. SUBJECT TERMS
Immunodiagnosis, Tumor Antigens, Immune Complexes, Monoclonal Antibodies, Immunoprognosis, Early Detection, Humans, Anatomical Samples

15. NUMBER OF PAGES
150

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

X RKG Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

X In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Rishab K. Gupta 9-28-99

PI - Signature Date

TABLE OF CONTENTS

FRONT COVER 1

SF 298 REPORT DOCUMENTATION PAGE 2

FOREWORD 3

TABLE OF CONTENTS 4

INTRODUCTION 5

BODY:

Materials and Methods 7

Results 12

Discussion 19

KEY RESEARCH ACCOMPLISHMENTS 23

REPORTABLE OUTCOMES 24

CONCLUSION 26

REFERENCES 27

APPENDIX:

Tables (14) 34-47

Figures (20) 48-64

INTRODUCTION

The overall objective of this research proposal was to develop laboratory tests that are meaningful diagnostic/prognostic indicators for the physician's use in the battle against human breast cancer. We worked towards the establishment of sensitive immunologic assays, which could reliably detect and quantify antigen-specific IC in the sera of patients with breast cancer. Therefore, our objectives were to define breast tumor associated antigens to which patients respond immunologically, and develop human and murine monoclonal antibodies to these immunogenic breast cancer antigens. It was expected that human monoclonal antibodies would be used to increase the level of tumor-associated antigen (TAA)-specific IC in samples from breast cancer patients who might have no or low levels of anti-tumor antibodies, and thus no or low levels of TAA-specific IC. We also proposed to develop an IC detection assay that would be specific for tumor associated antigen. Development of such methodologies was expected to greatly increase the diagnostic and prognostic significance of IC measurement. Also, our objective was to apply this technology to answer the following specific questions:

a. Do circulating IC values increase before clinically detectable tumor recurrence in breast cancer patients? If they do, how far in advance?

b. Can changes in antigen-specific IC levels be used as effective indicators of successful radiotherapy and chemotherapy?

c. How frequently are TAA-specific IC elevated for patients with newly diagnosed breast cancer?

d. Can the TAA-specific IC detection assay be of potential usefulness in screening women with high risk of developing cancer for early breast cancer?

We expected that these questions would be answered by analyzing sequential serum samples of patients with breast cancer and age matched normal women in a sensitive immunologic assay that detected tumor cell products. After review and approval of the grant, the scope of the proposal was reduced on August 18, 1994, to answer the above questions and obtain meaningful results within the approved duration of this proposal. We are pleased to report that significant progress has been made in each of the approved area of investigations.

BODY

SUMMARY OF THE SCOPE OF WORK (SOW):

The actual scope of this research project in terms of SOW is smaller than that proposed originally. This was done in response to a communication of July 29, 1994, from the U.S. Army Medical Research Acquisition Activity to accommodate the reviewer's comments of the original proposal. The changed SOW is relisted below:

Task 1. Identify breast TAA that are expressed by breast cancer cells and are immunogenic in breast cancer patients:

a. Analyze sera from breast cancer patients and control groups in a systematic manner for the levels of IC by PEG-CIC assay to select positive sera.

b. Isolate antibodies (IgG) from sera of breast cancer patients that react specifically with breast carcinoma cells at high titers after absorption with appropriate control cells, and use the antibodies in affinity chromatographic procedures to purify TAA from breast carcinoma cells grown in serum free medium.

Task 2. Use the purified TAA to develop monoclonal antibodies and TAA-specific IC assays:

a. Develop murine monoclonal antibodies.

b. Develop TAA-specific IC detection assay.

c. Isolate B-cells from the blood of breast cancer patients obtained at the time of high antibody levels, transform with Epstein Barr virus, and clone anti-TAA antibody producing cells.

Task 3. Use murine and human anti-TAA antibodies to develop assays to detect TAA in serum of breast cancer patients:

a. Glycoprotein TAA-specific IC results will be compared to CEA and CA15-3 results.

b. The assay results will be correlated with clinical course (recurrence and treatment) of patients, and used for early detection of breast cancer.

Following is the progress made under each Task of this research project. Clearly, virtually all of the Tasks under the SOW have been completed. Specifically, we have completed both aspects (1a and 1b) of Task 1, and all aspects of Task 2 and Task 3 of the SOW. In addition, we have optimized a TAA-specific-IC detection assay that can be successfully applied to identify patients who may be harboring the occult breast cancer (Task 2b). We have isolated and immortalized B-cells from breast cancer patients (Task 2c). At least one of the lymphoblastoid cell line (LCL-4) continues to secrete IgM antibody that reacts with breast cancer cell extracts. We have documented that the immunologic assay developed under this grant is a potentially useful screening marker for patients with early breast cancer and appears to be a useful adjunct to mammography. Furthermore, prospective application of the immunologic assay in conjunction with mammography confirmed that accuracy of the mammographic screening could be improved by the addition of this simple immunologic test that measures 90kD tumor antigen specific immune complex (TA-90-IC) levels in blood of the test subject. For the reason of simplicity and convenience, we have broken down the BODY into "Materials and Methods", "Results", and "Discussion" sections under each Task.

MATERIALS AND METHODS:

TASK1.

a. Analysis and Selection of Breast Cancer Patient's sera for Immune Complexes (IC):

Serum and lymphocyte samples were obtained from breast cancer patients when they visited our clinic. The serum samples were kept frozen at -35C and lymphocytes were viably cryopreserved in liquid nitrogen in the specimen bank. The clinical information on these patients was maintained in a database. These retrospectively procured serum samples were used in the investigations described below to comply with the Statement of Work (SOW) of this grant.

PEG-IC assay to detect antigen non-specific immune complexes was performed as described by Riha et al (*1*) and Digeon et al (*2*). This assay was also used to select serum samples that were positive for immune complexes. In addition to TAA-specific-IC determination, the serum samples were also used as the source of antibody to determine the presence of antigen(s) in breast cancer and control cell line extracts by ELISA and Western blot technique.

Ninety serum samples from breast cancer patients taken post-operatively were used as the antibody source against ultrasonically disrupted breast cancer and control cell extracts in a conventional ELISA. Wells of the ELISA plate were sensitized with 200ug protein of the cell extracts. Ten serum samples from self-proclaimed healthy females were used as controls. Alkaline phosphatase conjugated goat anti-human IgG was used as the detecting reagent. The antibody titers of the sera from breast cancer patients ranged from less than 1:100 to 1:12,800; whereas, level of reactivity in the control normal sera was 1:300 or less. The serum samples were pre-adsorbed with human normal peripheral blood lymphocytes (equal packed cell volume) to eliminate any reactivity due to histocompatibility antigens.

b. Isolation of IgG from serum of a Breast Cancer Patient and Purification of TAA:

One of the breast cancer patient's serum which exhibited high antibody level in ELISA was used to isolate and purify IgG antibodies by DEAE Affi-Gel blue column chromatography (*3*). Five ml serum was dialized against 0.02M K_2HPO_4 supplemented with 0.02% sodium azide at pH 8.0. The dialyzed serum was applied to a 20 ml bed volume of the gel equilibrated with the phosphate buffer. The column was flushed with 40 ml of the phosphate buffer. The effluent containing IgG was collected, concentrated to 5 ml by ultrafiltration and analyzed for IgG concentration by radial immunodiffusion. The IgG concentration was 6.1 mg/ml. This purified IgG antibody fraction was used for its reactivity to tumor (breast and other cancer) and normal cell lysates prepared by NP-40 extracts in Western blot.

Five breast cancer, one colon cancer, one melanoma, one sarcoma, and one normal fibroblastic cell lines were grown in RPMI-1640 supplemented with 10% fetal calf serum and antibiotics. Cells were cultured as monolayer in T150 flasks. Cells were harvested by scrapping when growth reached about 80% confluency. The cells were washed five times with ice cold PBS (0.025M phosphate buffer supplemented with 0.15M sodium chloride at pH 7.2). The washed cells (5×10^6) were pelleted and lysed with 1.0% NP-40 in 50mM Tris and 150mM sodium chloride buffer (pH

8.0) for 30 minutes. The lysed cells were centrifuged at 10,000 x g for 10 minutes at 4C. The supernate was further centrifuged at 36,000 x g for 30 minutes, and the clear supernatant was used as target antigen in Western blot (*4*) following separation by SDS-PAGE as described by Laemmli (*5*). Alkaline phosphatase conjugated goat anti-human IgG was used as the detecting reagent, and the reactivity was visualized by NBT/BCIP substrate. To obviate the problems due to non-specific protein-protein interactions, the target antigen was pre-cleared with immobilized protein-A (Sigma Chemical Co., Saint Louis, MO).

Since urine samples of several breast cancer patients were positive for the glycoprotein TAA, we obtained 24 hour urine collections from the positive patients. The pool was used as the starting material to purify the antigen. Collection of urine was done in Tris-HCl (pH 8.3) buffer supplemented with sodium azide to give a final concentration of 0.1 M Tris and 0.02% azide, and processed as outlined in Figure 1 of the Appendix. A total volume of 14.4 liters of urine was centrifuged at 6,000 x g for 10 min and filtered through #1 Whatman filter paper. The clarified sample was concentrated 100-fold using a hollow-fiber concentrator (10kD exclusion limit, Amicon Corporation, Danvers, Mass.) and then subjected to pressure ultra-filtration through a PM-10 membrane (10kD cutoff limit, Amicon Corporation). The concentrate was centrifuged at 800 x g for 10 min. The clearified supernate was subjected to gel filtration chromatography through a calibrated Sephacryl S-200 column (1.5 X 100 cm). Sodium phosphate (0.025 M, pH 7.2) buffer supplemented with 0.15 M NaCl and 0.02% sodium azide (PBS) was used as the eluent at a flow rate of 20 ml per hour. Five ml fraction per tube were collected and the elution of the protein was monitored at 280_{nm} (Figure 2). Fractions exhibiting greater than 0.1 OD at 280nm were pooled, and the pooled peaks were concentrated back to the volume initially applied to the column and analyzed for protein concentration and the TAA activity. Protein concentration in each pool was assessed by the method of Lowery et al (*6*), and the TAA activity was determined by a capture ELISA using murine monoclonal antibody AD1-40F4 as the catcher.

The antigenic fraction (peak I) was treated with rabbit anti-human immunoglobulin antibodies that were immobilized to agarose beads (Immunobeads - BioRadiation Laboratories, Richmond, CA). Five ml of the peak was added to 5 ml packed volume of the immunobeads, and the mixture was incubated at room temperature with continuous end-over-end mixing on a circular rotator for one hr. The absorbed antigen was recovered by centrifugation at 800 x g for 10 min, and subjected to reduction and alkylation.

The immunoaffinity (immobilized human anti-Ig) purified TAA was reduced with dithiothreitol in the presence of 6M guanidine hydrochloride and alkylated with iodoacetic acid at pH 8.5 according to the procedure described by Ozole (*7*). The products were separated by Sephacryl S-200 column (1.0 x 60 cm) chromatography using 1 M propionic acid as eluent (Figure 3). To determine the homogeneity of the purified material, the material was subjected to one and two dimensional gel electrophoresis. One dimensional polyacrylamide gel electrophoresis under reducing conditions was performed according to the procedure described by Laemmli (*5*) using a 4 to 15% gradient gel. Two dimensional gel electrophoresis was performed using the isoelectric focusing gel which contained acrylamide (4% T and 4% C), 9M urea, 9.2mM CHAPS, 0.4% NP-40 and 5% ampholyte (60%, 3-10; 20%, 5-7; 20%, 6-8) in the first direction. The sample was electrofocused at 600 V for 20 hrs. The second dimension electrophoresis was a mass separation through SDS-acrylamide (10

to 20% T and 2.6% C) gel at 30 V and 12C for 4 hrs. Protein spots were visualized by silver staining.

Reverse phase HPLC analysis of the samples was performed using a Delta-Pak C4 (5um particle with 300 A pore size) column and 5% to 95% acetonitrile linear gradient in 0.1% trifluoroacetic acid. The separation was performed in H-P model 1090M HPLC system. The identity of peaks were determined on the basis of retention time.

Western blotting technique (*4*) was used to determine immunoreactivity of various monoclonal antibodies to the purified 90kD TAA. Two microgram protein of the purified 90 kD glycoprotein per lane was subjected to SDS-PAGE and electroblotted to nitrocellulose membrane. After washing and blocking with 5% non-fat milk, the membrane was cut into 5mm wide strips. The strips were reacted with monoclonal antibodies at 1:100 dilution for ascites or 1:25 dilution for hybridoma culture supernates at 4C for 12 hrs. Goat anti-mouse Ig conjugated to alkaline phosphatase (Sigma Chemical Co., Saint Louis, MO) at 1:500 dilution and NBT/BCIP were used invariably to determine reaction of the murine monoclonal antibody.

TASK 2.

a. ***Preparation of murine monoclonal antibody to glycoprotein TAA:*** The glycoprotein TAA prepared as described below was used as an immunogen to develop a murine IgM monoclonal antibody, AD1-40F4, with specificity to the antigen according to the procedures described by Kohler and Milstein (*8*). The monoclonal antibody did not exhibit any reactivity with pooled human IgM, pooled human IgG, ferritin, B2-microglobulin, B2-glycoprotein, apolipoprotein B, apolipoprotein CII, apolipoprotein CIII, or human serum albumin. Western blot analysis revealed that the AD1-40F4 antibody recognized the 90kD subunit of the glycoprotein antigen (*9*). After specificity analysis, the murine monoclonal antibody was mass produced as ascites in BALB/c mice, and used as the source of antibody to develop the 90kD-TAA-specific IC detection assay as described below. The glycoprotein TAA which is expressed by 82% (18/22) carcinomas, was purified as described elsewhere (*10*) from a 24 h urine sample of a melanoma patient. Urine from melanoma patient (Je 8504) was used because the glycoprotein TAA is expressed by solid tumors of various histologic types and this patient's urine had relatively high antigenic activity (*10*). Briefly, the 24 h urine samples were collected and filtered through a Whatman no. 1 filter-paper (Whatman International, Maidstone, England) to remove all sediments. The clarified urine was concentrated 100-fold using an Amicon hollow-fiber concentrator equipped with an H1P10-8 cartridge (Amicon Corp., Beverly, Mass.). Concentrated material was passed through a Sephacryl S-200 column (Pharmacia LKB, Piscataway, N.J.) using 0.025 M phosphate buffered saline supplemented with 0.02% sodium azide as eluent at a flow rate of 25ml/h. Fractions under each peak observed at 280 $_{nm}$ were pooled separately, concentrated and tested for antigenic activity using an allogeneic double-determinant enzyme-linked immunosorbent assay as described previously (*9*). The antigenic pool was quantitatively absorbed with immobilized rabbit anti-human Ig antibodies until free of detectable human IgG in an enzyme immunoassay (*11*). The purified antigenic pool was used to develop murine monoclonal antibodies.

b. Development of TAA-specific-IC detection assay.

We developed a tumor antigen specific IC detection assay which utilizes an immobilized murine monoclonal antibody, AD1-40F4, directed to the 90kD subunit of a glycoprotein TAA. The murine monoclonal antibody, AD1-40F4, and the glycoprotein antigen were prepared as described below. Details of the assay have been described elsewhere (*15*). In brief, one hundred microliters of the AD1-40F4 ascites diluted to a protein concentration of 100 ug/ml were dispensed into each of the appropriate wells of glutaraldehyde activated microtiter plates (Dynatech Laboratories, Chantilly, VA). The plates were incubated at 4C for 16 h and then washed with PBS supplemented with 0.5% Triton X-100 (PBS-TX). The washed plates were blocked with 100 ul per well of 1% bovine serum albumin (BSA) in PBS-TX at 23C for 1.0 h. Test serum samples were diluted 1:60 with PBS-TX supplemented with 1% BSA, 0.5% normal mouse serum and 0.01M ethylene diamine tetraacetic acid (EDTA). One hundred microliters of the diluted sample were dispensed into duplicate wells of the activated plates and incubated at 37C for 45 min. At the end of incubation the wells were washed with PBS-TX. One hundred microliters of alkaline phosphatase conjugated to Fab fragment of goat anti-human IgG (Sigma Chemical Company, Saint Louis, MO) at 1:500 dilution were added to each test and control wells of the plate. The plates were incubated at 37C for 45 min and washed with PBS-TX. Each well of the plate then received 200 ul of p-nitrophenyl phosphate (1.0 mg/ml) in 10% diethanolamine buffer as the substrate and the plates were incubated in the dark for 1.0 h at 23C. The absorbance was read at 405_{nm}. Each sample was tested in duplicate with positive and negative controls and blanked individually in the same microtiter plate. Each test plate also included controls for non-specific protein binding and binding of conjugate to the immobilized murine monoclonal (capturing) antibody. The net optical densities of the control samples were used to generate a correction factor to normalize the net optical density of the test samples analyzed on that particular test plate. If the correction factor for a test plate fell outside the range from 0.8 to 1.2, the assay was considered invalid. The upper limit of normal for the glycoprotein TAA marker was set at 0.410 (mean + 3 SD ELISA values of 59 normal sera determined from previous studies).

c. Isolation of Peripheral Blood Lymphocytes and Transformation with EBV:

Peripheral blood lymphocytes (PBL) were obtained from breast cancer patients at the time when they had high anti-breast antibody levels in ELISA. The lymphocytes were separated by Ficoll-Hypaque centrifugation and partially depleted of T lymphocytes and cytophilic Ig by incubation at 37C for 1 hr in RPMI by the AET-rosetting method (*12*). The enriched B-lymphocytes were suspended (10×10^6 cells/ml) in cell free culture filtrate of Epstein Barr virus (EBV) producing marmoset lymphoblastoid cells to transform with the EBV as described by Irie et al (*13*). After 24 hr incubation, the growth medium was replaced by RPMI-FCS medium. Supernates from wells with visual growth of hybrids was analyzed for human immunoglobulin secretion by an enzyme immunofiltration assay (*14*) and then tested for anti-breast TAA activity by ELISA.

TASK 3.

a. CEA and CA15-3 Assays for Comparison with TA-90-IC assay

i Carcinoembryonic antigen (CEA) assay. CEA measurements on 68 of the 106 serum samples were performed by Dianon Systems, Inc., Stratford, Connecticut, using Abbott CEA-EIA procedures that followed manufacturer's instructions. Results were expressed as ng CEA/ml. A value of greater than 2.5 ng CEA/ml was considered positive.

ii CA15-3 assay. CA15-3 was measured by the radioimmunoassay kit (CA15-3 RIA) commercially available from Centocor, Malvern, PA. The RIA was performed according to the manufacturer's instructions by the Dianon Systems, Inc. Results were expressed as CA15-3 units/ml (U/ml). A value of greater than 30 U of CA15-3/ml was considered positive.

b. Correlation of Immunologic Assay with Clinical Course and Other Screening tests:

(i) Patient Population for Retrospective Studies:

In this investigation serum samples were procured from 106 women who were diagnosed to have breast cancer. The mean age of the patients was 51 years with a range of 25 to 82 years. Histopathologically, 90 patients had invasive ductal carcinoma (IDC) and 16 patients had ductal carcinoma in situ (DCIS). In addition, serum samples were procured from 107 self-proclaimed apparently healthy normal females. The age of normal controls ranged from 28 to 74 years with a mean of 45. All serum samples were stored frozen without any preservative at -35C until used.

(ii) Patient Population for Prospective Studies:

After obtaining informed consent, serum samples for various analyses TA-90-IC ELISA were procured preoperatively in a blinded fashion from 138 women scheduled to undergo open biopsy for an abnormal mammogram, breast mass, or bloody nipple discharge. All biopsy procedures were performed by the surgical staff at the John Wayne Cancer Institute. Biopsy specimens were examined for tumor size, tumor grade, DNA ploidy, S-phase, estrogen and progesterone receptors, and HER2neu expression. Serum samples were tested for TA-90-IC in a blinded fashion (without knowledge of pathology findings). Pathology findings were then correlated with TA-90-IC values in the Statistical Coordinating Unit (SCU).

STATISTICAL PROCEDURES:

Fisher's exact test as implemented by the Instat Biostatistics program from the GraphPad Software, Inc., San Diego, CA was used to determine statistically significant differences among the 90kD glycoprotein TA-90-IC assay values of normal and breast cancer patients, and for comparison between TA-90-IC results and other tumor marker results. All comparisons were two-tailed and a p value of less than 0.05 was considered statistically significant.

Fisher's exact test as implemented by the Instat Biostatistics program from the GraphPad Software, Inc., San Diego, Ca, was used to determine statistically significant differences among the post-

operative 90kD glycoprotein TA-90-IC positive and negative breast cancer patients with respect to recurrent disease over a follow-up period of 60 months. All comparisons were two-tailed and a p value of less than 0.05 was considered statistically significant. Statistical analysis for disease free survival was performed by the Mantel-Cox method.

The Kruskal-Wallis test was used to examine the difference between mean TA-90-IC values of sera from patients with invasive carcinomas and benign lesions. The Spearman correlation coefficient was used to analyze the relationship between TA-90-IC values and tumor size, tumor grade, DNA ploidy, and S-phase. The Kappa test was used to examine the consistency of TA-90-IC status and estrogen receptor status, progesterone receptor status, and HER2neu expression. Pearson chi-square test was used to investigate the relationship between TA-90-IC status and tumor size (< 1 cm versus ≥ 1 cm).

RESULTS

Purity of 90kD TAA (TA-90):

The glycoprotein TAA purified by the procedures outlined in Figure 1 appeared to be comprised of several bands by SDS-PAGE analysis (Figure 4, lane 2). Therefore, peak I of Figure 2 was subjected to reduction and alkylation, and the reduced product separated by Sephacryl S-200 column (1.0 x 60 cm) chromatography. The second peak (Figure 3) of the reduced and alkylated material clearly showed a single band in SDS-PAGE after silver staining (Figure 4, lane 4) and this band was reactive with the murine monoclonal antibody, AD1-40F4, (Figure 4, lane 6) in Western blot. The overall results of 90kD TAA purification achieved by the steps outlined in Figure 1 are summarized in Table 1. Clearly, this procedure allowed 1,000-fold purification with 13.9% yield of the antigen from the starting material.

Comparison of densitometric scans of the silver stained gels using a gel scanner (Shimadzu, Model CS9000U) of the starting material (100-fold concentrated urine) and the purified 90kD TAA revealed that while 90kD TAA was only 0.4% of total protein in the starting material, this component was greater than 89.4% of total protein in the most purified fraction (Figure 5). This level of purity (about 90%) was confirmed by two dimensional gel-electrophoresis where only one spot could be revealed by silver staining.

To further determine the level of purity and to compare the protein profile of final preparation of 90 kD subunit of U-TAA with that of starting material (100-fold concentrated urine), the samples were subjected to reverse phase HPLC analysis using Delta-Pak C4 (5um particle with 300 A pore size) column and 5% to 95% acetonitrile linear gradient in 0.1% trifluoroacetic acid. The separation was performed in H-P model 1090M HPLC system. The starting material (100-fold concentrated urine) was reduced with DTT and alkylated with iodoacetic acid as described above. Ten microliter sample was injected in each case. Figure 6A illustrates the 214nm absorbing profile of the starting material. Clearly at least 17 peaks are visible after 20 min of retention time. On the contrary, only a single peak with a slight shoulder on the left side with a retention time of about 36 min was seen in the profile of the purified 100 kD subunit of U-TAA (Figure 6B). Only a very minor peak was detected at this position in the profile of the starting material (Figure 6A).

Reactivity of Various Murine Monoclonal Antibodies to TA-90 in Western Blot:

The 90kD TAA is expressed by various solid neoplasms including breast carcinoma and melanoma. We realize that murine monoclonal antibodies that react with tumor cells or their lysate have been developed using tumor cells or partially purified tumor antigen fractions as the immunogen. Therefore, a possibility existed that the 90kD TAA which we purified could be an antigen recognized by the already available antibodies. To rule out this possibility, we procured several murine monoclonal antibodies either directly from the investigators or from commericial sources. These murine monoclonal antibodies were used in Western blot to determine their reactivity with purified 90kD TAA (material depicted in Figure 6B). Two microgram protein of the 90kD TAA per lane was subjected to SDS-PAGE and electroblotted onto nitrocellulose membrane. After washing and blocking with 5% non-fat milk, the membrane was cut into 5mm wide strips. The strips were reacted with monoclonal antibodies at 1:100 dilution for ascites or 1:25 dilution for hybridoma culture supernates at 4C for 12 hrs. Goat anti-mouse Ig conjugated to alkaline phosphatase (Sigma Chemical Co.) at 1:500 dilution was used invariably to determine reaction of the murine monoclonal antibody.

None of the seven murine monoclonal antibodies to either melanoma or breast cancer cells developed by various investigators reacted with the 90kD TAA; however, under similar experimental conditions, AD1-40F4 (at 1:500 dilution of ascites) -- an IgM murine monoclonal antibody developed by us using the TAA as immunogen -- showed positive reaction (Figure 7). These results denote that 90kD TAA does not bears any epitope which is recognizable by any of the seven murine monoclonal antibodies we have tested thus far.

Reactivity of Breast Cancer Sera with Tumor Cell Extract in ELISA:

Figure 8 represents the reactivity of sera from a breast cancer patient and a normal individual against the extract of a breast cancer cell line in ELISA. Antibody titers in 100 serum samples from breast cancer patients ranged from <1:100 to >1:12,000; whereas, such titers in normal control sera were less than 1:300.

Isolation of IgG by DEAE Affi-Gel Blue:

As described in the methods section, an IgG fraction was prepared from a high titered serum from a breast cancer patient. The isolated fraction was devoid of other serum proteins that could be detected by immunoelectrophoresis using goat antibodies to whole human serum. The protein concentration of the isolated fraction was 6.1 mg/ml. This fraction was used as the antibody source in Western blot.

SDS-PAGE and Western Blot Analysis of NP-40 Extracts of Tumor and Fibroblastic Cell Lines:

Figure 9 depicts the Coomassie blue stainable protein heterogeneity of extracts of NP-40 lysed cells of breast cancer, colon cancer, sarcoma, melanoma, and normal fibroblasts. The extracts were prepared from 5 x 10^6 cells, and 20ul amounts of the extracts after reduction with 2-

mercaptoethanol were loaded into lane of 6 to 20% gradient gels (Novex, San Diego, CA). After electrophoresis, the gel was stained with Coomassie blue. Similar gels were run in duplicate; but after electophoretic separation, the proteins were electo-transferred to nitrocellulose. One of the nitrocellulose membrane after blocking with non-fat milk was reacted with 1:200 dilution (3ug IgG protein) of antibody from serum of a breast cancer patient (Figure 10), and the other was reacted with IgG fraction of the normal serum (Figure 11). While no appreciable reactivity was seen on the blot reacted with IgG from normal serum (Figure 11), a prominent band at about 53kDa region was visible in lanes loaded with breast cancer cell extracts but not in lanes loaded with other tumor cell or fibroblastic cell extracts. In the preliminary studies, this appears to be a breast tumor-associated antigen (TAA).

Preparation of murine monoclonal antibody to glycoprotein TAA.

It is described in the "Material and Methods" section (Task 2a) above.

Detection of <u>in vitro</u> formed TA-90-IC-Immune Complexes by the Murine Monoclonal antibody Based ELISA (Immunologic Assay)

A polyclonal anti-glycoprotein TAA antibody purified from a baboon antiserum that was raised against the glycoprotein-TAA (*16*), was used to generate IC <u>in vitro</u>. For this purpose the baboon polyclonal antibodies were mixed with the purified glycoprotein TAA in different protein proportions. After incubation at 37C for 30 min, the mixtures were tested to determine if a positive signal was generated in the AD1-40F4 murine monoclonal antibody capture assay. Goat anti-human IgG conjugated to alkaline phosphatase was used as the signal developer. We have previously documented that the goat anti-human IgG enzyme conjugate reacts equally well with the baboon IgG. Table 2 denotes that binding of the enzyme conjugate was the highest when the immobilized AD1-40F4 murine monoclonal antibody was incubated and reacted with the mixture of purified glycoprotein TAA and purified polyclonal baboon anti-TAA IgG. Furthermore, this signal was consistently high over a wide range of antibody (baboon anti-TAA) to antigen (glycoprotein TAA) at protein concentration ratios (3:1 to 90:1). Neither of the two immune reactants (antigen or antibody) alone or pre-immune baboon IgG or human serum albumin exhibited a signal greater than 0.200 O.D. at 405_{nm}. These data confirmed our previous (*9*) observations that the AD1-40F4 murine monoclonal antibody had no significant reactivity with baboon IgG and human serum albumin, and that the enzyme conjugate had no affinity (specific or non-specific) with either the murine monoclonal antibody or with the glycoprotein TAA. These results clearly denote that the AD1-40F4 murine monoclonal antibody captured baboon anti-glycoprotein TAA IgG via the glycoprotein TAA only, i.e., when the IgG antibody was in the form of glycoprotein TAA-specifc immune complexes.

Reproducibility studies to assess the assay variability using a serum from breast cancer patients in ten replicates revealed that inter-assay variations ranged from 0.806 to 1.311 ELISA value with a mean of 1.010, standard deviation of 0.139 and coefficient of variation of 13.7%. These values for intra-assay variations ranged from 0.849 to 1.214 with a mean of 1.007, standard deviation of 0.105 and coefficient of variation of 10.4%.

Incidence of TA-90-IC in normal control vs breast cancer patients.

Initially we analyzed serum samples from self-proclaimed healthy normals and breast cancer patients. Figure 13 illustrates the distribution of TA-90-IC ELISA values of sera from 107 healthy normal females and from 106 breast cancer patients. In this experiment, the procurement of the sera from breast cancer patients was random, i.e., no criterian with respect to pre- or post-surgery, evidence or no evidence of disease, etc., was used in selecting the serum samples. Comparative analysis of the data in normal and breast cancer group revealed that the normalized ELISA value (mean $\pm$ SD) of the normal control sera (0.212 ± 0.088) was significantly ($p < 0.05$) lower than that of breast cancer patients (0.570 ± 0.438). Furthermore, when an ELISA value of 0.41 or greater was considered positive for the presence of the TAA-specific IC, the incidence of the glycoprotein TAA-specific IC was significantly ($p < 0.05$) greater in the breast cancer group (67/106, 63%) than the normal group (3/107, 2.8%).

While the incidence of TA-90-IC in the normal group was not affected by age, it was significantly higher ($p < 0.05$) in breast cancer patients that were over 60 years old (88%, 23/26) compared to those that were under 60 years old (55%, 44/80) (Fig. 14).

Of the 90 IDC breast cancer patients, 58 (64%) were positive for the glycoprotein TA-90-IC; whereas, this incidence was lower 56% (9/16) in DCIS breast cancer patients (Table 3). The lower incidence in DCIS patients may be due to the non-aggressive nature of the ductal carcinoma *in situ*.

Isolation of B-Cells from Blood of Breast Cancer Patients and Transformation with Epstein Barr Virus:

Peripheral blood lymphocytes (PBL) were isolated from ten breast cancer patients when they had high antibody levels (titer of >1:10,000) against breast cancer extract by ELISA. PBLs were treated to enrich for B-cells and transfected with EBV as described in the methods section. Despite success in the transformation of all of the B-cells, supernatant of only one lymphoblastoid cell line (LCL-4) showed reactivity to breast cancer cell extracts and its spent culture medium in ELISA and Western blot (Figure 12). The band visible in Western blot is of high molecular weight. The immunoglobulin isotype secreted by LCL-4 is IgM.

Association among Glycoprotein TA-90-IC, CEA and CA15-3.

CEA and CA15-3 have been considered useful tumor markers in the prognosis and monitoring of breast cancer patients. We compared the glycoprotein TAA-specific IC results using 68 serum samples of breast cancer patients selected on the basis of the presence of the disease. The two tumor markers, CEA and CA15-3, are not known to be immunogenic in cancer patients. Of the 68 serum samples 55 (80.9%) were positive for the glycoprotein TAA-specific IC, 16 (23.5%) were positive for CEA, and 23 (33.8%) were positive for CA15-3. Despite higher incidence of glycoprotein TAA-specific IC than CEA or CA15-3, it was observed that some serum samples that were positive for CEA or CA15-3 were not necessarily positive for the glycoprotein TA-90-IC.

As shown in Table 4, statistical evaluation of the data by Fisher's exact test revealed that there was no significant associations between the glycoprotein TAA and CEA or CA15-3 ($p > 0.05$). However, when either of the three or all of the three positive markers were taken into consideration, the incidence of positivity increase from 80.9% to 91% (Table 5).

Application of TA-90-IC ELISA to Analyze Pre- and Post-operative Serum Samples:

From a group of 128 breast cancer that were positive for TA-90-IC pre-operatively, serum samples were obtained retrospectively 2 to 12 weeks after surgical resection of all of the accessible tumor. The serum samples (256 total) obtained before surgery and after surgery were analyzed by the TA-90-IC ELISA. Of the 128 patients, 76 (59%) became negative after surgery for the marker, suggesting either a complete resection of the tumor or a reduction in tumor burden to a level below the threshold level that could result in the serum to become TA-90-IC positive. Despite surgical resection of the accessible tumor, there were 52 patients who remained positive for the TA-90-IC marker. Longitudinal clinical follow-up of the patients revealed that 34 of 52 (65%) post-operatively positive patients developed recurrent disease within five years. On the contrary, 9 of 76 (12%) of the post-operatively TA-90-IC negative patients developed recurrent disease ($p < 0.05$). Thus, it is apparent that in the group of patients in whom the TA-90-IC values remained positive, all of the disease was not resected and these patients harbored micrometastases. This translates to the fact that TA-90-IC marker can effectively identify patients with poor prognosis. Many patients who remained disease free for greater than 5 years were consistently negative for the TA-90-IC (Figure 15) and patients who had developed recurrent disease either remained positive after removal of tumor or became positive for the marker before clinically detectable disease (Figure 16).

Statistical analysis of TA-90-IC positive and negative patients by the Mantel-Cox method revealed that the two groups differed significantly ($p< 0.05$) in terms of recurrence rate. The incidence of TA-90-IC positivity was not related to the duration of post-operative recurrence. The time to recurrence in TA-90-IC positive patients ranged from 1 to 58 months with a mean $\pm$ SD of 29 ± 16 (Table 6). These results again suggest a positive correlation between postoperative presence of TAA-specific IC and subclinical residual or recurrent disease.

Clinical Utility of TA-90-IC in Conjunction with Results of Biopsy of Abnormal Mammography:

We are continuing to analyze serum samples taken at the time of biopsy of suspicious breast nodules that are identified by mammography. As depicted in Table 7, the correlation of TA-90-IC results with histopathological findings is excellent. Of the 151 abnormal mammograms, 75 patients had no malignancy by histopathology. These patients were considered clinically free of disease; however, 7% (5/75) sera of these patients were positive for TA-90-IC. Eleven percent (17/151) patients had histopathologically proven ductal carcinoma in situ and 39% (59/151) had invasive ductal carcinoma. The incidences of TA-90-IC in sera of these two groups of patients at the time of biopsy were 47% and 64%, respectively. Overall, 34% (51/151) patients with abnormal mammography were positive for the TA-90-IC and 66% (100/151) were negative. These results clearly denote that the TA-90-IC marker could be a valuable adjunct to mammographic screening of women.

Biopsy specimens were characterized as benign, ductal carcinoma in situ (DCIS), or invasive carcinoma. The distribution of positive and negative TA-90-IC values for each pathology is shown in Table 8. Mean TA-90-IC was 0.254 ± 0.239 OD in the 82 patients with benign lesions, 0.315 ± 0.230 OD in the 14 patients with DCIS, and 0.436 ± 0.209 OD in the 42 patients with invasive lesions; the difference between benign and invasive groups was highly significant (p=0.0001).

Of the 138 patients studied, 42 had invasive carcinoma, 31 (74%) with a positive TA-90-IC; 14 patients had DCIS, 4 (29%) with a positive TA-90-IC; and 82 had benign lesions, 6 (7%) with a positive TA-90-IC.

Of the 82 patients with benign pathology report, 72% had abnormal mammogram and 7% were positive for TA-90-IC. Of the 14 patients with DCIS pathology report, all (14/14) had abnormal mammogram and 29% were positive for TA-90-IC. On the contrary, of the 42 patients whose pathology reports revealed invasive carcinoma of the breast, 86% had abnormal mammogram and 74% were positive for TA-90-IC.

Twenty-six patients had a normal mammogram in the face of a palpable mass, and one had a normal mammogram and a bloody nipple discharge. Of these 27 patients, none had DCIS; 6 (22%) had invasive carcinoma, 4 with a positive TA-90-IC; and 21 (88%) had benign lesions, none with a positive TA-90-IC. Mammography was positive in 9 patients with invasive carcinoma and negative in six patients with invasive carcinoma. Mammography was positive in 14 patients with benign lesions and negative in 21 patients with benign lesions (Table 9).

Among the 42 patients with invasive breast cancer, 27 (64%) had a positive mammogram plus a positive TA-90-IC, 4 (10%) had a positive TA-90-IC only, 9 (21%) had a positive mammogram only, and 2 (5%) had neither a positive mammogram nor a positive TA-90-IC.

Of the 56 patients with invasive carcinoma or DCIS, 52 had lesions evaluable by size. Of the 15 patients with tumors <1 cm, 6 (40%) had a positive TA-90-IC. Of the 37 patients with tumors ≥ 1 cm, 28 (82%) had a positive TA-90-IC (p=0.014). Thirty-one of the 56 tumors were tested for S-phase. Of the 20 patients with a positive TA-90-IC, 8 (40%) had tumors with an S-phase greater than 5%; of the 11 patients with a negative TA-90-IC, only 3 (27%) had tumors with an S-phase greater than 5%. However, statistical analysis using S-phase and TA-90-IC as continuous variables revealed a positive correlation between TA-90-IC and S-phase (Spearman correlation coefficient =0.4011; p=0.0253) (Figure 17). There was no demonstrable association between TA-90-IC value and tumor grade among 45 patients with invasive or noninvasive tumors (Spearman correlation coefficient = 0.2789, p=0.0635) or among the subgroup of 34 patients with invasive tumors (Spearman correlation coefficient=0.3040, p=0.0804).

Of the 38 tumors tested for estrogen receptors (ER) and progesterone receptors (PR), 7 were ER-negative: 6 (86%) of these were TA-90-IC positive, compared with 21 (68%) of the 31 ER-positive tumors (p=0.8282). Of the 7 PR-negative tumors, 5 (71%) were TA-90-IC positive, compared with 22 (70%) of the 31 PR-positive tumors (p=0.5097). Of the 34 tumors tested for HER2/*neu* expression, 8 were positive: 6 (75%) of these were TA-90-IC positive, compared with 17 (65%) of the 26 HER2/*neu*-negative tumors (p=0.3056).

The serum of 36 patients with a malignancy was tested for CEA and/or CA15-3 in addition to TA-90-IC. CA15-3 was positive in 3 patients (one invasive carcinoma, 2 DCIS) out of 32 patients (26 invasive carcinoma, 6 DCIS). CEA was positive in 2 patients (invasive carcinoma) of 33 patients (26 invasive carcinoma, 7 DCIS). Of the 36 patients, 24 (67%) were positive for TA-90-IC, whereas only 3 (8%) were positive for CEA or CA15-3 (Table 10). One patient with DCIS had a positive CA15-3 but a negative CEA and TA-90-IC. Of the 22 patients with a positive TA-90-IC and invasive carcinoma, only 2 were positive for CEA or CA15-3. This confirms our earlier study demonstrating the superior sensitivity of the TA-90-IC assay (*17*).

Of the 42 invasive carcinomas, 17 were American Joint Committee on Cancer (AJCC) Stage I, 18 were AJCC stage IIa, 5 were AJCC stage IIb, and 2 were AJCC stage IIIa. TA-90-IC values were positive in 11 (65%) stage I tumors, 14 (78%) stage IIa tumors, 5 (100%) stage IIb tumors, and 1 (50%) stage IIIa tumor (Figure 18).

One hundred and fifty-seven breast biopsies were performed in the same number of patients. Benign disease was found in 88 patients (56%), ductal carcinoma in situ (DCIS) was found in 17 patients (11%), and invasive adenocarcinoma was found in 52 patients (33%). Of the 52 patients with invasive carcinoma, 23 had American Joint Committee on Cancer stage I disease, 21 had stage IIA, 3 had stage IIB, and 5 patients had stage IIIA disease (Table 11).

Serum samples for the TA-90-IC assay were available for all 157 patients. Average TA-90-IC values were 0.282 for benign disease (median 0.189), 0.327 for DCIS (median 0.182), and 0.460 for invasive carcinoma (median 0.462). In those patients with invasive cancers, the mean TA-90-IC values were 0.534 for stage I, 0.370 for stage IIA, 0.452 for stage IIB, and 0.455 for stage III. The percentage of patients with elevated TA-90-IC values increased from benign disease to DCIS to invasive cancer (Figure 19, $p<0.0001$).

Table 12 shows the pathologic findings of open biopsy according to the results of mammography and TA-90-IC assay. Of the 105 patients with suspicious or indeterminate mammograms, 64 (62%) had normal levels of TA-90-IC. Thirteen of these 64 patients had invasive disease, 9 had DCIS, and 42 had benign disease on pathologic examination. Forty-one patients had both a positive mammogram and an elevated TA-90-IC level: 30 had invasive cancer, 6 had DCIS, and only 5 had benign disease. Therefore, in the group of patients with positive mammograms, TA-90-IC assay correctly identified 42 of the 47 benign biopsies (89%), but missed 13 of the 43 (30%) invasive cancers and 9 of the 15 (60%) DCIS cases (Table 12).

Of the 52 patients with a negative mammogram, 41 had normal TA-90-IC levels: on pathologic examination, 3 had invasive cancer, 1 had DCIS, and 37 (90%) had benign disease. Of the 11 patients with elevated TA-90-IC, 6 (55%) had a cancer, 1 (9%) had DCIS, and only 4 (36%) were benign on pathologic examination. Therefore, the addition of TA-90-IC to a negative mammogram correctly identified 7 of the 11 cancers missed by mammography (Table 12). In total, of the 69 cases of DCIS and invasive cancers, 11 (16%) were missed by mammography and 26 (37.7%) were missed by TA-90-IC assay but only 4 (5.8%) were missed by both tests.
The corresponding sensitivity, specificity, positive predictive value (PPV), false positive rate (FPR), and overall accuracy of the two screening tests are listed in Table 13. The patients who presented with indeterminate and/or suspicious mammograms were considered positive radiographically; the patients who presented with DCIS and/or with invasive adenocarcinoma

pathologically were considered positive for these calculations. Although the sensitivity of mammography alone was higher than that of TA-90-IC (84% vs. 77%, p=0.0038), all other parameters, specifically FPR (54% vs 10%, p=0.0038) were superior when TA-90-IC was used as the screening test. A combination of tests, defined as an elevation of either test, yielded a superior sensitivity (94%) than each test alone, but at the expense of increasing FPR and decreasing overall accuracy.

Excluding the 20 indeterminate mammograms, which potentially could have biased the accuracy of mammography in a negative fashion, did not significantly affect the analysis (Table 13). Mammography remained slightly more sensitive than TA-90-IC assay (82% vs. 61% respectively, p=0.007), but at the expense of higher FPR (46% vs. 21% respectively, p=0.007) and lower overall accuracy (66% vs. 77% respectively, p=0.0056).

Looking only at the subset of patients with benign disease, the number of positive TA-90-IC tests was significantly lower than the number of positive mammograms (Table 14); this discrepancy was significant in all age groups except patients over 70 years of age. Among all 56 patients younger than 50 years of age, 6 of the 16 cases of invasive and noninvasive cancers were missed by mammography, 5 were missed by TA-90-IC assay, but only one was missed by both tests (Table 12). In this age group the overall accuracy of mammography decreased to 58% but the accuracy of TA-90-IC improved to 87% (Table 13).

DISCUSSION

Serological tumor markers are considered to be useful in the early detection and monitoring of metastases for early and effective treatment to increase the duration of disease free and/or overall survival (*18, 19*). However, with the exception of CA15-3, recent reports have questioned the value of many of these sensitive markers both in diagnosis of systemic disease and in assessing response to therapy (*20, 21*). In this investigation, we have analyzed serum samples from breast cancer patients to determine the usefulness of an antigen specific immune complex detection assay. This marker differs from the existing tumor markers in that it determines the presence of a glycoprotein TAA which is immunogenic in patients and circulates in the form of immune complexes in the blood. The detection assay can be considered as a form of double-antibody sandwich ELISA in which the immune complexes present in the test sample are captured by an immobilized murine monoclonal antibody, AD1-40F4. This monoclonal antibody was specifically developed using the purified glycoprotein TAA defined by autologous and allogeneic antibodies, and recognizes an epitope different from those recognized by the autologous antibodies.

There is sufficient evidence in literature to suggest that estimation of immune complex levels or their fluctuations during the course of malignant disease might predict the outcome of the disease (*22*). However, unlike other tumor markers, one of the drawbacks, which inhibited the application of this technology in a clinical setting has been the use of antigen non-specific assays for the detection of immune complexes. The use of antigen-nonspecific assays resulted in inconsistent results, because some of the material detected by these assays were characterized to be aggregated IgG, reaction products of denatured self-proteins, polyamines, or bacterial

lipopolysaccharides (*23*). In general, immune complexes detected in sera of cancer patients have been characterized with respect to their size and the presence of anti-Ig and anti-tumor antibodies, and tumor or other antigens. A number of methods have been used to isolate and characterize the antibody and antigen components of the immune complexes (*24*). However, manipulations of the *in vivo* formed immune complexes are prone to introduce artifacts for characterization in subsequent studies; thus, providing inaccurate results. Therefore, development of an assay such as the one used in this investigation which detects antigen-specific immune complexes without any pre-treatment or manipulation of the test sample represents a significant and major advancement in the area of immunodiagnosis of human cancer.

It can be argued that immune complexes present in circulation may be composed of either IgG or IgM antibodies or both; however, anti-tumor antibodies of IgG type to macromolecular antigens are more prevalent (*25*), and the TA-90-IC assay can be modified to detect immune complexes containing IgM antibody by using anti-human IgM conjugate. We feel that the success of the TA-90-IC assay for the detection of cancer is for the following reasons. The immunogenic tumor antigens shed into circulation by growing tumor cells are in small quantities and are neutralized by the humoral antibodies (*26*). Therefore, the results of any sensitive methods applied to detect free antigens in serum or plasma are generally negative; however, detection of human antibody (immunoglobulin) molecules via the antigen captured by the immobilized MuMoAb gives an amplification effect. This is because the unreduced antigen is a complex of at least four different subunits (*10*), each of which is immunogenic in the cancer host and thus can bear multiple *in vivo* reacted immunoglobulin molecules. Furthermore, glycoprotein TAA being immunogenic in cancer patients should circulate in the bloodstream in the form of immune complexes, particularly at the time when the source of the antigen (tumor) is present only in small amounts.

The serum level of 90kD glycoprotein TAA, as assessed in the form of immune complexes, was uniformly low in the control group of 107 apparently healthy females. The values (0.212 ± 0.088 OD_{405nm}) observed in this investigation were comparable to those reported earlier (0.249 ± 0.080 OD_{405nm}) from our laboratory where the control group was comprised of 250 normal males and females (*15*). Furthermore, the incidence of positive values were comparable (2.8% vs 3.2%). In this investigations where serum samples were obtained from patients with a history of breast cancer, elevated serum levels of 90kD glycoprotein TAA were observed at a frequency of about 63%. These results confirm and significantly expand the initial observation that the 90kD glycoprotein TAA marker could be detected in greater proportion of breast cancer patients as well (*27*). It is of particular interest to note that the incidence of 90kD glycoprotein TAA in breast cancer patients was affected by age. It has been reported that the mortality rate in younger breast cancer patients is significantly lower in contrast to older breast cancer patients (*28, 29*). Patients with an age of greater than 60 years showed significantly greater incidence than those patients who were younger than 60 years. It would appear as if the tumors of younger women do not express this antigen or the tumor cells do not release it into circulation. Is it due to dormancy or different metabolic turnover rates of surface molecules? We are currently in the process of correlating the expression of the glycoprotein TAA with the level of expression of estrogen/progesterone receptors by the breast cancer cells. These hormones have been reported to modulate the expression of surface macromolecules, e.g. cerebellar responses to amino acid neurotransmitters (*30*).

It is obvious that the TA-90-IC detection assay described here is not 100% accurate in identifying breast cancer sera. Furthermore, as low it may be, certain proportion of sera from normal controls was positive for the marker. The presence of 90kD glycoprotein TAA in normal population is unexplainable at this time; however, it may be possible that these apparently healthy individuals had occult neoplasm at the time of serum sampling. This possibility of existence of occult disease is difficult to prove or disprove.

Despite the fact that we have not reached 90% or greater accuracy in predicting the outcome of breast cancer patients using the TA-90-IC assay, the results presented here are highly encouraging. This study describes one of the unique approaches to immunodiagnosis of human cancer via detecting an immunogenic tumor antigen in circulation, its specificity can be significantly enhanced by incorporating other existing tumor markers, such as CEA (*31*) and CA15-3 (*32-34*). Extensive evaluation of CEA in combination with other tumor markers or alone for clinical correlations with the clinical course of breast cancer patients has resulted in conflicting reports [*34-38*]. In fact, it has been suggested that CA15-3 correlated with the stage of disease and in metastatic patients with the response to treatment (*39-44*). Use of this novel marker (tumor antigen-specific immune complexes) in conjunction with CEA and\or CA15-3 may prove to be more sensitive than when used alone for immunodiagnosis and immunoprognosis of breast cancer.

Our continued efforts in the application of the 90kD glycoprotein TA-90-IC assay have revealed that this marker can indicate the presence of residual disease after surgical resection in breast cancer patients. This is apparent from the observation that 41% post-operative patients were positive for the TA-90-IC. Also, longitudenal clinical follow-up of the TA-90-IC positive patients revealed that post-operative presence of TA-90-IC in serum identified patients that were at high risk of developing recurrence. Clearly, the TA-90-IC alone is not a perfect marker because there were some patients (12%) in the post-operative TA-90-IC negative group that developed recurrent disease. This could be due to the fact that recurrent tumor of these patients did not express the 90kD glycoprotein antigen. Furthermore, some patients that were positive post-operatively did not develop recurrent disease for at least up to five years of follow-up. We feel that residual disease in these patients either regressed or remained dormant for the duration of their clinical follow-up. Therefore, it is necessary to identify tumor-associated antigens that are more or less breast cancer specific and are immunogenic in the host.

At the present time breast mammography is recommended for asymptomatic women, every one to two years between the ages of 40 to 49, and once a year for those over 50. However, abnormal mammograms do not necessarily mean the presence of malignant disease. Often, histopathologic examination of biopsy of the lesions revealed them to be benign. Correlation of the results of histopathologic analysis of biopsy and analysis of the serum taken at the same time for TA-90-IC from women with abnormal mammograms, revealed that TA-90-IC or similar marker could be a valuable adjunct to mammography. However, as indicated above, like any tumor marker, the TA-90-IC marker could not be solely relied upon for clinical judgements in the care of patients until the present results are validated by further studies.

We selected a serum with an IgG antibody titer of 1:12,800. This serum sample from a breast cancer patient was used to successfully isolate IgG antibodies by the DEAE Affi-Gel blue chromatography. The purity of the antibody was such that no arc other than IgG was seen in immunoelectrophoresis using a goat antiserum directed to whole human serum. Using the purified IgG from sera of breast cancer patient and normal control volunteer, we have identified a band of about 53kDa (TAA) in the NP-40 extract of breast cancer cell lines using the Western blot technique. In addition, we have been able to isolate and immortalize B-cells from breast cancer patients. One of the lymphoblastoid cell line (LCL-4) established in this manner continues to produce an IgM antibody that reacts with a band in the high molecular weight region in breast cancer cell extract and its spent culture medium in Western blot and ELISA.

Investigations undertaken during the past year have evaluated the utility of TA-90-IC for identifying patients with early breast cancer in a prospective manner. Results indicate that TA-90-IC shows promise as an adjunct to mammographic screening. Eighty-five percent of patients with a positive TA-90-IC had a breast malignancy. Of the 97 patients with a negative TA-90-IC, 76 (78%) had benign disease and 21 (22%) had a malignancy. Only 6 of 82 (7%) patients with a benign lesion had a positive TA-90-IC, whereas 4 of 6 patients with invasive carcinoma and a normal mammogram had a positive TA-90-IC (Figure 20). Of 29 patients with a normal mammogram, 6 (21%) had a malignancy; similarly, 21 of 97 (22%) TA-90-IC values <0.41 OD were falsely negative. Five (45%) of the 11 patients with a positive mammogram had a malignancy. When TA-90-IC and/or mammographic results were combined, 54 of 56 (96%) breast neoplasms were detected.

Although mammography has reduced the mortality of breast cancer by about one-third in patients 50 to 69 years old, it has limitations. Not all breast cancers are demonstrable on mammography, in which case the mammogram would be falsely negative. In our study, 4 of 42 (10%) patients with invasive carcinoma had a negative mammogram. Coveney et al (*46*) found a 16.5% false-negative rate for mammography in 291 patients of all ages with palpable cancers. Retrospective review of false-negative mammograms showed that 30% were normal (true negatives), 20% were obvious oversights, and 50% had radiographic abnormalities that were indeterminate. A serum marker indicating whether or not a lesion was malignant should decrease the rate of false-negative mammography. This would be particularly useful in 40-49 year old patients without a palpable mass (*45*).

False positives are also a problem with mammography. Only 17% to 32% of non-palpable lesions found on mammography are malignant (*46-55*). Therefore, a large percentage of open biopsies reveal benign disease. Because women younger than 50 years have a much higher rate of false-positive mammograms (*48, 56*), the cost of screening mammography is five times higher in the 40-49 year age group than the 50-69 year group (*57*). The current consensus of the National Institutes of Health is that mammography can not universally be recommended in women between 40 and 49 years old (*58*). A serum marker that could distinguish between benign and malignant lesions on a mammogram should make mammography in this age group more cost-effective and beneficial.

Serum tumor markers have been described for the early detection and monitoring of metastases from a primary breast cancer (*59, 60*). However, many of these markers are questionable, with

the possible exception of CA15-3, in regards to both diagnosis of systemic disease and response to therapy (*61, 62*). Tomlinson et al (*63*) reported 70% sensitivity, 96% specificity, and an 87% predictive value for CA15-3 in metastatic disease, but this marker does not appear to be useful in screening. Our study confirmed this finding – only 3 of 32 patients with a malignancy had a positive CA15-3. Serum markers such as TPS (*64*), alpha-1-antiprotease (*65*), YKL-40 (*66*), MCA (*67*), and c-*erb*B-2 (*68-71*) have shown promise in predicting survival and monitoring disease regression or recurrence, but are not suitable for screening.

KEY RESEARCH ACCOMPLISHMENTS

- A 90kD glycoprotein tumor associated antigen (TA-90) has been isolated, purified and characterized with respect to its:
  - expression by breast cancer cell and detection in tissue culture spent culture medium
  - immunogenic characteristic in cancer patients
  - patients may have high levels of antibodies to this antigen
  - any release of the antigen by the growing or dormant breast cancer cells into circulation of the patients is not immediately realized because of neutralization of the antigen

- A new tumor associated antigen which is recognized by allogeneic serum samples from breast cancer patients has been identified in cell free extracts of breast cancer cell lines.

- A lymphoblastoid cell line was established from Epstein Barr virus (EBV) transformed B-cells of a breast cancer patient.

- A murine monoclonal to the 90kD glycoprotein TAA was developed.

- The murine monoclonal antibody was used to develop an assay that is capable of detecting antigen-specific immune complexes in serum samples of breast cancer patients.

- After optimization the new immunologic assay was found to be useful in identifying breast cancer patients who harbored occult micrometastases.

- Like most other tumor markers, the immunologic (TA-90-IC) assay did not appear to be 100% accurate. There were some false positive and some false negative results; however, false predictive values were less prominent than many other markers that are commonly used currently.

- Comparison of the TA-90-IC results in breast cancer patients with evidence of disease with the results of CEA and CA15-3 revealed that the incidence of abnormal values could be increase to about 90%. Thus, the newly developed assay could be used in conjunction with other markers with increased sensitivity for immunodiagnosis and prognosis.

- Seventy-four percent of patients with invasive breast cancers had elevated TA-90-IC levels compared with 7% of patients with benign disease; thus, it may be of benefit in identifying a neoplasm missed by mammography.

- The TA-90-IC levels in breast cancer patients did not correlate with estrogen and progestrone receptor status or with Her-2-Nu expression.

- Levels of TA-90-IC decrease significantly for patients who undergo successful resection of the disease.

REPORTABLE OUTCOMES

- Manuscripts, abstracts and presentations

Zavotsky J, Gupta RK, Brennan MB, Yee R, Giuliano AE, Morton DL: Prospective Evaluation of TA90 Immune Complex Assay for Preoperative Diagnosis of Benign and Malignant Breast Lesions (*Submitted*).

Habal N, Zavotsky J, Gupta RK, Yee R, Johnson T, Brennan MB, Brenner RJ, Hansen N, Giuliano AE, Morton DL: Accuracy of TA90 Immune Complex Assay Compared with Mammography for the Detection of Breast Cancer (*Submitted*).

Gupta RK, Morton DL: Detection and comparison of a 90kD glycoprotein tumor-associated antigen specific immune complex with CEA and CA15-3 in breast cancer. Int J Oncol, 7:741-747, 1995.

Gupta RK, Giuliano AE, Kelley MC, Yee R, Leopoldo Z, Morton DL: Clinical significance of a 90kD glycoprotein tumor-associated antigen specific immune complexes in sera of breast cancer patients. J Tumor Marker Oncol, 11:44, 1996.

Gupta RK, Morton DL: Glycoprotein tumor-associated antigen specific immune complexes in sera of breast cancer patients. In *XVI International Cancer Congress*, Monduzzi Editore, Bologna, Italy, pp 1333-1337, 1994.

Gupta RK, Yee R, Leopoldo ZC, Morton DL: Detection of occult breast cancer by a novel immunologic approach. Era of Hope - Department of Defence. Vol. I, pp 181-182, 1997.

Gupta RK, Morton DL: Association between a 90kD glycoprotein tumor-associated antigen specific immune complexes and breast cancer. (*In preparation*).

Gupta RK, Giuliano AE, Kelley MC, Yee R, Leopoldo Z, Morton DL: Clinical significance of a 90kD glycoprotein tumor-associated antigen specific immune complexes in sera of breast cancer patients. Invited speaker at the International Academy of Tumor Marker Oncology, Singapore, June - 1996.

Gupta RK, Giuliano AE, Yee R, Morton DL: Detection of breast cancer by a novel immunologic technique using the TA90 specific immune complexes. Satellite Symposium on Tumor Immunology, Chittaranjan National Cancer Institute, Calcutta, India, Invited guest speaker. October 28-30, 1998 (*Invited speaker*).

Gupta RK: A novel approach to detect immunogenic tumor-associated antigen in sera of breast cancer patients. K.G. Medical College, Lucknow, India, October 20, 1998. (*Invited lecturer*).

Gupta RK: Giuliano AE, Yee R, Morton DL: A new immunologic approach involving antigen (TA90) specific immune complexes to detect occult breast cancer. Invited guest speaker at the Mini Symposium on Mechanisms of Tumor Invasion, Metastasis and Host-tumor interactions at the Cancer Research Institute, Tata Memorial Centre, Mumbai, India, November 9-11, 1998.

Gupta RK, Morton DL: Detection of a 90kD glycoprotein tumor-associated antigen specific immune complexes in sera of patients with breast cancer. Presented at the XXIII Meeting of the International Society for Oncodevelopmental Biology and Medicine, Montreal, Canada, 1995.

Gupta RK, Leopoldo Z, Yee R, Morton DL: Isolation and purification of a 90kDa subunit from a glycoprotein complex of a urinary tumor-associated antigen. Proc Am Assoc Cancer Res 37:465, 1996.

Gupta RK, Morton DL: A novel approach to detect tumor-antigen specific immune complexes in sera of cancer patients. Clin Ligand Assay Soc. 1996.

Gupta RK, Morton DL: A new method to detect tumor antigen specific immune complexes in lung cancer patients. Clin Immunol Soc. 1996.

Gupta RK, Giuliano AE, Kelley MC, Yee R, Leopoldo Z, Morton DL: Clinical significance of a 90kD glycoprotein tumor-associated antigen specific immune complexes in sera of breast cancer patients. Internatl Conf on Human Tumor Markers. 11:44, 1996.

Gupta RK, Hsueh EC, Brosman SA, Yee R, Leopoldo ZC, Morton DL: An ELISA to detect tumor-associated antigen specific immune complexes in sera of cancer patients. Am Soc Microbiol 97: May, 1997.

Gupta RK, Giuliano AE, Yee R, Leopoldo ZC, Morton DL: Detection of a glycoprotein tumor-associated antigen specific immune complexes in breast cancer patients. Presented at the 20th Annual San Antonio Breast Cancer Symposium. Breast Cancer Treatment Report. Dec. 3-6, 1997.

Zavotsky J, Gupta RK, Brennan MB, Yee R, Giuliano AE, Morton DL. Prospective evaluation of TA90 immune complex assay in the preoperative diagnosis of benign and malignant breast lesions. Accepted for poster presentation at the American Society for Clinical Oncology, 17:106a, 1998.

- patents and licenses applied for and/or issued: None.

- degrees obtained that are supported by this grant: Not applicable

- development of cell lines, tissue or serum repositories:
  In this project we have used two different breast cancer cell line that were established at JWCI before initiation of this project. However, 37 breast cancer tissues were processed to prepare antigen targets in various experiments during the period of this grant. Of these 22 tissues were invasive ductal or lobular carcinomas of the breast, and 15 were ductal carcinoma in situ. In addition, we have obtained and used 327 blood samples from breast cancer patients as the source of antibody and peripheral blood lymphocytes.

- informatics such as databases and animal models, etc.: None.

- funding applied for based on work supported by this award: Yes, to the California Breast Cancer Research Program. However, the grant was not funded.

- employment or research opportunities applied for and/or received on experiences/training by this award: Not applicable.

CONCLUSIONS

Investigations conducted in this award resulted in many useful reagents and information. As outlined in the key research accomplishment section above, a 90kD glycoprotein has been purified to near homogeneity. This antigen is immunogenic in breast cancer patients. A murine monoclonal antibody of IgM isotype was developed. This monoclonal antibody recognized an epitope on the antigen that is different from the ones recognized by the allogeneic (patient's) antibodies. This murine monoclonal antibody was used to develop an immunologic assay to assess the TA-90 specific immune complexes (TA-90-IC) as a marker in the sera of breast cancer patients. Results of investigations conducted in this grant award indicate that TA-90-IC is a potentially useful screening marker for patients with early breast carcinoma. Although a higher proportion of stage IIa patients were TA-90 positive, 11 of 17 stage I patients were positive for TA-90-IC as well. This means that TA-90-IC is detectable in the blood of patients with smaller, pre-metastatic tumors. Although this marker is not 100% sensitive or specific, it complements mammography by identifying cancers by an independent means. In our study, we showed that TA-90-IC could identify 67% of tumors not visualized on a mammogram. Of course, these tumors were palpable and did not present a diagnostic dilemma. In this group of patients, all of whom had an indication of breast biopsy, TA-90-IC proved to be a useful adjunct to mammography. Furthermore, comparison of the immunologic assay results with those of CEA and CA15-3 revealed that there was no significant association between TA-90-IC, CEA and CA15-3. When either of the three positive markers were taken into consideration incidence, the incidence of positivity increased from 80.9% to 91%.

REFERENCES

1. Riha I, Haskova V, Kaslik J, Maierova M, Stansky J: The use of polyethylene glycol for immune complex detection in human sera. Molecular Immunol 16:489-493, 1979.

2. Digeon M, Laver M, Riza J and Bach JF: Detection of circulating immune complexes in human sera by simplified assays with polyethylene glycol. J Immunol Methods 16:165-183, 1977.

3. Hunt KK, Masahiko S, Gupta RK, Morton DL: Complement - dependent lysis of tumor cells by a baboon IgM antibody to a tumor-associated antigen. Cancer Immunol Immunother 34:377-382, 1992.

4. Towbin H, Gordon J: Immunobloting and dot immunobinding - Current status and outlook. J Immunol Methods 72:313-340, 1984.

5. Laemmli EK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685, 1970.

6. Lowery OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275, 1951.

7. Ozole S: Guide to protein purification. Methods in Enzymology 182:598, 1990.

8. Kohler G and Milstein C: Continuous culture of fused cells secreting antibody of predefined specificity. Nature 256:495-497, 1975.

9. Euhus DM, Gupta RK and Morton DL: Detection of a tumor associated glycoprotein antigen in serum and urine of melanoma patients by murine monoclonal antibody (AD1-40F4) in enzyme immunoassay. J Clin Lab Analysis 3:184-190, 1989.

10. Euhus DM, Gupta RK and Morton DL: Association between allo-immunoreactive and xeno-immunoreactive subunits of a glycoprotein tumor associated antigen. Cancer Immunol Immunother 32:214-220, 1990.

11. Euhus DM, Gupta RK and Morton DL: Characterization of a 90-100 kDa tumor associated antigen in sera of melanoma patients. Int J Cancer 45:1065-1070, 1990.

12. Kaplan ME, Woodson M, and Clark C: Detection of human T lymphocytes rosette formation with AET-treated sheep red cells. In: In vitro Methods in Cell-Mediated and Tumor Immunity. (BR Bloom and JR David, eds), Academic Press, New York pp. 83-88, 1976.

13. Irie RF, Sze LL, and Saxton RE: Human antibody to OFA-I, a tumor antigen, produced in vitro by Epstein-Barr virus-transformed human B-lymphoblastoid cell lines. Proc Natl Acad Sci USA 69:6651-6655, 1982.

14. Glassy MC, and Cleveland PH: Use of mouse and human monoclonal antibodies in enzyme immunofiltration. Methods in Enzymonl 121:525-541, 1986.

15. Gupta RK and Morton DL: Monoclonal antibody based ELISA to detect glycoprotein tumor-associated antigen specific immune complexes in cancer patients. J Clin Lab Analysis 6:329-336, 1992.

16. Hunt KK, Shibata M, Gupta RK and Morton DL: Complement dependent lysis of tumor cells by a baboon IgM antibody to a tumor-associated antigen. Cancer Immunol Immunother 34:377-382, 1992.

17. Gupta RK, Morton DL: Detection and comparison of a 90 kD glycoprotein tumor-associated antigen specific immune complexes with CEA and CA15-3 in breast cancer. Int J Oncol 7:741-747, 1995.

18. Foekens JAA, van Putten WLJ, Potengen H, de Koning HYWCM, Thirion B, Alexieva-Figusch and Kijn GM: Prognostic value of PS2 and cathepsin D in 710 human primary breast tumors: multivariate analysis. J clin Oncol 11:899-908, 1993.

19. Ng JSY, Sturgeon CM, Seth J, Paterson GM, Roulston JE and Leonard RCF: Serological Markers for metastatic breast cancer. Disease Markers 11:217-223, 1993.

20. Tondini C, Hayes DF, Gelman R, Henderson IC and Kufe DW: Comparison of CA15-3 and carcinoembryonic antigen in monitoring the clinical course of patients with metastatic breast cancer. Cancer Res 48:4107-4112, 1988.

21. Salinas FA, Wee KH and Silver HKB: Immune complexes and human neoplasia. Biomed Pharmacol 37:119-125, 1983.

22. Robertson JFR, Pearson D, Price MR, Selby C, Blamy RW and Howell A: Objective measurement of therapeutic response in breast cancer using tumor markers. Br J Cancer 64:757-763, 1991.

23. Salinas FA, Wee KH and Silver HKB: Clinical relevance of immune complexes, associated antigen, antibodies in cancer. In: Immune Complexes and Human Cancer, FA Salinas, MA Hanna, (eds.), Plenum Press, New York, pp 55-109, 1985.

24. Gupta RK, and Morton DL: Circulating immune complexes in malignant melanoma. Disease Markers 6:81-95, 1988.

25. Vlock DR, DerSimonian R and Kirkwood DR: Prognostic role of antibody reactivity to melanoma. J Clin Invest 77:1116-1121, 1986.

26. Euhus DM, Gupta RK and Morton DL: Induction of antibodies to a tumor associated antigen by immunization with a whole melanoma cell vaccine. Cancer Immunol Immunother 26:247-254, 1989.

27. Gupta RK and Morton DL: Prognostic value of a 90kd subunit containing glycoprotein tumor-associated antigen specific immune complexes in lung cancer patients. Disease Markers 12:51-61, 1994.

28. Albain KS, Allerd DC and Clark GM: Breast cancer outcome and predictors of outcome: Are there age differences? Monogr Natl Cancer Inst 16:35-42, 1994.

29. Hankey BF, Miller B, Curtis R and Kosary C: Trends in breast cancer in younger women in contrast to older women. Monogr Natl Cancer Inst 16:7-14, 1994.

30. Smith SS, Waterhouse BB and Woodward DJ: Sex steroid effects on extrahypothalamic CNS. Progesterone, alone and in combination with estrogen, modulates cerebellar responses to amino acid *neurotransmitters*. Brain Res 422:52-62, 1987.

31. Buamah PK, Bent DJ, Bodger WA and Skillen AW: A profile of serum CA15-3, carcinoembryonic antigen, alkaline phosphatase, and gamma-glutamyl transferase levels in patients with breast cancer. J Surg Oncol 53:84-87, 1993.

32. Zamagni C, Martoni A, Cacciari N, Bellanava B, Vecchi F and Pannuti F: CA-549 serum levels in breast cancer monitoring. Int J Biol Markers 7:217-221, 1992.

33. Gion M, Ruggeri G, Mione R, Marconato R, Casella C, Nasadini A, Simoncini E, Belloli S, Da Zennaro E and Bruseagnin G: A new approach to tumour marker assessment by preoperative determination in breast and colorectal cancer. Int J Biol Markers 8:8-13, 1993.

34. Eskelinen M, Hippelainen M, Carlsson L, Jonsson P and Alhava E: A decision support system for predicting a recurrence of breast cancer, a prospective study of serum tumour markers TAG 72, CA15-3 and MCA. Anticancer Res. 12:1439-1442, 1992.

35. Bates SE and Longo DL: Tumor markers: Value and limitation in the management of cancer patients. Cancer Treat Rev 12:163-207, 1985.

36. Daly L, Ferguson J, Cram GP, Hars V, George SL, McCarty KS and Bast RC: Comparison of a novel assay for breast cancer mucin to CA15-3 and carcinoembryonic antigen. J Clin Oncol 10:1057, 1992.

37. Kalliionemi AP, Oksa H, Arran RK, Hietanen T, Lehtinen M and Kiovula T: Serum CA15-3 assay in the diagnosis and follow-up of breast cancer. Br J Cancer 58:213-215, 1988.

38. Repetto L, Onetto M, Gardin G, Costanzi B, Guidici S, Vitiello E, Merlini L, Naso C, Zannini C, Paganuzzi M, Rosso R and Santi L: Serum CEA, CA15-3, and MCA in breast cancer patients: A clinical evaluation. Cancer Detection & Prevention 17:411-415, 1993.

39. Colomer R, Ruibal A, Navarro M, Encabo G, Sole LA and Savador L: Circulating CA15-3 levels in breast cancer. Our present experience. Int J Biol Markers 1:89-95, 1986.

40. Gion M, Mione R, Dittadi R, Fassan S, Pallini A and Bruscaglin G: Evaluation of CA15-3 serum levels in breast cancer patients. J Nucl Med & All Sci 30:29-35, 1986.

41. Gion M, Mione R, Nascimben O, Valsecchi M, Gatti C, Leon A and Bruscagnin G: The tumour associated antigen CA15-3 in primary breast cancer: Evaluation of 667 cases. Br J Cancer 63:809-813, 1991.

42. Nicoloni A, Colombini C, Luciani L, Capri A and Guiliani L: Evaluation of serum CA15-3 determination with CEA and TPA in the post-operative follow-up of breast cancer patients. Br J Cancer 64:154-158, 1991.

43. Omar YT, Behbehani AE and Al-Naqeeb N: Carcinoembryonic antigen and breast carcinoma antigen (CA15-3) in pre-operative staging and post-operative monitoring of patients with carcinoma of the breast. Int J Biol Markers 3:165-169, 1988.

44. Schimidt-Rhode P, Schulz KD, Strum G, Raab-Frick A and Prinz H: CA15-3 as a tumor marker in breast cancer. Int J Biol Marker 2:135-141, 1987.

45. Chew SB, Hughes M, Kennedy C, Gillet D, Carmalt H: Mammographically negative breast cancer at the Strathfield Breast Centre. Aust N Z J Surg 66(3):134-137, 1996.

46. Coveney EC, Geraghty JG, O'Laoide R, Hourihane JB, O'Higgins NJ: Reasons underlying negative mammography in patients with palpable breast cancer. Clin Radiol 49(2):123-125,1994.

47. Griffen MM, Welling RE: Needle-localized biopsy of the breast. Surg Gynecol Obstet 170(2):145-148, 1990.

48. Lein BC, Alex WR, Zebley DM, Pezzi CM: Results of needle localized breast biopsy in women under age 50. Am J Surg 171:356-359, 1996.

49. Hall JA, Murphy DC, Hall BR, Hall KA: Mammographic abnormalities and the detection of carcinoma of the breast. Am J Obstet Gynecol 168(6 Pt 1):1677-1680, 1993.

50. Albert MP, Sachsse E, Coe NP, Page DW, Reed WP: Correlation between mammography and the pathology of nonpalpable breast lesions. J Surg Oncol 44(1):44-46, 1990.

51. O'Laoide R, Geraghty JG, Coveney EC, McDermott EW, Hourihane JB, McCabe M, O'Higgins NJ: Mammographic needle localisation of impalpable breast lesions. Ir Med Jun;(2):70-72, 1992.

52. Rissanen TJ, Makarainen HP, Mattila SI, Karttunen AI, Kiviniemi HO, Kallioinen MJ, Kaarela OI: Wire localised biopsy of breast lesions: a review of 425 cases found on screening or clinical mammography. Clin Radiol 47(1):14-22, 1993.

53. Johnston JA, Clee CZ: Analysis of 308 localisation breast biopsies in a New Zealand hospital. Australas Radiol 35(2):148-151, 1991.

54. Mueller X, Amery A, Lallemand RC: Biopsy of mammographically-detected breast lesions in a district hospital. Eur J Surg Oncol 19:415-419, 1993.

55. Alexander HR, Candela FC, Dershaw DD, Kinne DW: Needle-localized mammographic lesions. Results and evolving treatment strategy. Arch Surg 125:1441-1444, 1990.

56. Rothman LP, Harvald TB, Overgaard H: Impalpable breast lesions diagnosed by mammography: incidence of histologically confirmed malignancy. Eur J Surg 162:691-694, 1996.

57. Salzmann P, Kerlikowske K, Phillips K: Cost-effectiveness of extending screening mammography guidelines to include women 40 to 49 years of age. Ann Intern Med 127:955-965, 1997.

58. National Institutes of Health: Breast cancer screening for women ages 40-49. NIH Consensus Statement Jan 21-23, 1997. 15(1):1-35, 1997.

59. Ardavanis A, Gerakini F, Amanatidou A, Scorilas A, Pateras C, Garoufali A, Pissakas G, Stravolemos K, Apostolikas N, Yiotis I: Relationship between cathepsin-D, pS2 protein and hormonal receptors in breast cancer cytosols: inconsistency with their established prognostic significance. Anticancer Res 17:3665-3669, 1997.

60. Barna BP, Deodhar SD: Immunology, tumor markers, and breast cancer. Surg Clin North Am, 58:693-704, 1978.

61. Heinze T, Schurenkamper P, Minguillon C, Lichtenegger W: Mammary serum antigen (MSA), Ca 549, CA15-3 and CEA in breast cancer preoperative sensitivity and correlation to prognostic factors. Anticancer Res 17:2953-2954, 1997.

62. Shering SG, Sherry F, McDermott EW, O'Higgins NJ, Duffy MJ: Preoperative CA15-3 concentrations predict outcome of patients with breast carcinoma. Cancer 83:2521-2527, 1998.

63. Tomlinson IPM, Whyman A, Barrett JA, Kremer JK: Tumour marker CA15-3: Possible uses in the routine management of breast cancer. Eur J Cancer 31:899-902, 1995.

64. Bremer K, Eklund G, Bjorklund B: Notable relationship between the level of tumor marker TPS in serum and survival in breast cancer. Anticancer Res 16:905-910, 1996.

65. Varela AS, Saez JJBL: Utility of plasmatic levels of alpha-1-antiprotease (A1AP) as a cancer marker. Cancer Letters 89:15-21, 1995.

66. Johanson JS, Cintin C, Jorgenson M, Kamby C, Price PA: Serum YKL-40: a new potential marker of prognosis and location of metastases of patients with recurrent breast cancer. Eur J Cancer 31:1436-1442, 1995.

67. Muller-Brand J, Macke H: Clinical significance of the new tumor marker MCA in the follow-up of patients with mammory carcinoma. Int J Biol Markers 8:130-132, 1993.

68. Leitzel K, Teramoto Y, Konrad K, Chinchilli VM, Volas G, Grossberg H, Harvey H, Demers L, Lipton A: Elevated serum c-*erb*B-2 antigen levels and decreased response to hormone therapy of breast cancer. J Clin Oncol 5:1129-1135, 1995.

69. Volas GH, Leitzel K, Teramoto Y, Grossberg H, Demers L, Lipton A: Serial serum c-*erb*B-2 levels in patients with breast carcinoma. Cancer 78:267-272, 1996.

70. Carney WP, Hamer PJ, Petit C, Retos C, Greene R, Zabrecky JR, McKenzie S, Hayes D, Kufe D, DeLellis R, Naber S, Wolfe H: Detection and quantification of human neu oncoprotein. Tumor Marker Oncol 6:53-72, 1991.

71. Yu FZ, Sugano K, Ohkura H, Mori S: Development of sandwich radioimmunogenic assay for serum c-erbB-2 oncogene product and its significance in diagnosing breast carcinoma. Rinsho Byori 39:1087-1092, 1991.

72. Bumol TF, Chee DO, Reisfeld RA: Immunochemical and biosynthetic analysis of monoclonal antibody-defined melanoma-associated antigen. Hybridoma 1:283-292, 1982.

73. Morgan AC Jr., Gallaway DR, Reisfeld RA: Production and characterization of monoclonal antibody to a melanoma specific glycoprotein. Hybridoma 1:27-36, 1981.

74. Ruiter DJ, Dingjan GM, Steijlen PM, van Beveren-Hooyer M, de Graaff-Reitsma C, Bergman W, van Muijen GN, Warnaar SO: Monoclonal antibodies selected to discriminate between malignant melanomas and nevocellular nevi. J Invest Dermatol 85:4-8, 1985.

75. Nuti M, Teramoto Y, Mariana-Costanini R, Horan Hand P, Colcher D, Schlom J: A monoclonal antibody (B72.3) defines patterns of distribution of a novel tumor-associated antigen in human mammary carcinoma cell populations. Int J Cancer 29:539-545, 1982.

76. Mesa-Tejada R, Palakodety RB, Leon JA, Khatcherian AS, Greaton CJ: Immunocytochemical distribution of a breast carcinoma associated glycoprotein identified by monoclonal antibodies. Am J Pathol 130:305-314, 1988.

77. Edwards DP, Grzyb KT, Dressler LG, Mansel RE, Zava DT, Sledge Jr. GW, McGuire WL: Monoclonal antibody identification and characterization of a Mr 43,000 membrane glycoprotein associated with human breast cancer. Cancer Res 46:1306-1317, 1986.

78. Colcher D, Horan Hand P, Nuti M, Schlom JA: A spectrum of monoclonal antibodies reactive with human mammary tumor cells. Proc Natl Acad Sci 78:3199-3203, 1981.

APPENDICES

(Please see Tables (14) and Figures (20) on the following pages numbered 34 through 64)
(2 Published papers, 2 manuscripts, 6 abstracts, and 1 copy of Gupta's CV)

TABLE 1

YIELD AND FOLD-PURIFICATION OF 90kDa SUBUNIT OF TAA

Purification step	Total volume (ml)	Protein conc. (mg/ml)	Total protein (mg)	Antigenic activity (units/mg)*	Total antigen (units)	Purification (fold)	Yield (%)
100x concentrated urine	144	19.98	2877	25	71,925	1	100
Sephacryl S-200 peak-I	140	0.21	29.4	1190	35,000	47.6	48.7
Sephacryl S-200 peak-II after reduction and alkylation	10	0.04	0.4	25000	10,000	1,000	13.9

* One unit of antigenic activity is defined as the minimum amount of protein required to cause 50% inhibition of binding between TAA and AD1-40F4.

TABLE 2. Detection of purified glycoprotein TAA after mixing with purified baboon polyclonal IgG antibody by the murine monoclonal antibody, AD1-40F4, capture assay.

Test material	Absorbance at 405_{nm}
Immobilized murine monoclonal antibody (control)[a]	0.042
+ baboon anti-glycoprotein TAA (44 ug purified IgG/ml)	0.138
+ glycoprotein TAA (15 ug protein/ml)	0.068
+ human serum albumin (20 ug/ml)	0.162
+ pre-immune baboon IgG (50 ug/ml)	0.129
+ mixture of baboon anti-TAA IgG (44 ug/ml) and:	
glycoprotein TAA (15 ug/ml)	0.686
glycoprotein TAA (5 ug/ml)	0.869
glycoprotein TAA (1.6 ug/ml)	0.714
glycoprotein TAA (0.53 ug/ml)	0.753
human serum albumin (20 ug/ml)[b]	0.153
human serum albumin (10 ug/ml)[b]	0.218
human serum albumin (5 ug/ml)[b]	0.176
+ mixture of pre-immune baboon IgG (50 ug/ml) and:	
glycoprotein TAA (15 ug/ml)	0.188

[a]Anti-90kD glycoprotein TAA murine monoclonal antibody in the form of ascites (100 ug protein per ml) was immobilized to the wells of glutaraldehyde-activated microtiter plates.

[b]Human serum albumin was used as control to determine the effect of nonspecific protein control.

TABLE 3. Incidence and level of 90kD glycoprotein TAA-specifc IC in two different histologic types of breast carcinoma.

Histologic types	Total number	Number positive	Percent positive	ELISA values		
				Range	Mean	SD
Invasive ductal CA	90	58	64	0.000-2.038	0.453	0.365
Ductal CA in situ	16	9	56	0.046-1.357	0.412	0.314

TABLE 4. Association between serum glycoprotein TA-90-IC and CEA or CA15-3.

		Glycoprotein TA-90-IC		
		Positive[*]	*Negative*	*Total*
CEA	Positive[**]	13	3	16
	Negative	42	10	52
	Total	55	13	68
		$p > 0.05$		
CA15-3	Positive[***]	18	5	23
	Negative	37	8	45
	Total	55	13	68
		$p > 0.05$		

[*] An ELISA value of greater than 0.41 OD at 405_{nm} was considered positive.

[**] A value of greater than 2.5 ng CEA/ml was considered positive.

[***] A value greater than 30 U of CA15-3/ml was considered positive.

TABLE 5. Incidence of positivity for glycoprotein TA-90-IC, CEA and CA15-3 or their combination in sera from breast cancer patients.

Marker (Alone or in combination)	(n = 68)	
	Number positive	Percent positive
Glycoprotein TA-90-IC only	55	81
CEA only	16	24
CA15-3 only	23	34
CEA or CA15-3	30	44
TA-90-IC or CEA	48	71
TA-90-IC or CA15-3	60	88
TA-90-IC or CEA or CA15-3	62	91

TABLE 6: Correlation between 90kD glycoprotein TA-90-IC and post-operative recurrence in breast cancer patients.

Patient* recurrence group (Months)	Number of patients in group	Number of TA-90-IC positive patients	Time (months) between TA-90-IC positive and recurrence		
			Range	Mean	SD
<12	8	6	1 to 11	6	4
12 - 24	8	6	14 to 20	17	2
25 - 36	13	9	25 to 33	29	3
37 - 48	8	7	37 to 47	40	3
49 - 60	6	6	49 to 58	54	4
All patients	43	34	1 to 58	29	16

* Patients were grouped on the basis of recurrence of disease after removal of primary disease.

TABLE 7. Correlation between TAA-specific-IC and histopathologic results of biopsy tissue of patients with abnormal mammography (Total 151 patients).

Histopathology result	N	90kDa glycoprotein TA-90-IC	
		Positive	Negative
Ductal Ca in situ	17 (11%)	8 (47%)	9 (53%)
Invasive ductal Ca	59 (39%)	38 (64%)	21 (36%)
No malignancy	75 (50%)	5 (7%)	70 (93%)

TABLE 8. Correlation between TA-90-IC Levels and Pathological Findings.

TA-90-IC LEVELS	BENIGN	DCIS	INVASIVE	TOTAL
TA-90-IC <0.41	76 (93%)	10 (71%)	11 (26%)	97 (70%)
TA-90-IC ≥0.41	6 (7%)	4 (29%)	31 (74%)	41 (30%)
TOTAL	82	14	42	138

TABLE 9. Correlation between TA-90-IC and mammographic results according to pathology of the breast lesion

	Pathology of the Breast Lesion		
Screening test results	*Benign*	*DCIS*	*Invasive cancer*
TA-90-IC (+) Mammography+	6	4	27
TA-90-IC (+) Mammography-	0	0	4
TA-90-IC (-) Mammography+	53	10	9
TA-90-IC (-) Mammography-	23	0	2

TABLE 10. Incidence of three serum markers (TA-90-IC, CEA, CA15-3) in sera of patients with DCIS or invasive breast cancer.

	Histopathology of breast lesion	
Marker status	*DCIS*	*Invasive cancer*
TA-90-IC (+) CEA/CA15-3+	0	2
TA-90-IC (+) CEA/CA15-3-	2	20
TA-90-TC (-) CEA/CA15-3+	1	0
TA-90-IC (-) CEA/CA15-3-	4	7

TABLE 11. Mammographic Findings, TA-90-IC Levels, and Final Histopathologic Outcome in All 157 Patients Studied

	Pathologic Findings *(number of patients)*					
	Invasive Cancer *(by AJCC§ Stage)*				DCIS‡	Benign
Test Results	**I**	**II**	**III**	***All***		
Mammogram only †						
Suspicious	**18**	**18**	**2**	***38***	**13**	**34**
Indeterminate	**4**	**0**	**1**	***5***	**2**	**13**
Benign	**1**	**6**	**2**	***9***	**2**	**41**
TA-90-IC only †						
Elevated	**15**	**17**	**4**	***36***	**7**	**9**
Normal	**8**	**7**	**1**	***16***	**10**	**79**

§ American Joint Committee on Cancer; ‡ Ductal Carcinoma in Situ

† McNemar's test (for paired data): compares TA-90-IC elevation with positive mammogram results (suspicious and indeterminates combined), $p < 0.0001$

TABLE 12. Results Subdivided First by Mammography and Then by TA-90-IC Level, for the Entire Group of 157 Patients and Then for the 56 Patients Below the Age of 50

		Pathologic Findings *(number of patients in each group)*		
		Cancer		Benign
		Invasive	DCIS	
All Patients				
Mammo + *(N=105)*	TA90+ *(N=41)*	30	6	5
	TA90- *(N=64)*	13	9	42
Mammo – *(N=52)*	TA90+ *(N=11)*	6	1	4
	TA90- *(N=41)*	3	1	37

Number of invasive tumors missed by a negative TA90 = 16/52
Number of DCIS tumors missed by a negative TA90 = 10/17
Number of benign tumors missed by a positive TA90 = 9/88

Patients < 50 years old				
Mammo + *(N=28)*	TA90+ *(N=7)*	5	1	1
	TA90- *(N=21)*	2	2	17
Mammo – *(N=28)*	TA90+ *(N=6)*	4	1	1
	TA90- *(N=22)*	1	0	21

Number of invasive tumors missed by a negative TA90 = 3/12
Number of DCIS tumors missed by a negative TA90 = 2/4
Number of benign tumors missed by a positive TA90 = 2/40

'Mammo +' includes suspicious and indeterminate mammograms combined into one group, 'Mammo –' includes negative mammograms only; 'TA90 +' includes elevated TA-90-IC levels, 'TA90-' includes normal TA-90-IC levels.

TABLE 13. Accuracy of Mammography Compared with TA-90-IC Assay

	Sensitivity	Specificity	PPV	Accuracy	FPR
All Patients					
Mammogram	84%	46%	54%	62%	54%
TA-90-IC	77% *	90% §	82% §	84% *	10% *
Patients < 50 years old					
Mammogram	67%	55%	34%	58%	45%
TA-90-IC	67%	93% §	83%	87%	7%
Indeterminate Mammograms Excluded					
Mammogram	82%	54%	58%	66%	46%
TA-90-IC	61% ‡	79% §	82% §	77% ‡	21% ‡
Test Combinations					
Either Positive	94%	41%	56%	64%	59%
Both Positive	50%	95%	89%	75%	5%

§ $p < 0.0001$ ***‡ $p < 0.007$*** **** $p < 0.005$***

TABLE 14. Analysis of False Positive TA-90-IC Levels or Mammograms (Suspicious or Indeterminates Combined) in the Subset of Patients with Benign Histopathology

Age Group	n	TA-90-IC Elevated		Mammogram Suspicious		Indeterminate		Combined	*p-value* *
		n	*%*	*n*	*%*	*n*	*%*	*%*	
25-39	13	1	7.7	2	15.4	5	38.5	53.8	0.0313
40-49	27	1	3.7	5	18.5	6	22.2	40.7	0.0063
50-59	25	5	20.0	15	60.0	0	0.0	60.0	0.0063
60-69	14	1	7.1	8	57.1	1	7.1	64.3	0.0215
70+	9	1	11.1	4	44.4	1	11.1	55.6	0.2188
Total	**88**	**9**	**10.2**	**34**	**38.6**	**13**	**14.8**	**53.4**	**0.0001**

* McNemar's test for paired data comparing TA-90-IC elevation with positive mammographic results (combined suspicious and indeterminate readings)

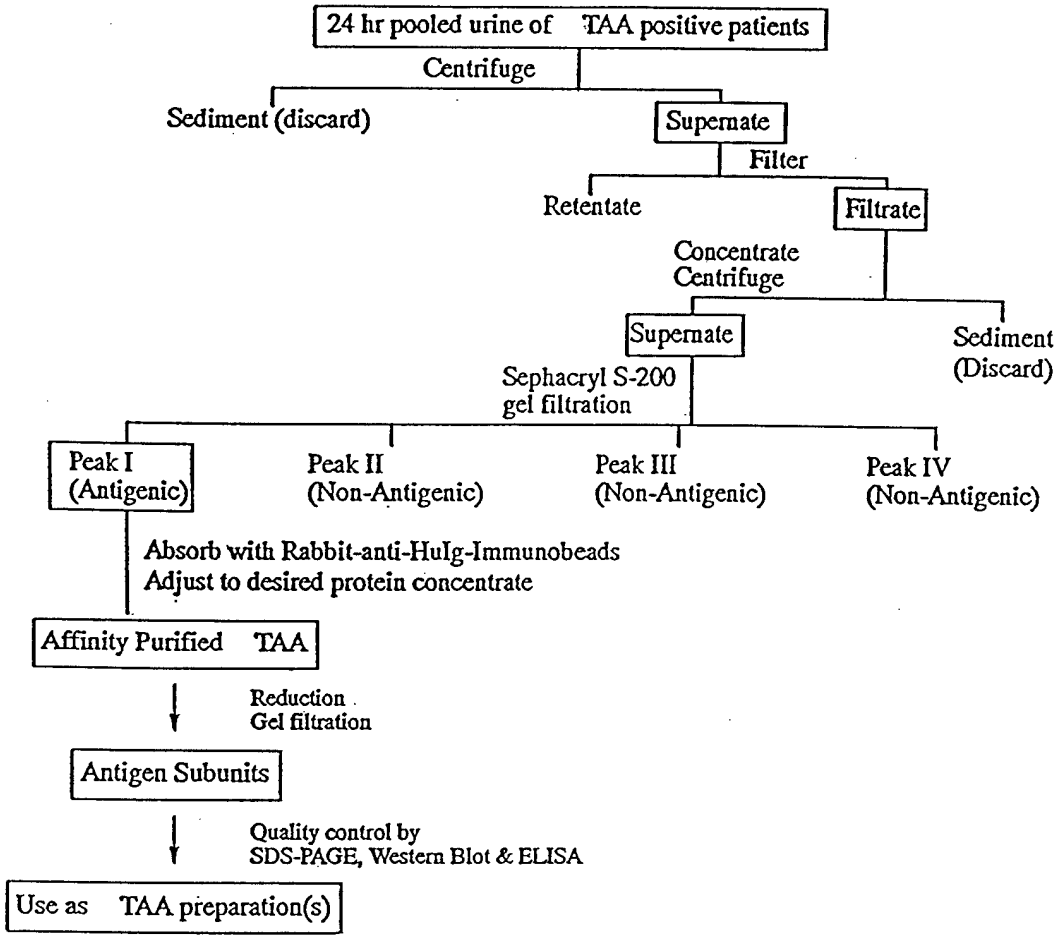


FIGURE 1: Purification protocol for 90kD tumor-associated antigen (TAA) from positive breast cancer patients. Details are given on page 12 of the text.

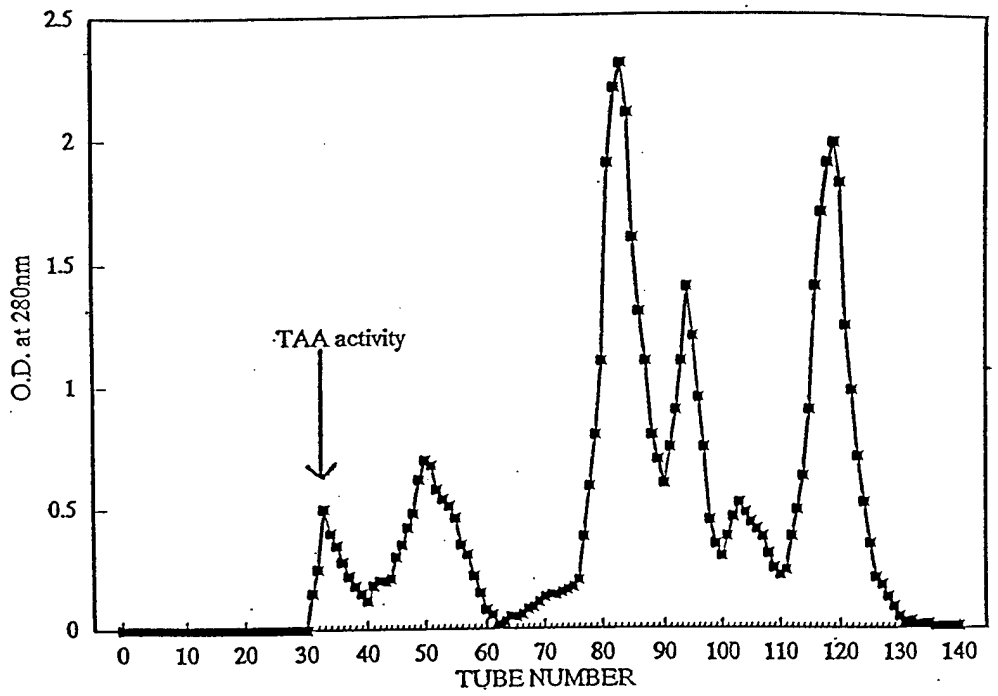


FIGURE 2: Elution profile of 100-fold concentrated urine from breast cancer patients from a Sephacryl S-200 column (1.5 x 100 cm). Phosphate buffer (0.025M) supplemented with 0.15M NaCl and 0.02% sodium azide was used as an eluent at a flow rate of 20 ml per hr. Five ml fractions per tube were collected and elution of protein was monitored at 280_{nm}. Tubes with OD_{280nm} of >0.1 under each peak were pooled, concentrated to the original volume and assessed for TAA activity against the murine monoclonal antibody, AD1-40F4, in ELISA.

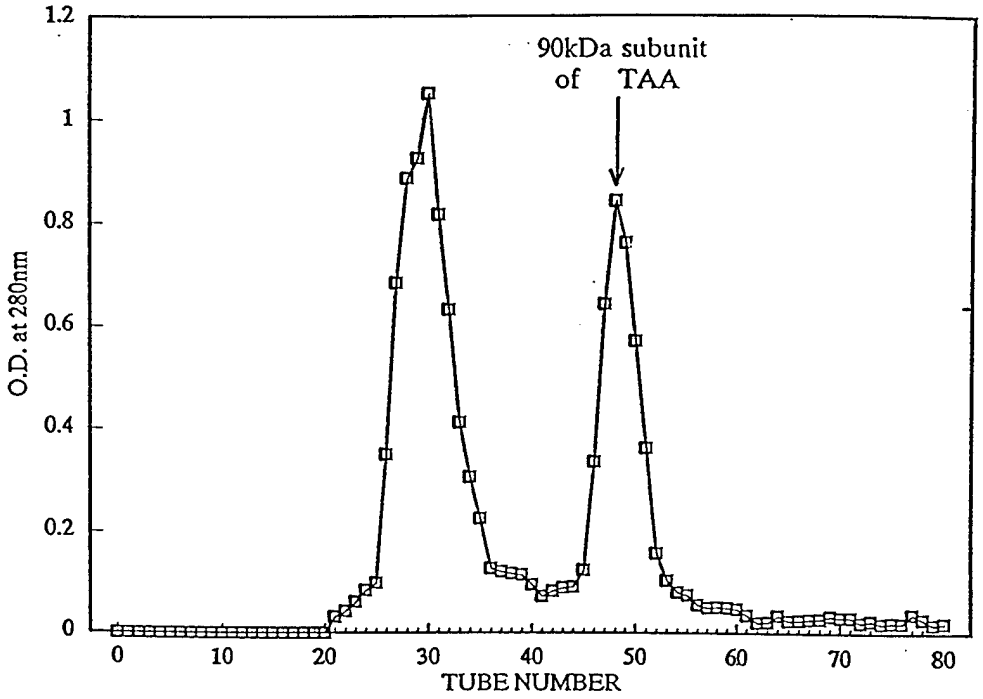


FIGURE 3: Elution profile from Sephacryl S-200 column (1.0 x 60 cm) of reduced and alkylated TAA preparation. 1.0M propionic acid was used as the eluent, and 2 ml fractions per tube were collected. Protein elution profile was monitored at 280_{nm}. Tubes under peak with >0.25 OD were pooled, dialyzed against PBS at pH 7.2, and tested for TAA activity by competitive inhibition in ELISA using AD1-40F4 as the target antibody.

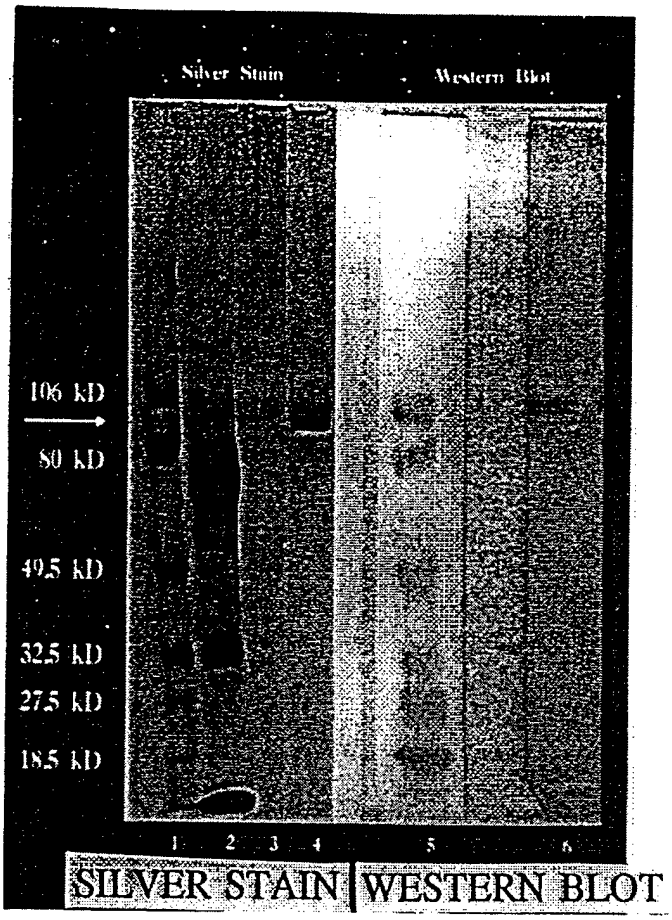


FIGURE 4: SDS-PAGE (4-15% gradient) and Western blot analysis under reducing conditions (2-mercaptoethanol, 5%) using Tris-glycine buffer at ph 8.3.

SDS-PAGE RESULTS:

Lane 1: Molecular weight markers.
Lane 2: 100x concentrated breast urine.
Lane 3: Sephacryl S-200 peak-I (Figure 2).
Lane 4: Sephacryl S-200 peak-II of reduced and alkylated TAA representing the murine monoclonal antibody immunoreactive 90kD subunit (Figure 3).

WESTERN BLOT RESULTS:

Lane 5: Molecular weight markers.
Lane 6: 90kD TAA (same material as in lane 4) reacted with the murine monoclonal antibody, AD1-40F4, after transfer to nitrocellulose membrane.

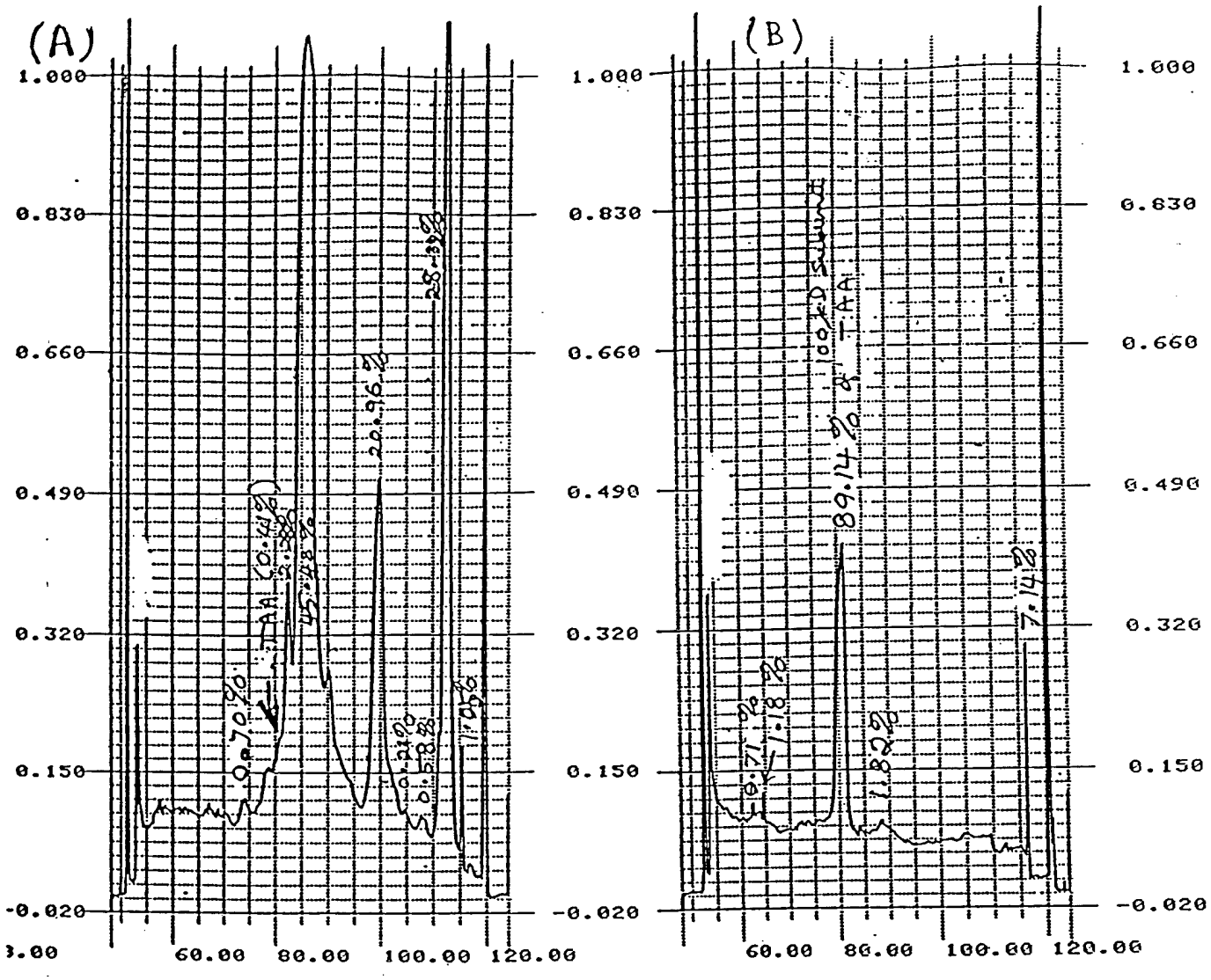


FIGURE 5: Densitometric scan of SDS-PAGE performed under reducing conditions. (A) Starting material (100x concentrated urine). (B) Purified 90kD TAA (Purity level: about 90%).

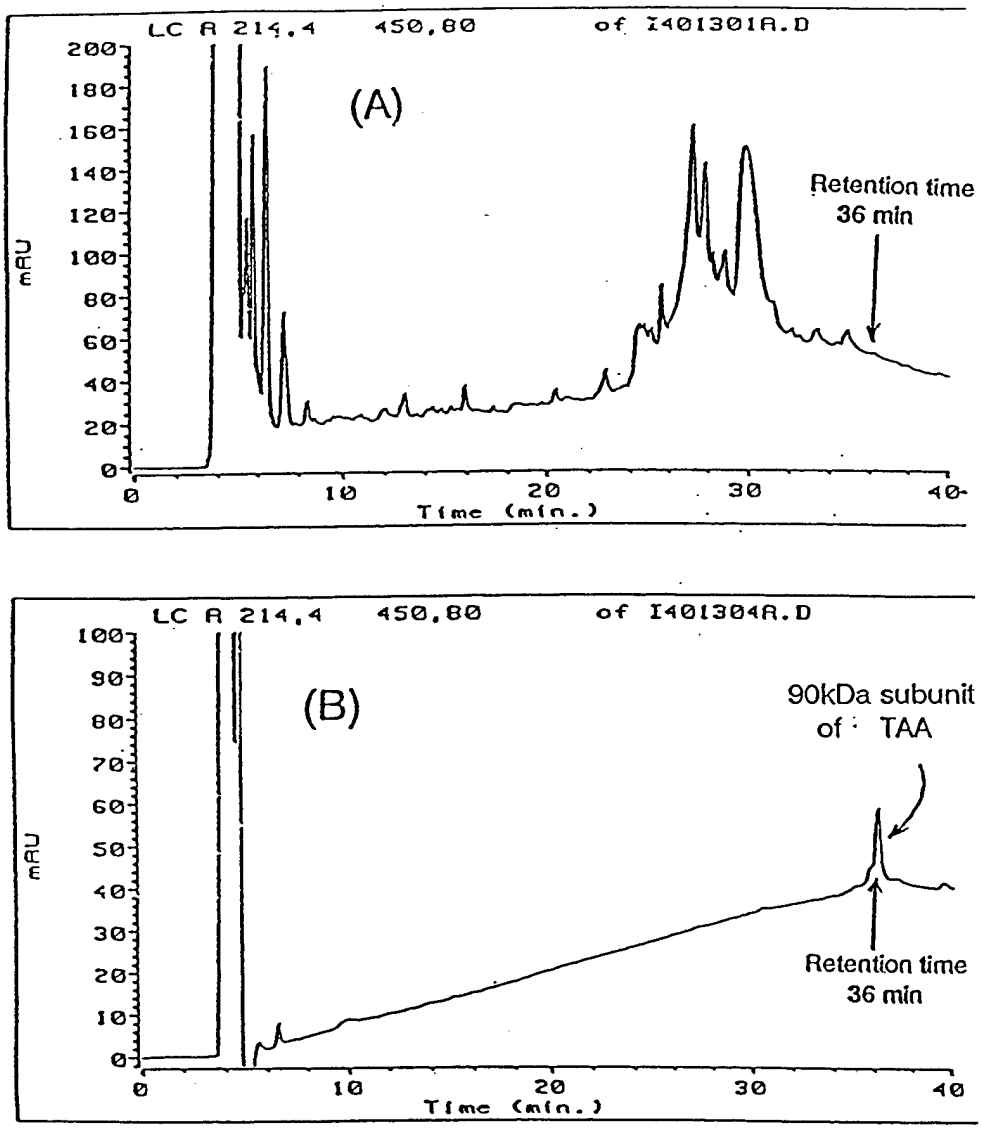


FIGURE 6: Reverse phase HPLC profiles of (A) starting material (100x concentrated urine, and (B) purified 90kD TAA using a Delta-Pack C4 column and 5-95% acetonitrile gradient in 0.1% trifluoroacetic acid.

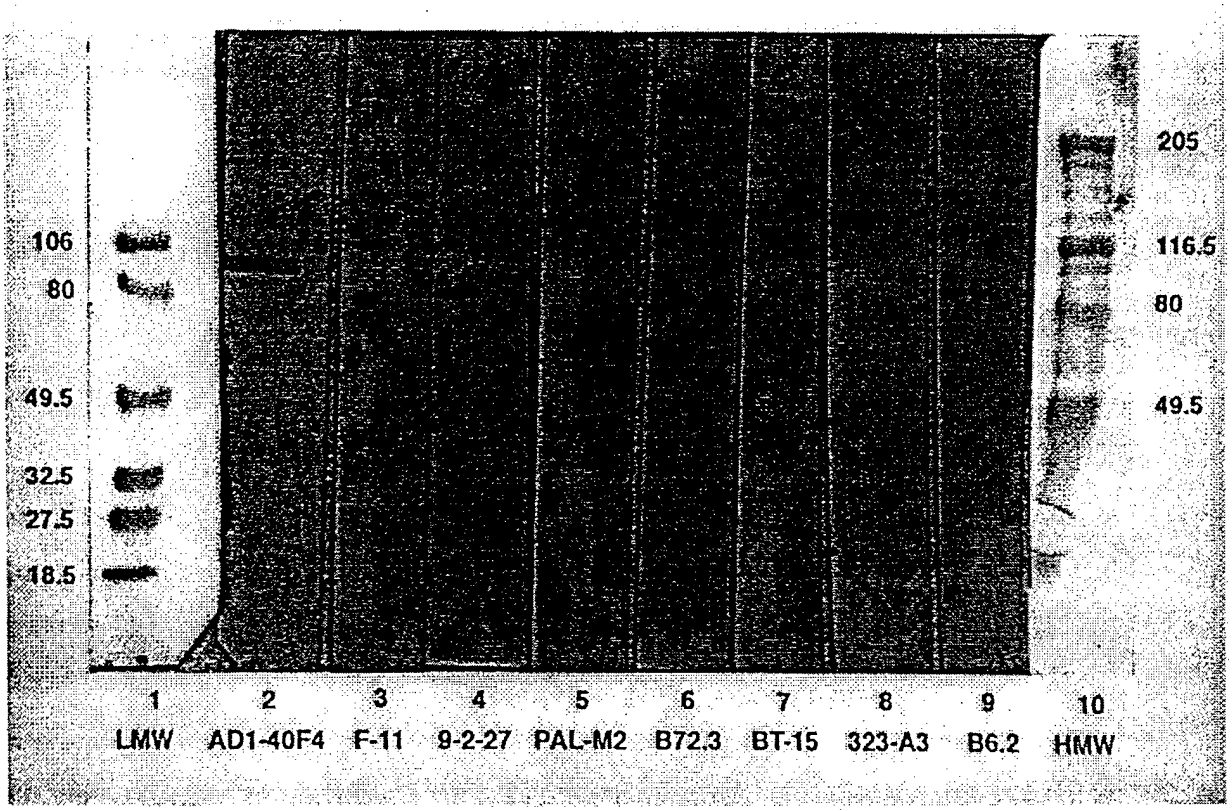


FIGURE 7: Immunoreactivity of 90kD TAA with various murine monoclonal antibodies in Western blot.

Lane #	Immunogen to develop MuMoAb	MuMoAb recognizes	Reference
1	Low molecular weight markers		
2	Glycoprotein TAA	90kD	(19)
3	Partially purified spent medium	75/77 & 100kD molecules	(20)
4	Melanoma cell extract	240kD	(21)
5	Melanoma cells	95-100kD	(22)
6	Breast cancer cells	TAG-72	(23)
7	Breast cancer cells	80-85kD	(24)
8	Breast cancer cells	43kD	(25)
9	Breast cancer cells	90kD	(26)
10	High molecular weight markers		

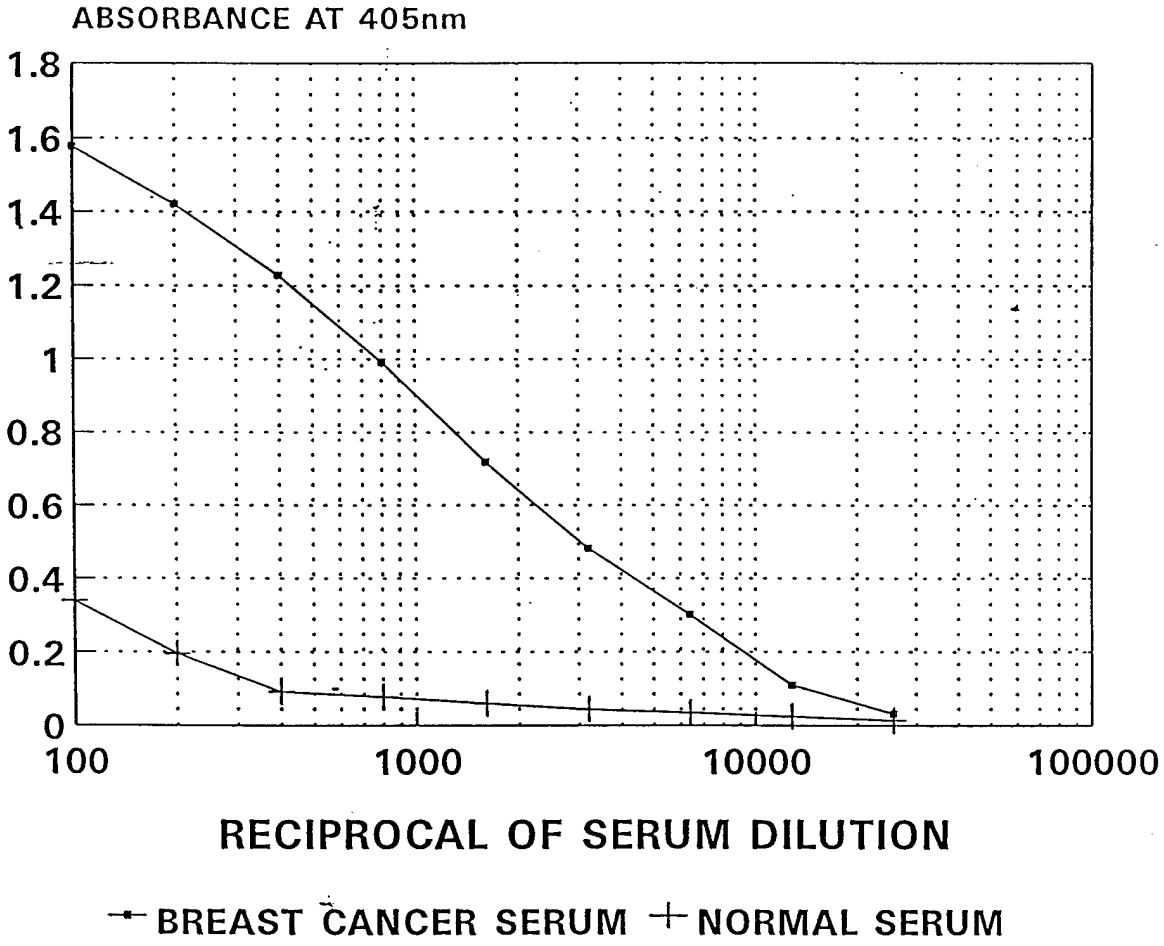


FIGURE 8: Immunoreactivity of representative serum samples from a breast cancer patient and a self-proclaimed normal individual to ultrasonically disrupted tissue culture breast cancer cell extracts in a conventional ELISA. Serum samples were diluted in PBST supplemented with 1% fetal calf serum. Two hundred microgram protein of the cell extract was used as the target per well of the ELISA plate. Alkaline phosphatase conjugated goat anti-human IgG was used as the detecting reagent. A highest dilution of the serum resulting in an optical density of 0.1 at 405_{nm} was considered as the antibody titer.

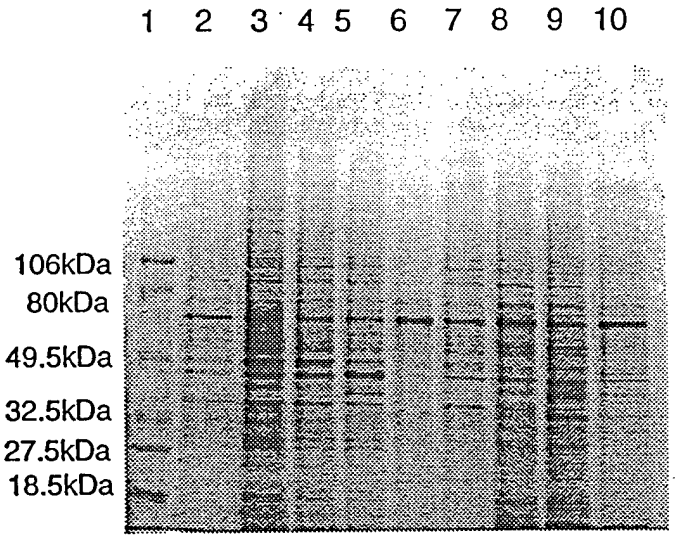


FIGURE 9: SDS-PAGE of NP-40 extracts of various cell line. Extracts were loaded onto lanes of 6 to 20% gradient gel. After electrophoresis, the gels were stained with Coomassie. Lane 1: molecular weight standards; lane 2: melanoma (M14) cell extract; lane 3: breast cancer (157) cell line; lane 4: breast cancer (231) cell line; lane 5: breast cancer (734B) cell line; lane 6: sarcoma (LV) cell line; lane 8: colon (LS174T) cell line; lane 9: breast cancer (Br-2) cell line; and lane 10: normal fibroblast cell line.

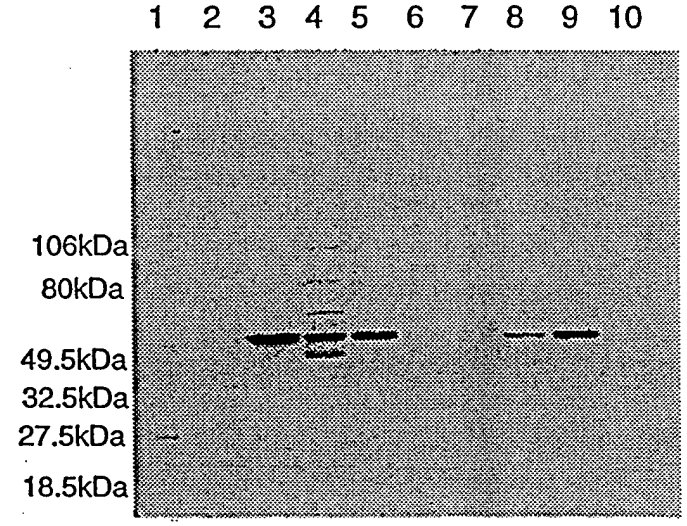


FIGURE 10: Reactivity of purified IgG from serum of a breast cancer patient in Western blot. The IgG was used at a concentration of 3ug/ml, and the reactivity of the antibody was realized using alkaline phosphatase conjugated Fab fragment of goat anti-human IgG. The target in various lanes are as follows: Lane 1: molecular weight standards; lane 2: melanoma (M14) cell extract; lane 3: breast cancer (157) cell line; lane 4: breast cancer (231) cell line; lane 5: breast cancer (734B) cell line; lane 6: sarcoma (LV) cell line; lane 8: colon (LS174T) cell line; lane 9: breast cancer (Br-2) cell line; and lane 10: normal fibroblast cell line.

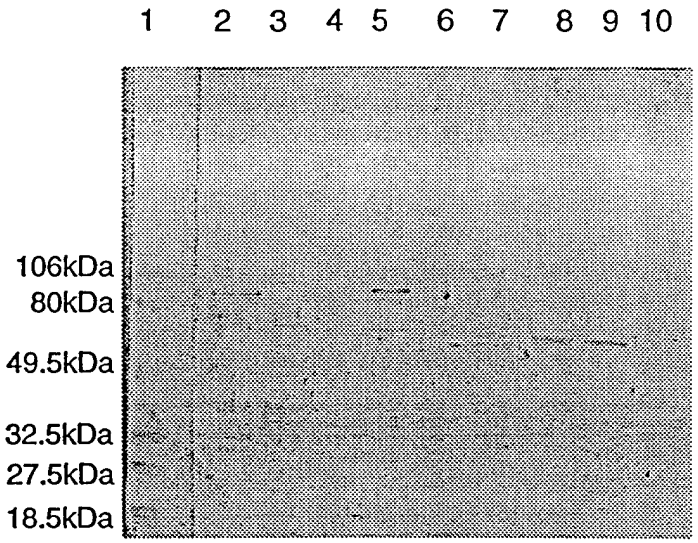


FIGURE 11: Reactivity of normal serum with targets in Figure 4 using Western blot. Similar results were observed when IgG fraction of the normal serum was used as the source of antibody at 200 ug/ml concentration. (Lane numbers are defined in Figure 9).

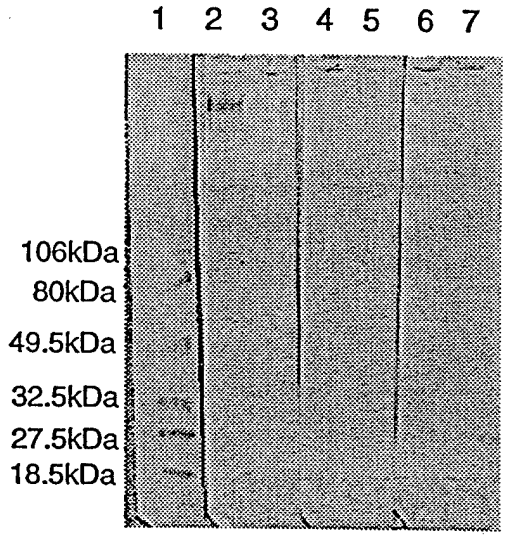


FIGURE 12: Reactivity of LCL-4 culture supernate (antibody source) in Western blot. Lane 1: Molecular weight standards; lane 2: LCL-4 reacted with Br-2 (breast carcinoma cell line) spent medium; lane 3: LCL-4 reacted with normal fibroblast spent medium; lane 4: RPMI instead of LCL-4 culture medium as no antibody control reacted with Br-2 spent medium; lane 5: RPMI instead of LCL-4 culture medium reacted with fibroblast spent medium; lane 6: alkaline phosphatase conjugated goat anti-human IgM control (conjugate control) with spent medium of Br-2; and lane 7: same as lane 6 but against spent medium of normal fibroblasts.

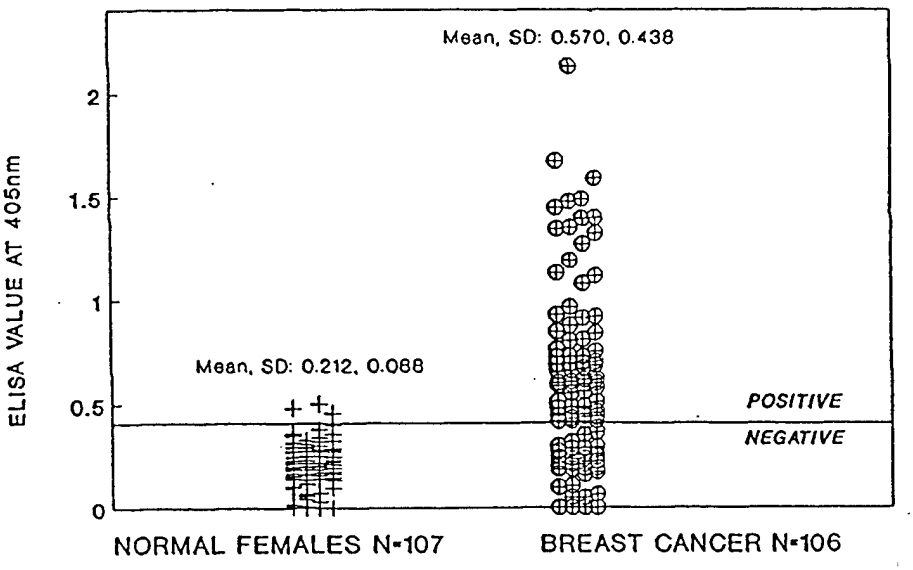


FIGURE 13: Incidence and distribution of 90kD glycoprotein TAA specific IC (TA-90-IC) in sera of normal controls and randomly selected breast cancer patients. Horizontal solid line denotes the positive cut off level.

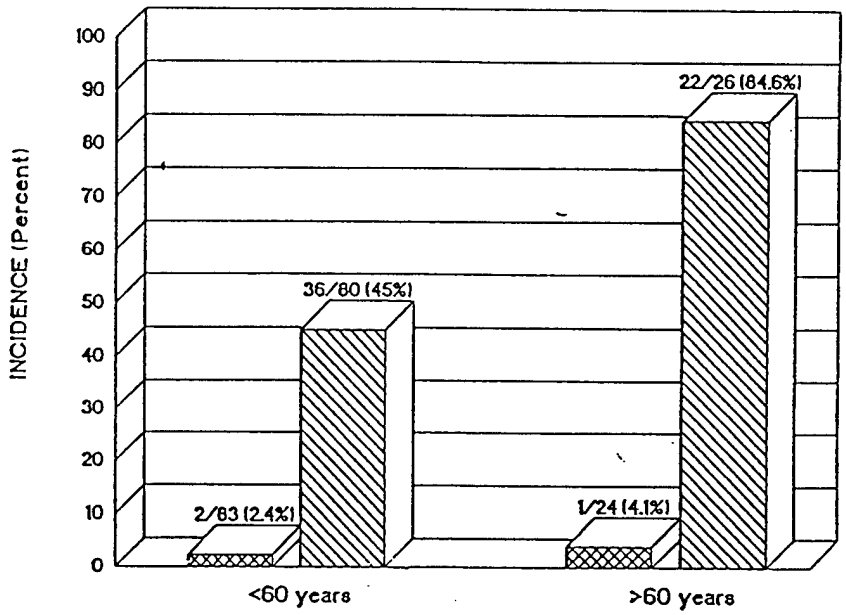


FIGURE 14: Age related incidence of the 90kD glycoprotein TAA specific IC (TA-90-IC) in breast cancer patients and normal controls. The samples were segregated on the basis of age, <60 years and equal to or >60 years. An ELISA value of greater than 0.410 OD_{405nm} was considered positive.

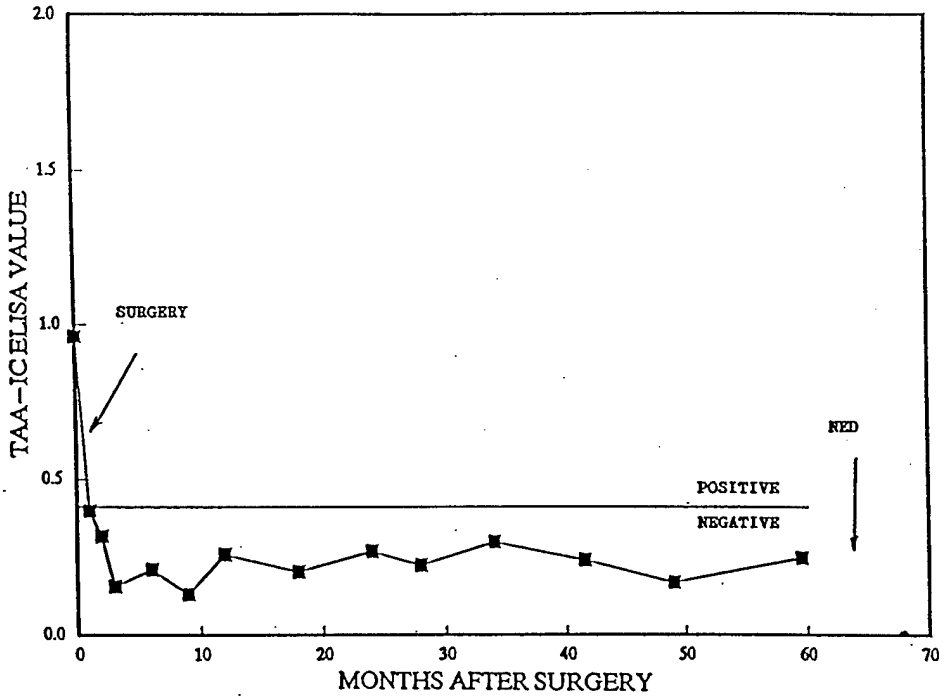


FIGURE 15: 90kD glycoprotein TAA-specific-IC during the clinical follow-up of a breast cancer patient who remained disease free for more than 5 years. The post-operative serum

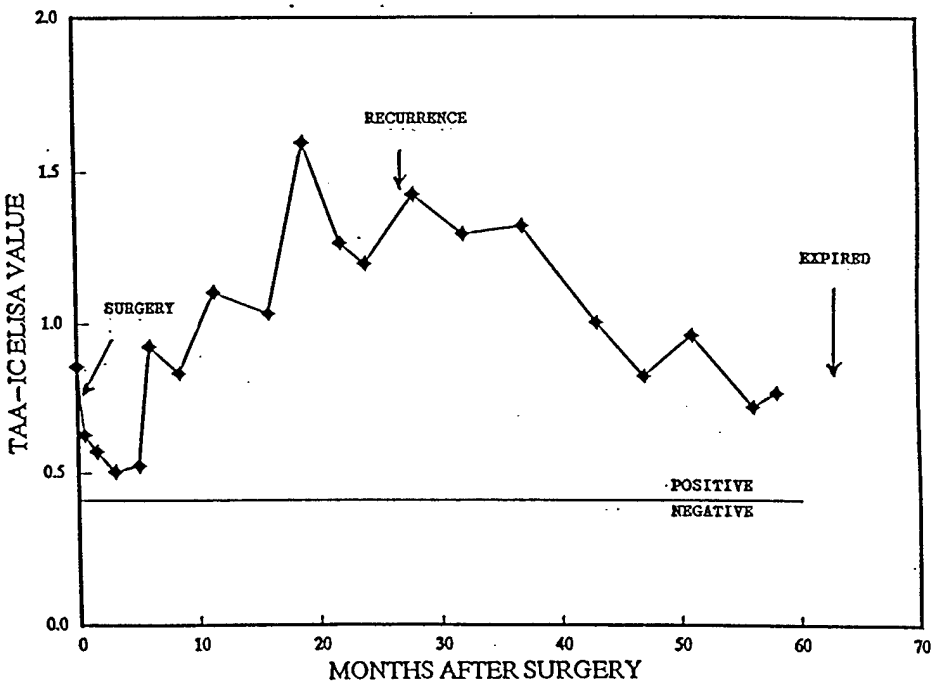


FIGURE 16: 90kD glycoprotein TAA-specific-IC during the clinical follow-up of a breast cancer patient who developed recurrent disease within 26 months of surgery. The TAA-specific-IC values of sequential serum samples of this patient were positive several months before clinically detectable disease.

FIGURE 17: RELATIONSHIP BETWEEN S-PHASE AND TA-90-IC ELISA VALUE

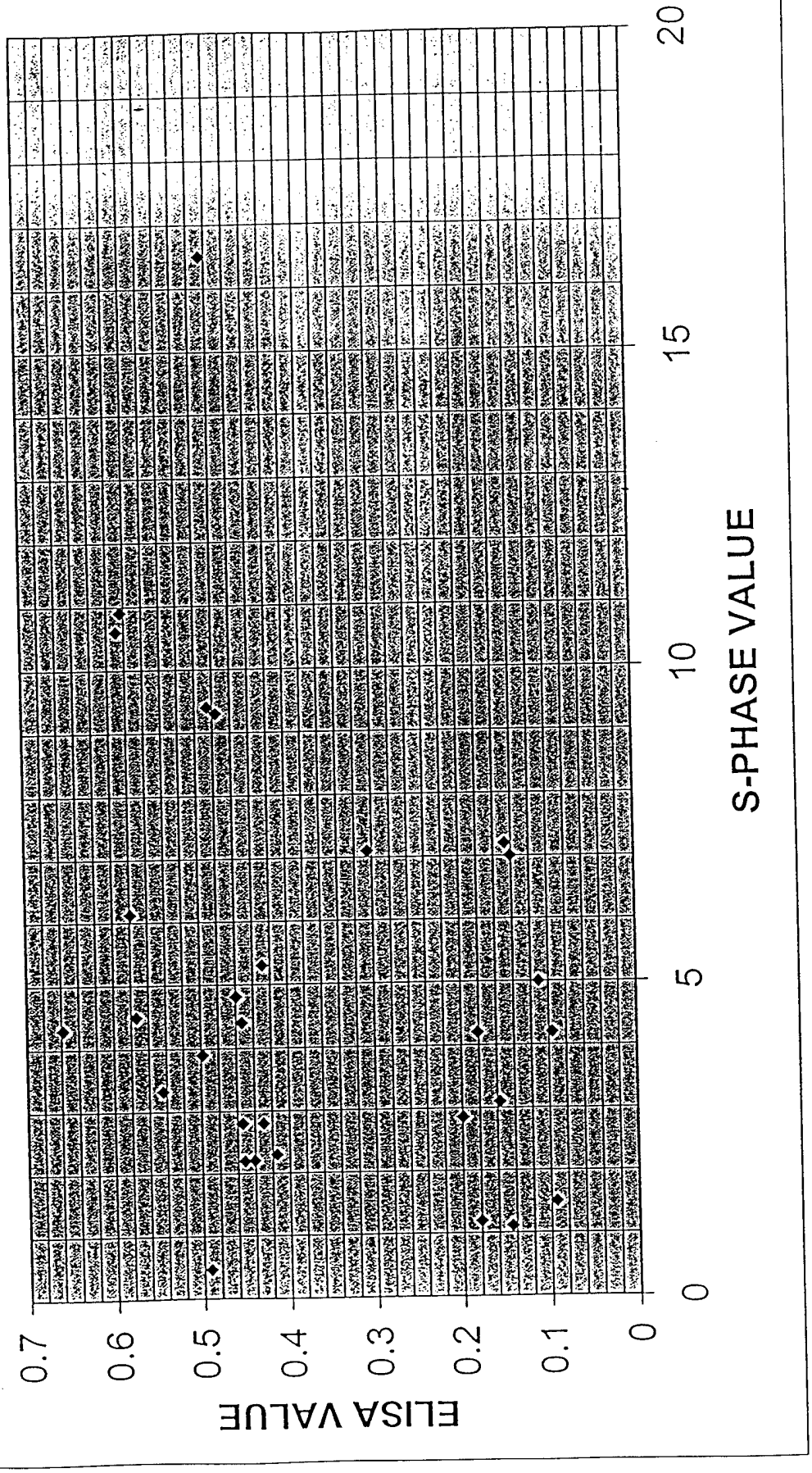
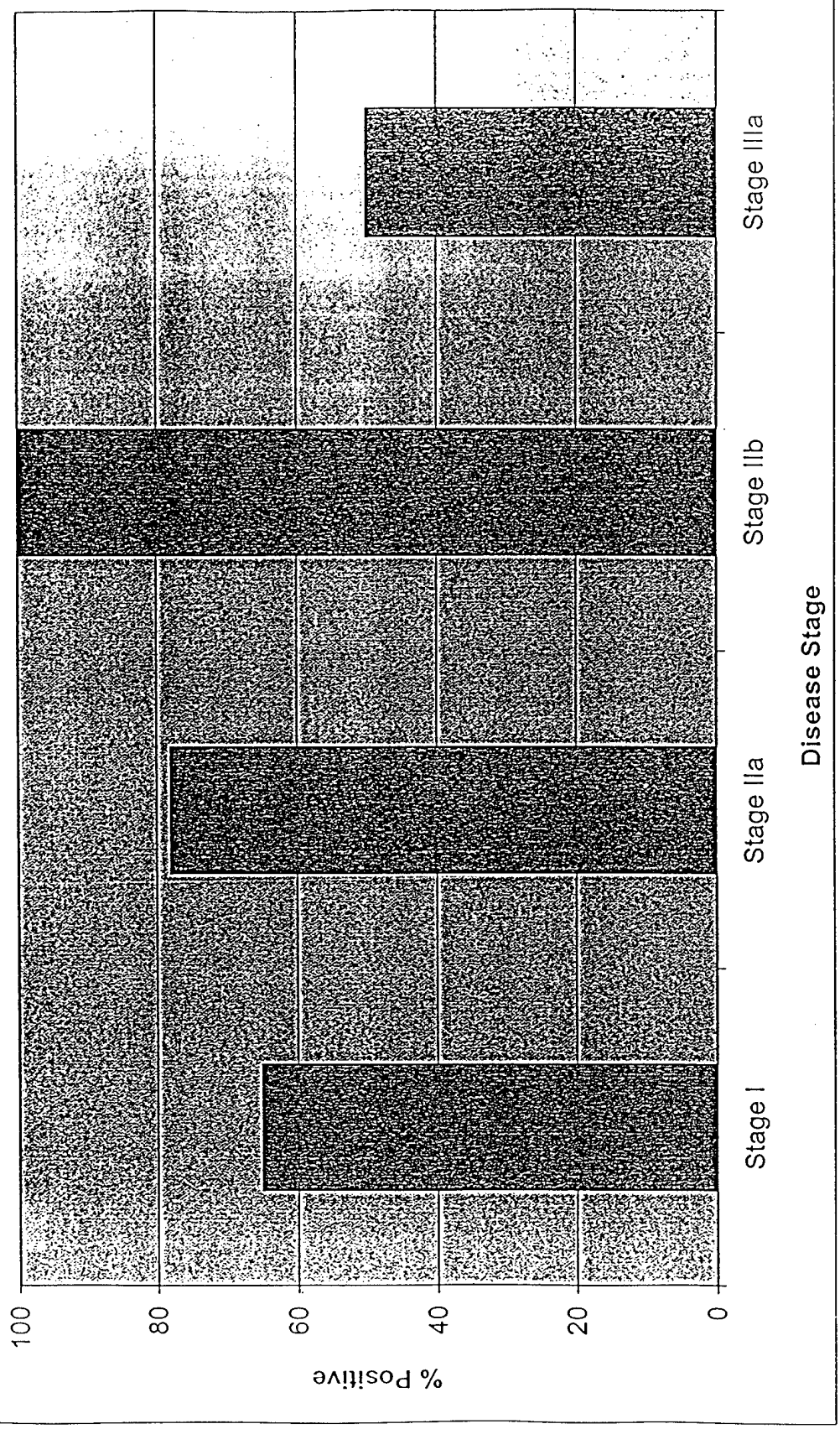
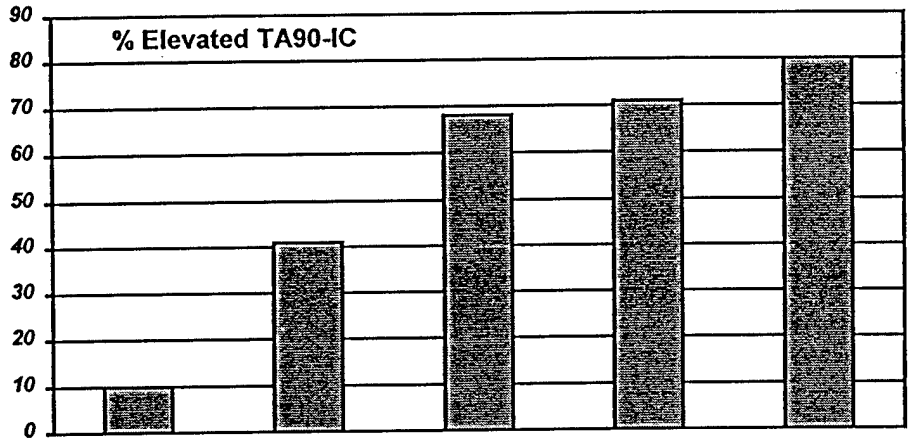


FIGURE 18: INCIDENCE OF TA-90-IC POSITIVITY INCREASES LINEARLY WITH AJCC STAGE OF DISEASE





	BENIGN	*DCIS*	*Stage I*	*Stage II*	*Stage III*
% Elevated TA90-IC	10	41	68	71	80
mean TA90-IC	0.282	0.327	0.534	0.452	0.455
n	*88*	*17*	*23*	*24*	*5*

FIGURE 19: Percent of patients with elevated TA-90-IC levels based on pathologic analysis.

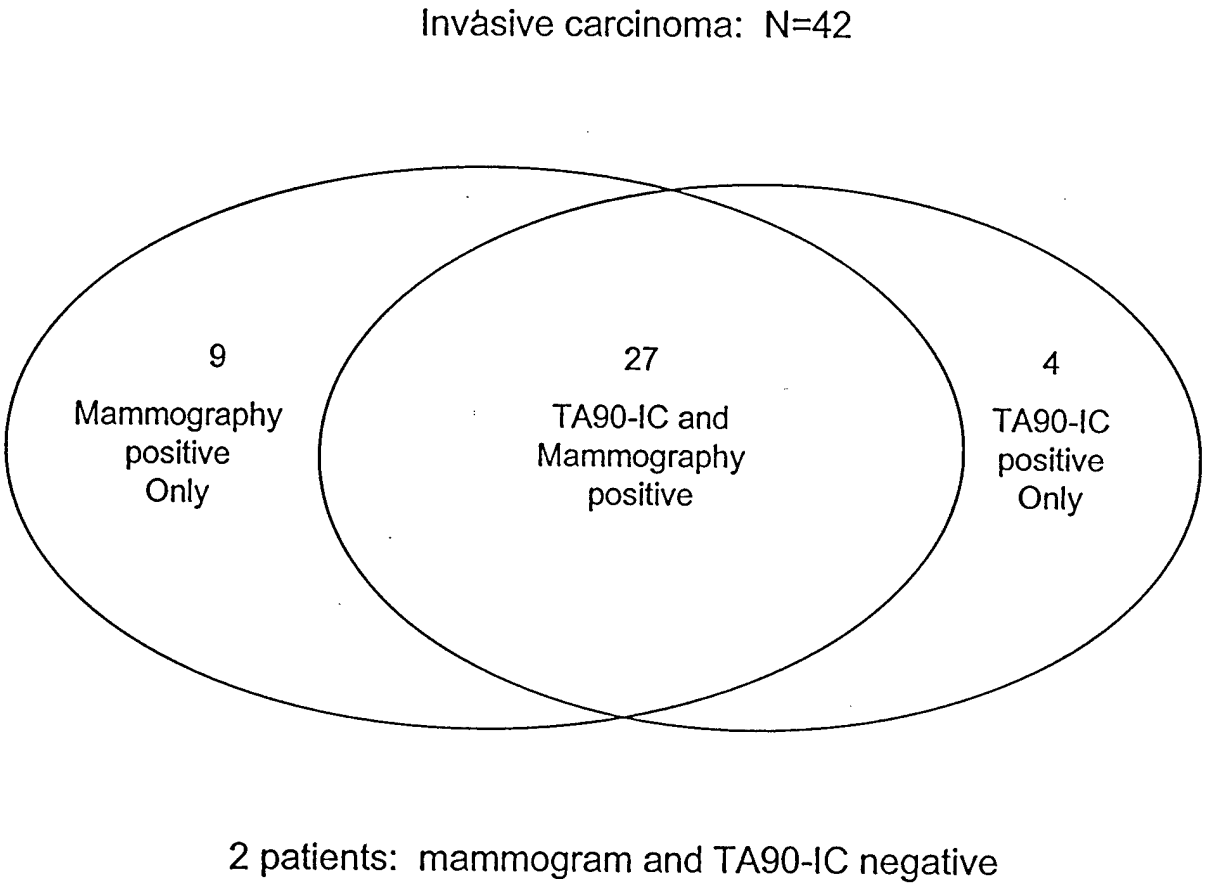


FIGURE 20: Overlap between results of mammography and TA-90-IC ELISA in 40 patients with invasive breast carcinoma. Two additional patients with invasive carcinoma had negative mammographic findings and a negative TA-90-IC ELISA.

Detection and comparison of a 90 kD glycoprotein tumor-associated antigen specific immune complexes with CEA and CA15-3 in breast cancer

RISHAB K. GUPTA and DONALD L. MORTON

John Wayne Cancer Institute at Saint John's Hospital and Health Center, 2200 Santa Monica Blvd., Santa Monica, CA 90404, USA

Communicated by B. Bonavida, July 10, 1995

Abstract. Serum samples selected randomly from 106 patients that had histopathologically proven breast cancer, and from 107 self-proclaimed and apparently healthy females were analyzed for the presence of a 90 kD subunit containing glycoprotein TAA-specific immune complexes (IC) by a murine monoclonal antibody based ELISA. The incidence of the glycoprotein antigen specific IC in breast cancer patients was 63% (67/106), as indicated by the normalized ELISA value above 0.410 OD_{405nm}. On the contrary, only 3 (2.8%) of 107 apparently healthy controls had positive ELISA value ($p<0.05$). Comparison of the glycoprotein TAA-specific IC results in breast cancer patients with evidence of disease with the results of CEA and CA15-3 revealed that the incidence of abnormal values was increased to 91%. Thus, use of the glycoprotein TAA specific-IC marker in conjunction with CEA and/or CA15-3 may prove to be more sensitive than when used alone for immunodiagnosis and immunoprognosis.

Introduction

Breast cancer is the second leading cause of death among females with cancer (1,2). The overall survival for breast cancer patients is considerably low, which is in part due to the fact that by the time the disease is diagnosed, it may have already metastasized (3). A number of tumor markers have been developed for diagnosis, prognosis, and early detection of recurrence in breast cancer patients (4-6). Most of the markers described thus far are not immunogenic in breast cancer patients.

The presence of tumor-associated antigens that are immunogenic in breast cancer patients has now been confirmed by various investigators (7-9). Therefore, it is logical to assume that circulating immune complexes (IC) should form *in vivo* each time a humoral immune response is made to the antigens, and the source of the antigen is present in breast cancer patients.

Salinas *et al* (10) reported that immune complexes (IC) could be demonstrated in 50% sera of breast cancer patients at the time of diagnosis. Patients with metastatic disease had more IC than patients with limited disease. The IC values were correlated with other markers, e.g., carcinoembryonic antigen (CEA), and it was suggested that IC measurements might provide additional prognostic information, particularly in patients who do not have elevation of other markers (10,11). Subsequent reports from various investigators revealed that results of IC detection and correlation with malignant disease varied from investigator to investigator due to the antigen non-specific nature of the IC detection assays (12). This is because the cancer patients might have had apparent and not apparent infection or autoimmune disease at the time of serum collection. These situations most likely caused some false positive IC values, and thus lowering of prognostic significance of the assay. Therefore to ascertain whether the presence of IC detected in the circulation of a cancer patient at a given point in time was due to the presence of tumor, it was necessary to use cumbersome procedures to confirm that the antigen portion of the IC was indeed the defined TAA (13). To overcome this problem, we have isolated immune complexes from IC positive sera and after dissociation, characterized antibody and antigen components (14). One of the antigen identified in the antigenic fraction of cancer patient's IC was similar to a heat stable glycoprotein expressed by cancer cells of various histologic types (15). The antigen was purified from urine of a melanoma patient, because its presence was in a relatively high concentration, and was used as immunogen to develop a murine monoclonal antibody (MuMoAb), AD1-40F4, of IgM isotype (16). Analysis by Western blot revealed that AD1-40F4 monoclonal antibody recognized a 90 kD subunit of the antigen (17). The MuMoAb showed no immunologic reactivity with human serum proteins and the epitope recognized by the monoclonal antibody resided in the protein part of the glycoprotein TAA (16). Blocking studies revealed that the epitope on the 90 kD subunit recognized by the MuMoAb was different from those recognized by the allogeneic anti-glycoprotein-TAA antibodies (18).

The present study investigates the presence of a 90 kD subunit containing glycoprotein tumor-associated antigen (TAA) specific IC in the sera of breast cancer patients, and its correlation with other tumor markers, such as CEA and CA15-3.

Correspondence to: Dr Rishab K. Gupta, John Wayne Cancer Institute, 2200 Santa Monica Blvd., Santa Monica, CA 90404, USA

Key words: glycoprotein tumor-associated antigen, breast cancer, immune complexes, CEA, CA15-3, ELISA, murine monoclonal antibody

Materials and methods

Patient population and follow-up. In this investigation serum samples were procured from 106 women who were diagnosed to have breast cancer. The mean age of the patients was 51 years with a range of 25 to 82 years. Histopathologically, 90 patients had invasive ductal carcinoma (IDC) and 16 patients had ductal carcinoma *in situ* (DCIS). In addition, serum samples were procured from 107 self-proclaimed apparently healthy normal females. The age of normal controls ranged from 28 to 74 years with a mean of 45. All serum samples were stored frozen without any preservative at -35°C until used.

90 kD TAA-specific IC detection assay. We developed a tumor antigen specific IC detection assay which utilizes an immobilized murine monoclonal antibody, AD1-40F4, directed to the 90 kD subunit of a glycoprotein TAA. The murine monoclonal antibody, AD1-40F4, and the glycoprotein antigen were prepared as described below. Details of the assay have been described elsewhere (8). In brief, one hundred microliters of the AD1-40F4 ascites diluted to a protein concentration of 100 µg/ml were dispensed into each of the appropriate wells of glutaraldehyde activated microtiter plates (Dynatech Laboratories, Chantilly, VA). The plates were incubated at 4°C for 16 h and then washed with PBS supplemented with 0.5% Triton X-100 (PBS-TX). The washed plates were blocked with 100 µl per well of 1% bovine serum albumin (BSA) in PBS-TX at 23°C for 1.0 h. Test serum samples were diluted 1:60 with PBS-TX supplemented with 1% BSA, 0.5% normal mouse serum and 0.01 M ethylene diamine tetraacetic acid (EDTA). One hundred microliters of the diluted sample were dispensed into duplicate wells of the activated plates and incubated at 37°C for 45 min. At the end of incubation the wells were washed with PBS-TX. One hundred microliters of alkaline phosphatase conjugated to Fab fragment of goat anti-human IgG (Sigma Chemical Company, Saint Louis, MO) at 1:500 dilution were added to each test and control wells of the plate. The plates were incubated at 37°C for 45 min and washed with PBS-TX. Each well of the plate then received 200 µl of p-nitrophenyl phosphate (1.0 mg/ml) in 10% diethanolamine buffer as the substrate and the plates were incubated in the dark for 1.0 h at 23°C. The absorbance was read at 405_{nm}. Each sample was tested in duplicate with positive and negative controls and blanked individually in the same microtiter plate. Each test plate also included controls for non-specific protein binding and binding of conjugate to the immobilized murine monoclonal (capturing) antibody. The net optical densities of the control samples were used to generate a correction factor to normalize the net optical density of the test samples analyzed on that particular test plate. If the correction factor for a test plate fell outside the range from 0.8 to 1.2, the assay was considered invalid. The upper limit of normal for the glycoprotein TAA marker was set at 0.410 (mean±3 SD ELISA values of 59 normal sera determined from previous studies).

Preparation of glycoprotein TAA. The glycoprotein TAA which is expressed by 82% (18/22) of carcinomas, was purified as described elsewhere (18) from a 24 h urine sample of a melanoma patient. Urine from melanoma patient (Je 8504) was used because the glycoprotein TAA is expressed by solid tumors of various histologic types and this patient's urine had relatively high antigenic activity (18). Briefly, the 24 h urine samples were collected and filtered through a Whatman no. 1 filter-paper (Whatman International, Maidstone, England) to remove all sediments. The clarified urine was concentrated 100-fold using an Amicon hollow-fiber concentrator equipped with an H1P10-8 cartridge (Amicon Corp., Beverly, MA). Concentrated material was passed through a Sephacryl S-200 column (Pharmacia LKB, Piscataway, NJ) using 0.025 M phosphate buffered saline supplemented with 0.02% sodium azide as eluent at a flow rate of 25 ml/h. Fractions under each peak observed at 280_{nm} were pooled separately, concentrated and tested for antigenic activity using an allogeneic double-determinant enzyme-linked immunosorbent assay as described previously (16). The antigenic pool was quantitatively absorbed with immobilized rabbit anti-human Ig antibodies until free of detectable human IgG in an enzyme immunoassay (17). The purified antigenic pool was used to develop murine monoclonal antibodies.

Preparation of murine monoclonal antibody to glycoprotein TAA. The glycoprotein TAA prepared as described above was used as an immunogen to develop a murine IgM monoclonal antibody, AD1-40F4, with specificity to the antigen according to the procedures described by Kohler and Millstein (19). The monoclonal antibody did not exhibit any reactivity with pooled human IgM, pooled human IgG, ferritin, B2-microglobulin, B2-glycoprotein, apolipoprotein B, apolipoprotein CII, apolipoprotein CIII, or human serum albumin. Western blot analysis revealed that the AD1-40F4 antibody recognized the 90 kD subunit of the glycoprotein antigen (16). After specificity analysis, the murine monoclonal antibody was mass produced as ascites in BALB/c mice, and used as the source of antibody to develop the 90 kD-TAA-specific IC detection assay as described above.

Carcinoembryonic antigen (CEA) assay. CEA measurements on 68 of the 106 serum samples were performed by Dianon Systems, Inc., Stratford, CT, using Abbott CEA-EIA procedures that followed manufacturer's instructions. Results were expressed as ng CEA/ml. A value of greater than 2.5 ng CEA/ml was considered positive.

CA15-3 assay. CA15-3 was measured by the radioimmunoassay kit (CA15-3 RIA) commercially available from Centocor, Malvern, PA. The RIA was performed according to the manufacturer's instructions by the Dianon Systems, Inc. Results were expressed as CA15-3 units/ml (U/ml). A value of greater than 30 U of CA15-3/ml was considered positive.

Statistical analysis. Fisher's exact test as implemented by the Instat Biostatistics program from the GraphPad Software, Inc., San Diego, CA, was used to determine statistically significant differences among the 90 kD glycoprotein TAA-specific IC assay values of normal and breast cancer patients, and for comparison between 90 kD TAA-specific results and other tumor marker results. All comparisons were two-tailed and a p-value of less than 0.05 was considered statistically significant.

Table I. Detection of purified glycoprotein TAA after mixing with purified baboon polyclonal IgG antibody by the murine monoclonal antibody, AD1-40F4, capture assay.

Test material	Absorbance at 405_{nm}
Immobilized murine monoclonal antibody (control)[a]	0.042
+ baboon anti-glycoprotein TAA (44 μg purified IgG/ml)	0.138
+ glycoprotein TAA (15 μg protein/ml)	0.068
+ human serum albumin (20 μg/ml)	0.162
+ pre-immune baboon IgG (50 μg/ml)	0.129
+ mixture of baboon anti-TAA IgG (44 μg/ml) and: glycoprotein TAA (15 μg/ml)	0.686
glycoprotein TAA (5 μg/ml)	0.869
glycoprotein TAA (1.6 μg/ml)	0.714
glycoprotein TAA (0.53 μg/ml)	0.753
human serum albumin (20 μg/ml)[b]	0.153
human serum albumin (10 μg/ml)[b]	0.218
human serum albumin (5 μg/ml)[b]	0.176
+ mixture of pre-immune baboon IgG (50 μg/ml) and: glycoprotein TAA (15 μg/ml)	0.188

[a]Anti-90 kD glycoprotein TAA murine monoclonal antibody in the form of ascites (100 μg protein per ml) was immobilized to the wells of glutaraldehyde-activated microtiter plates. [b]Human serum albumin was used as control to determine the effect of nonspecific protein control.

Results

Detection of in vitro formed 90 kD glycoprotein TAA-specific IC. A polyclonal anti-glycoprotein TAA antibody purified from a baboon antiserum that was raised against the glycoprotein-TAA (20), was used to generate IC *in vitro*. For this purpose the baboon polyclonal antibodies were mixed with the purified glycoprotein TAA in different protein proportions. After incubation at 37°C for 30 min, the mixtures were tested to determine if a positive signal was generated in the AD1-40F4 murine monoclonal antibody capture assay. Goat anti-human IgG conjugated to alkaline phosphatase was used as the signal developer. We have previously documented that the goat anti-human IgG enzyme conjugate reacts equally well with the baboon IgG. Table I denotes that binding of the enzyme conjugate was the highest when the immobilized AD1-40F4 murine monoclonal antibody was incubated and reacted with the mixture of purified glycoprotein TAA and purified polyclonal baboon anti-TAA IgG. Furthermore, this signal was consistently high over a wide range of antibody (baboon anti-TAA) to antigen (glycoprotein TAA) at protein concentration ratios (3:1 to 90:1). Neither of the two immune reactants (antigen or antibody) alone or pre-immune baboon IgG or human serum albumin exhibited a signal greater than 0.200 O.D. at 405_{nm}. These data confirmed our previous (16) observations that the AD1-40F4 murine monoclonal antibody had no significant reactivity with baboon IgG and human serum albumin, and that the enzyme conjugate had no affinity (specific or non-specific) with either the murine monoclonal antibody or with the glycoprotein TAA. These results clearly denote that the AD1-40F4 murine monoclonal antibody captured baboon anti-glycoprotein TAA IgG via the glycoprotein TAA only, i.e., when the IgG antibody was in the form of glycoprotein TAA-specifc immune complexes.

Reproducibility studies to assess the assay variability using a serum from breast cancer patients in ten replicates revealed that inter-assay variations ranged from 0.806 to 1.311 ELISA value with a mean of 1.010, standard deviation of 0.139 and coefficient of variation of 13.7%. These values for intra-assay variations ranged from 0.849 to 1.214 with a mean of 1.007, standard deviation of 0.105 and coefficient of variation of 10.4%.

Incidence of 90 kD TAA-specific IC in normal control vs breast cancer patients. Initially we analyzed serum samples from self-proclaimed healthy normals and breast cancer patients. Fig. 1 illustrates the distribution of 90 kD TAA-specific IC ELISA values of sera from 107 healthy normal females and from 106 breast cancer patients. In this experiment, the procurement of the sera from breast cancer patients was random, i.e., no

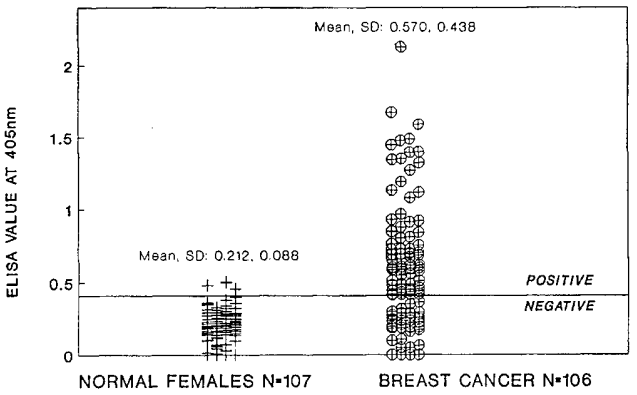


Figure 1. Incidence and distribution of 90 kD glycoprotein-TAA-specific IC in sera of normal controls and randomly selected breast cancer patients. Horizontal solid line denotes the positive cut-off level.

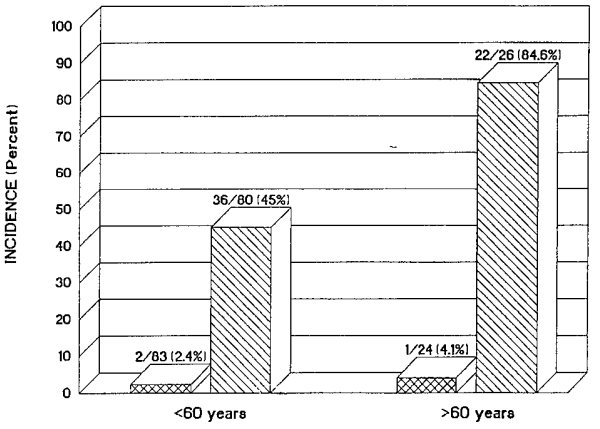


Figure 2. Age related incidence of the 90 kD glycoprotein TAA-specific IC in breast cancer patients and normal controls. The samples were segregated on the basis of age, <60 years and equal to or >60 years. An ELISA value of greater than 0.410 OD_{405nm} was considered positive.

Table II. Incidence and level of 90 kD glycoprotein TAA-specifc IC in two different histologic types of breast carcinoma.

Histologic type	Total number	Number positive	Percent positive	ELISA values Range	mean	SD
Invasive ductal carcinoma	90	58	64	0.000-2.038	0.453	0.365
Ductal carcinoma *in situ*	16	9	56	0.046-1.357	0.412	0.314

criterion with respect to pre- or post-surgery, evidence or no evidence of disease, etc., was used in selecting the serum samples. Comparative analysis of the data in normal and breast cancer group revealed that the normalized ELISA value (mean±SD) of the normal control sera (0.212±0.088) was significantly ($p<0.05$) lower than that of breast cancer patients (0.570±0.438). Furthermore, when an ELISA value of 0.41 or greater was considered positive for the presence of the TAA-specific IC, the incidence of the glycoprotein TAA-specific IC was significantly ($p<0.05$) greater in the breast cancer group (67/106, 63%) than the normal group (3/107, 2.8%).

While the incidence of 90 kD TAA-specific IC in the normal group was not affected by age, it was significantly higher ($p<0.05$) in breast cancer patients that were over 60 years old (88%, 23/26) compared to those that were under 60 years old (55%, 44/80) (Fig. 2).

Of the 90 IDC breast cancer patients, 58 (64%) were positive for the glycoprotein TAA-IC; whereas, this incidence was lower 56% (9/16) in DCIS breast cancer patients (Table II). The lower incidence in DCIS patients may be due to the non-aggressive nature of the ductal carcinoma *in situ*.

Association among glycoprotein TAA-IC, CEA and CA15-3. CEA and CA15-3 have been considered useful tumor markers in the prognosis and monitoring of breast cancer patients. We compared the glycoprotein TAA-specific IC results using 68 serum samples of breast cancer patients selected on the basis of the presence of the disease. The two tumor markers, CEA and CA15-3, are not known to be immunogenic in cancer patients. Of the 68 serum samples 55 (80.9%) were positive for the glycoprotein TAA-specific IC, 16 (23.5%) were positive for CEA, and 23 (33.8%) were positive for CA15-3. Despite higher incidence of glycoprotein TAA-specific IC than CEA or CA15-3, it was observed that some serum samples that were positive for CEA or CA15-3 were not necessarily positive for the glycoprotein TAA-IC.

As shown in Table III, statistical evaluation of the data by Fisher's exact test revealed that there was no significant associations between the glycoprotein TAA and CEA or CA15-3 ($p>0.05$). However, when either of the three or all of the three positive markers were taken into consideration, the incidence of positivity increased from 80.9% to 91% (Table IV).

Discussion

Serological tumor markers are considered to be useful in the early detection and monitoring of metastases for early and effective treatment to increase the duration of disease-free and/or overall survival (21,22). However, with the exception of CA15-3, recent reports have questioned the value of many of these sensitive markers both in diagnosis of systemic disease and in assessing response to therapy (23,24). In this

Table III. Association between serum glycoprotein TAA-IC and CEA or CA15-3.

		Glycoprotein TAA-IC Positive[a]	Negative	Total
CEA	Positive[b]	13	3	16
	Negative	42	10	52
	Total	55	13	68
		p>0.05		
CA15-3	Positive[c]	18	5	23
	Negative	37	8	45
	Total	55	13	68
		p>0.05		

[a]An ELISA value of greater than 0.41 OD at 405_{nm} was considered positive. [b]A value of greater than 2.5 ng CEA/ml was considered positive. [c]A value greater than 30 U of CA15-3/ml was considered positive.

Table IV. Incidence of positivity for glycoprotein TAA-IC, CEA and CA15-3 or their combination in sera from breast cancer patients.

Marker (Alone or in combination)	(n=68) Number positive	Percent positive
Glycoprotein TAA-IC only	55	81
CEA only	16	24
CA15-3 only	23	34
CEA or CA15-3	30	44
TAA-IC or CEA	~~48~~ 58	~~71~~ 84
TAA-IC or CA15-3	60	88
TAA-IC or CEA or CA15-3	62	91

investigation, we have analyzed serum samples from breast cancer patients to determine the usefulness of an antigen specific immune complex detection assay. This marker differs from the existing tumor markers in that it determines the presence of a glycoprotein TAA which is immunogenic in patients and circulates in the form of immune complexes in the blood. The detection assay can be considered as a form of double-antibody sandwich ELISA in which the immune complexes present in the test sample are captured by an immobilized murine monoclonal antibody, AD1-40F4. This monoclonal antibody was specifically developed using the purified glycoprotein TAA defined by autologous and allogeneic antibodies, and recognizes an epitope different from those recognized by the autologous antibodies.

There is sufficient evidence in literature to suggest that estimation of immune complex levels or their fluctuations during the course of malignant disease might predict the outcome of the disease (10). However, unlike other tumor markers, one of the drawbacks which inhibited the application of this technology in a clinical setting has been the use of antigen non-specific assays for the detection of immune complexes. The use of antigen-nonspecific assays resulted in inconsistent results, because some of the material detected by these assays were characterized to be aggregated IgG, reaction products of denatured self-proteins, polyamines, or bacterial lipopolysaccharides (25). In general, immune complexes detected in sera of cancer patients have been characterized with respect to their size and the presence of anti-Ig and anti-tumor antibodies, and tumor or other antigens. A number of methods have been used to isolate and characterize the antibody and antigen components of the immune complexes (26). However, manipulations of the *in vivo* formed immune complexes are prone to introduce artifacts for characterization in subsequent studies; thus, providing inaccurate results. Therefore, development of an assay such as the one used in this investigation which detects antigen-specific immune complexes without any pre-treatment or manipulation of the test sample represents a significant and major advancement in the area of immuno-diagnosis of human cancer.

It can be argued that immune complexes present in circulation may be composed of either IgG or IgM antibodies or both; however, anti-tumor antibodies of IgG type to macromolecular antigens are more prevalent (27), and the 90 kD glycoprotein TAA-specific IC assay can be modified to detect immune complexes containing IgM antibody by using anti-human IgM conjugate. We feel that the success of the 90 kD glycoprotein TAA-IC assay for the detection of cancer is for the following reasons. The immunogenic tumor antigens shed into circulation by growing tumor cells are in small quantities and are neutralized by the humoral antibodies (28). Therefore, the results of any sensitive methods applied to detect free antigens in serum or plasma are generally negative; however, detection of human antibody (immunoglobulin) molecules via the antigen captured by the immobilized MuMoAb gives an amplification effect. This is because the unreduced antigen is a complex of at least four different subunits (18), each of which is immunogenic in the cancer host and thus can bear multiple *in vivo* reacted immunoglobulin molecules. Furthermore, glycoprotein TAA being immunogenic in cancer patients should circulate in the bloodstream in the form of immune complexes, particularly at the time when the source of the antigen (tumor) is present only in small amounts.

The serum level of 90 kD glycoprotein TAA, as assessed in the form of immune complexes, was uniformly low in the control group of 107 apparently healthy females. The values (0.212 ± 0.088 OD_{405nm}) observed in this investigation were comparable to those reported earlier (0.249 ± 0.080 OD_{405nm}) from our laboratory where the control group was comprised of 250 normal males and females (8). Furthermore, the incidence of positive values were comparable (2.8% vs 3.2%).

In the investigations where serum samples were obtained from patients with a history of breast cancer, elevated serum levels of 90 kD glycoprotein TAA were observed at a frequency of about 63%. These results confirm and significantly expand the initial observation that the 90 kD glycoprotein TAA marker could be detected in greater proportion of breast cancer patients as well (29). It is of particular interest to note that the incidence of 90 kD glycoprotein TAA in breast cancer patients was affected by age. It has been reported that the mortality rate in younger breast cancer patients is significantly lower in contrast to older breast cancer patients (30,31). Patients with an age of more than 60 years showed significantly greater incidence than those patients who were younger than 60 years. It would appear as if the tumors of younger women do not express this antigen or the tumor cells do not release it into circulation. Is it due to dormancy or different metabolic turnover rates of surface molecules? We are currently in the process of correlating the expression of the glycoprotein TAA with the level of expression of estrogen/progesterone receptors by the breast cancer cells. These hormones have been reported to modulate the expression of surface macromolecules, e.g. cerebellar responses to amino acid neurotransmitters (32).

It is obvious that the 90 kD glycoprotein TAA-IC detection assay described herein is not 100% accurate in identifying breast cancer sera. Furthermore, as low it may be, certain proportion of sera from normal controls was positive for the marker. The presence of 90 kD glycoprotein TAA in normal population is unexplainable at this time; however, it may be possible that these apparently healthy individuals had occult neoplasm at the time of serum sampling. This possibility of existence of occult disease is difficult to prove or disprove.

Despite the fact that we have not reached 90% or greater accuracy in predicting the outcome of breast cancer patients using the 90 kD glycoprotein TAA assay, the results presented are highly encouraging. This study describes one of the unique approaches to immunodiagnosis of human cancer via detecting an immunogenic tumor antigen in circulation, its specificity can be significantly enhanced by incorporating other existing tumor markers, such as CEA (33) and CA15-3 (34-36). Extensive evaluation of CEA in combination with other tumor markers or alone for clinical correlations with the clinical course of breast cancer patients has resulted in conflicting reports (37-40). In fact, it has been suggested that CA15-3 correlated with the stage of disease and in metastatic patients with the response to treatment (41-46). Use of this novel marker (tumor antigen-specific immune complexes) in conjunction with CEA and/or CA15-3 may prove to be more sensitive than when used alone for immunodiagnosis and immunoprognosis of breast cancer.

Acknowledgements

We gratefully acknowledge the critical review of the manuscript by Dr Steven J. O'Day and expert technical assistance of Mr Reynold Yee, Mr Zacarias Leopoldo and Mr Thomas Sumobay. These investigations were supported by the Department of Army grant DAMD17-94-J-4459, by the Department of Human Health Services grants CA29605, CA30019 and CA12582, by the Associates for Breast Cancer Study, by the John Wayne Cancer Institute Auxiliary and by the Jack Green Fund.

References

1. American Cancer Society: Cancer Facts and Figures. Atlanta, pp9, 1992.
2. Boring CC, Squires TS and Tony T: Cancer statistics. CA Cancer J Clin 42: 19-36, 1992.
3. Seidman H, Gelb SK, Silverberg E, LaVerda N and Lubera JA: Survival experience in the breast cancer detection demonstration project. CA Cancer J Clin 37: 258-290, 1987.
4. De Jong-Bakker M, Hart AA, Persijn JP and Cleton FJ: Prognostic significance of CEA in breast cancer: A statistical study. Eur J Cancer 17: 1307-1313, 1981.
5. Eskelinen M, Lippoen P, Hippelainen M, Seveneus L and Syrajanen K: Expression of tumour-associated antigens 12H12, 2E11, 7A9, CEA and DF3 in human breast cancer. Anticancer Res 13: 2399-2406, 1993.
6. Cannon PM, Ellis IO, Blamey RW, Bell J, Elston CW and Robertson JF: Expression of tumour-associated antigens in breast primary tissue compared with serum levels. Eur J Surg Oncol 6: 523-527, 1993.
7. Gorsky Y, Vanky F and Sulitzeanu D: Isolation from patients with breast cancer of antibodies specific for antigens associated with breast cancer and other malignant disease. Proc Natl Acad Sci USA 73: 2101-2105, 1976.
8. Gupta RK and Morton DL: Monoclonal antibody based ELISA to detect glycoprotein tumor-associated antigen specific immune complexes in cancer patients. J Clin Lab Analysis 6: 329-336, 1992.
9. Maidment BW, Papsidero LD, Nemoto T and Ming C: Recovery of immunologically reactive antibodies and antigens from breast cancer immuno-complexes by preparative isoelectric focusing. Cancer Res 41: 795-800, 1981.
10. Salinas FA, Wee KH and Silver HKB: Immune complexes and human neoplasia. Biomed Pharmacol 37: 119-125, 1983.
11. Dent PB, Louis JA, McCulloch PB, Dunnett CW and Cerottini J: Correlation of elevated C1q binding activity and carcinoembryonic antigen levels with clinical features and prognosis in bronchogenic carcinoma. Cancer 45: 130-136, 1980.
12. Herberman RB, Bordes M, Lambert PH, Luthra HS, Robins RA, Sizaret P and Theofilopoulus A: A report on international comparative evaluation of possible value of assays for immune complexes for diseases of human breast cancer. Int J Cancer 27: 569-576, 1981.
13. Gupta RK, Leitch AM and Morton DL: Nature of antigen and antibodies in immune complexes isolated by staphylococcal protein A from plasma of melanoma patients. Cancer Immunol Immunother 16: 40-47, 1983.
14. Gupta RK, Silver HKB, Reisfeld RA and Morton DL: Isolation and immunochemical characterization of antibodies from the sera of cancer patient which are reactive against human melanoma cell membranes by affinity chromatography. Cancer Res 39: 1683-1695, 1979.
15. Gupta RK, Huth JF, Korn EL and Morton DL: Prognostic significance of urinary antigen analysis by enzyme linked immunosorbent assay in melanoma patients. Diag Immunol 1: 303-309, 1983.
16. Euhus DM, Gupta RK and Morton DL: Detection of a tumor associated glycoprotein antigen in serum and urine of melanoma patients by murine monoclonal antibody (AD1-40F4) in enzyme immunoassay. J Clin Lab Analysis 3: 184-190, 1989.
17. Euhus DM, Gupta RK and Morton DL: Characterization of a 90-100 kDa tumor associated antigen in sera of melanoma patients. Int J Cancer 45: 1065-1070, 1990.
18. Euhus DM, Gupta RK and Morton DL: Association between allo-immunoreactive and xeno-immunoreactive subunits of a glycoprotein tumor associated antigen. Cancer Immunol Immunother 32: 214-220, 1990.
19. Kohler G and Milstein C: Continuous culture of fused cells secreting antibody of predefined specificity. Nature 256: 495-497, 1975.
20. Hunt KK, Shibata M, Gupta RK and Morton DL: Complement dependent lysis of tumor cells by a baboon IgM antibody to a tumor-associated antigen. Cancer Immunol Immunother 34: 377-382, 1992.
21. Foekens JA, van Putten WLJ, Portengen H, de Koning HYWCM, Thirion B, Alexieva-Figusch J and Klijn JGM: Prognostic value of PS2 and cathepsin D in 710 human primary breast tumors: multivariate analysis. J Clin Oncol 11: 899-908, 1993.
22. Ng JSY, Sturgeon CM, Seth J, Paterson GM, Roulston JE and Leonard RCF: Serological markers for metastatic breast cancer. Disease Markers 11: 217-223, 1993.

23. Robertson JFR, Pearson D, Price MR, Selby C, Blamy RW and Howell A: Objective measurement of therapeutic response in breast cancer using tumor markers. Br J Cancer 64: 757-763, 1991.
24. Tondini C, Hayes DF, Gelman R, Henderson IC and Kufe DW: Comparison of CA15-3 and carcinoembryonic antigen in monitoring the clinical course of patients with metastatic breast cancer. Cancer Res 48: 4107-4112, 1988.
25. Salinas FA, Wee KH and Silver HKB: Clinical relevance of immune complexes, associated antigen, antibodies in cancer. In: Immune Complexes and Human Cancer. Salinas FA, Hanna MA (eds). Plenum Press, New York pp55-109, 1985.
26. Gupta RK and Morton DL: Circulating immune complexes in malignant melanoma. Disease Markers 6: 81-95, 1988.
27. Vlock DR, Der Simonian R and Kirkwood DR: Prognostic role of antibody reactivity to melanoma. J Clin Invest 77: 1116-1121, 1986.
28. Euhus DM, Gupta RK and Morton DL: Induction of antibodies to a tumor associated antigen by immunization with a whole melanoma cell vaccine. Cancer Immunol Immunother 26: 247-254, 1989.
29. Gupta RK and Morton DL: Prognostic value of a 90 kD subunit containing glycoprotein tumor-associated antigen specific immune complexes in lung cancer patients. Disease Markers 12: 51-61, 1994.
30. Albain KS, Allerd DC and Clark GM: Breast cancer outcome and predictors of outcome: Are there age differences? Monogr Natl Cancer Inst 16: 35-42, 1994.
31. Hankey BF, Miller B, Curtis R and Kosary C: Trends in breast cancer in younger women in contrast to older women. Monogr Natl Cancer Inst 16: 7-14, 1994.
32. Smith SS, Waterhouse BB and Woodward DJ: Sex steroid effects on extrahypothalamic CNS. Progesterone, alone and in combination with estrogen, modulates cerebellar responses to amino acid neurotransmitters. Brain Res 422: 52-62, 1987.
33. Buamah PK, Bent DJ, Bodger WA and Skillen AW: A profile of serum CA15-3, carcinoembryonic antigen, alkaline phosphatase, and gamma-glutamyl transferase levels in patients with breast cancer. J Surg Oncol 53: 84-87, 1993.
34. Zamagni C, Martoni A, Cacciari N, Bellanava B, Vecchi F and Pannuti F: CA-549 serum levels in breast cancer monitoring. Int J Biol Markers 7: 217-221, 1992.
35. Gion M, Ruggeri G, Mione R, Marconato R, Casella C, Nasadini A, Simoncini E, Belloli S, Da Zennaro E and Bruscagnin G: A new approach to tumour marker assessment by preoperative determination in breast and colorectal cancer. Int J Biol Markers 8: 8-13, 1993.
36. Eskelinen M, Hippelainen M, Carlsson L, Jonsson P and Alhava E: A decision support system for predicting a recurrence of breast cancer, a prospective study of serum tumour markers TAG 72, CA15-3 and MCA. Anticancer Res 12: 1439-1442, 1992.
37. Bates SE and Longo DL: Tumor markers: Value and limitation in the management of cancer patients. Cancer Treat Rev 12: 163-207, 1985.
38. Daly L, Ferguson J, Cram GP, Hars V, George SL, McCarty KS and Bast RC: Comparison of a novel assay for breast cancer mucin to CA15-3 and carcinoembryonic antigen. J Clin Oncol 10: 1057-1065, 1992.
39. Kallioniemi OP, Oksa H, Aaran RK, Hietanen T, Lehtinen M and Koivula T: Serum CA15-3 assay in the diagnosis and follow-up of breast cancer. Br J Cancer 58: 213-215, 1988.
40. Repetto L, Onetto M, Gardin G, Costanzi B, Guidici S, Vitiello E, Merlini L, Naso C, Zannini C, Paganuzzi M, Rosso R and Santi L: Serum CEA, CA15-3, and MCA in breast cancer patients: A clinical evaluation. Cancer Detection Prev 17: 411-415, 1993.
41. Colomer R, Ruibal A, Navarro M, Encabo G, Sole LA and Savador L: Circulating CA15-3 levels in breast cancer. Our present experience. Int J Biol Markers 1: 89-95, 1986.
42. Gion M, Mione R, Dittadi R, Fassan S, Pallini A and Bruscagnin G: Evaluation of CA15-3 serum levels in breast cancer patients. J Nucl Med All Sci 30: 29-35, 1986.
43. Gion M, Mione R, Nascimben O, Valsecchi M, Gatti C, Leon A and Bruscagnin G: The tumour associated antigen CA15-3 in primary breast cancer: Evaluation of 667 cases. Br J Cancer 63: 809-813, 1991.
44. Nicoloni A, Colombini C, Luciani L, Capri A and Guiliani L: Evaluation of serum CA15-3 determination with CEA and TPA in the post-operative follow-up of breast cancer patients. Br J Cancer 64: 154-158, 1991.
45. Omar YT, Behbehani AE and Al-Naqeeb N: Carcinoembryonic antigen and breast carcinoma antigen (CA15-3) in pre-operative staging and post-operative monitoring of patients with carcinoma of the breast. Int J Biol Markers 3: 165-169, 1988.
46. Schimidt-Rhode P, Schulz KD, Strum G, Raab-Frick A and Prinz H: CA15-3 as a tumor marker in breast cancer. Int J Biol Marker 2: 135-141, 1987.

XVI International
Cancer Congress
1994

New Delhi, India
30 Oct. - 5 Nov. 1994

Glycoprotein tumor-associated antigen specific immune complexes in sera of breast cancer patients

R.K. GUPTA and D.L. MORTON

John Wayne Cancer Institute at
Saint John's Hospital and Health Center, Santa Monica, CA (USA)

SUMMARY

A murine monoclonal antibody, AD1-40F4, based ELISA that detected a 90kD subunit containing glycoprotein tumor-associated antigen (TAA) specific immune complexes (IC) was developed and applied to screen sera from normal individuals and breast cancer patients to determine its clinical usefulness. Sera from randomly selected breast cancer patients had a significantly higher ($p < 0.05$) incidence for positive TAA-specific IC (50.9% (58/114)) than the control (normal) group (3.2% (8/250)). This incidence in breast cancer patients that were selected on the basis of the presence of primary disease was 71.4% (25/35) and decreased significantly to 18.2% (6/33) after surgical resection of the disease ($p < 0.05$). These results suggest that the 90kD subunit containing glycoprotein TAA-specific IC is a useful marker for immunodiagnosis and immunoprognosis of breast cancer prior to conventional treatment procedures.

INTRODUCTION

The presence of tumor-associated antigens that are immunogenic in breast cancer patients has been documented by various investigators [1-3]. Therefore, it is logical to assume that circulating immune complexes (IC) should form in vivo each time a humoral immune response is made to the antigens and the source of the antigen is present in breast cancer patients. Immune complexes have been observed in the circulation of humans with various malignancies, including breast carcinoma [1, 4, 5]. Subsequent

Copyright 1994 by Monduzzi Editore S.p.A. - Bologna (Italy)

developments suggest that an assessment of IC levels may be useful for monitoring the clinical course in patients with malignancy [6-8].

Salinas et al [1] determined the levels and composition of IC in patients with breast carcinoma by fetal cell inhibition assay and ultracentrifugation. IC were demonstrated in 50% of the sera at the time of diagnosis. Patients with metastatic disease had more IC than patients with limited disease, and a good correlation between IC levels and the clinical course of the disease. In another report [9], elevation of IC and CEA in cancer patients beyond the immediate post-operative period were predictive of a significantly shorter median survival time than normal levels. Despite certain false positive IC results, it was suggested that IC measurements might provide additional prognostic information, particularly in patients who do not have elevation of other markers [1, 9]. Subsequent reports from various investigators revealed that results of IC detection and correlation with malignant disease varied from investigator to investigator due to the antigen non-specific nature of the IC detection assays [10]. Therefore to ascertain if the presence of IC detected in circulation of a cancer patient at a given point in time was due to the presence of tumor, it was necessary to use cumbersome procedures to confirm that the antigen portion of the IC was indeed the defined TAA [11]. Investigations described here circumvent this problem.

MATERIALS AND METHODS

Patient population. This study included a total of 114 women who had pathologically proven breast carcinoma. The age of the patients ranged from 25 to 84 years with a mean of 52 years. Per review of medical charts 35 patients had primary resectable disease. Where possible, serum samples were procured from these patients pre-operatively and post-operatively. In addition, serum samples were procured from 250 self-proclaimed apparently healthy normal individuals. The age of normal controls ranged from 19 to 74 years with a mean of 40.

90kD TAA-specific IC ELISA. We developed a tumor antigen specific IC detection assay that utilizes immobilized murine monoclonal antibody (MAb) directed to the 90kD subunit of a glycoprotein TAA. This antigen is expressed by 82% (18/22) carcinomas. Details of the assay and normalization of ELISA values have been described elsewhere [4].

Statistical Procedures. Mann-Whitney test as implemented by the Instat Biostatistics program from the GraphPad Software, Inc., San Diego, CA, was used to determine the statistically significant differences among the glycoprotein TAA ELISA values of normal and breast cancer groups. All tests were two-tailed and a p value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Incidence of 90kD glycoprotein TAA-specific IC. As illustrated in figure 1, the incidence of the glycoprotein TAA-specific-IC in sera from breast cancer patients (50.9% (58/114)) was significantly ($p < 0.05$) higher than the normal

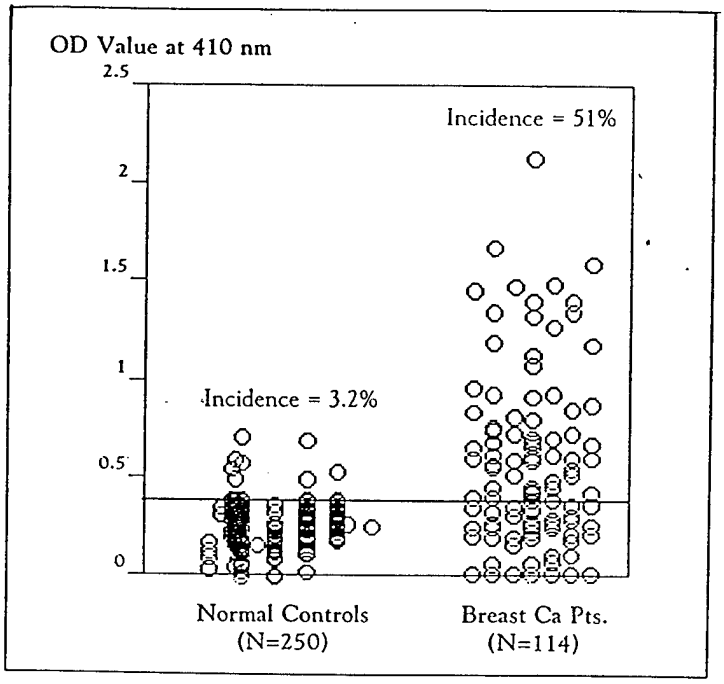


Figure 1. Incidence and distribution of 90kD glycoprotein-TAA-specific IC levels (ELISA values) in sera of 250 normal controls and 114 randomly selected breast cancer patients. Horizontal solid line denoted the positive cut-off level.

control group (3.2% (8/250)). Furthermore, the mean ± standard deviation (SD) ELISA values of control sera (0.169 ± 0.080) were significantly lower ($p < 0.05$) lower than those of sera from breast cancer patients (0.525 ± 0.449). A serum was considered positive when the TAA-specific-IC ELISA value was greater than 0.410 OD_{405nm}. The incidence of the TAA-specific-IC was not affected by age in the normal group; however, this incidence was significantly higher in breast cancer patients who were 60 years or older.

Incidence of 90kD glycoprotein TAA-specific IC in pre- and post-operative breast cancer patients. Analysis of serum samples obtained from breast cancer patients that were selected on the basis of the presence of primary disease, i.e., before removal of the primary tumor, revealed that 71.4% (25/35) were positive for the TAA-specific IC. Nineteen of the 25 breast cancer patients that were positive for the glycoprotein-TAA became negative post-operatively; however, 6 patients were positive for the glycoprotein-TAA both before and after surgical resection of the primary disease. Positivity of post-operative samples (after removal of all accessible disease) was perhaps due to the presence of micrometastases to the lymph nodes or elsewhere. These results clearly denote that TAA-specific-IC may be useful as a tumor marker for the detection of primary and/or metastatic disease in breast cancer patients.
Although this study describes a unique approach to immunodiagnosis of human cancer via detecting an immunogenic tumor antigen in circulation, its specificity can be significantly enhanced by incorporating other existing tumor markers, such as CEA [12], CA15-3, CA-549 [13], CA19-9 [14], TAG-72,

MCA [15], etc. Extensive evaluation of CEA in combination with CA15-3 or alone for clinical correlations with the clinical course of breast cancer patients has resulted in conflicting reports [16-18]. Use of the novel marker described here (tumor antigen-specific immune complexes) in conjunction with one or more of the tumor markers currently available may prove to be more sensitive than used thus far in immunodiagnosis, immunoprognosis, and improving the control of advanced breast cancer through appropriate treatment modality.

ACKNOWLEDGEMENT

These investigations were supported in part by the Department of Human Health Services grants CA29605, CA30019 and CA12582, by the U.S. Army Medical Research Acquisition Activity, by the John Wayne Cancer Institute Auxiliary, by Associates for Breast Cancer Studies, and by the Jack Green Fund. We gratefully acknowledge the expert technical assistance of Mr. Reynold Yee, Mr. Zacarias Leopoldo, and Mr. Thomas Sumobay.

REFERENCES

1. SALINAS FA, WEE KH, SILVER HKB: Clinical relevance of immune complexes, associated antigen, antibodies in cancer. In: Immune Complexes and Human Cancer, FA Salinas, MA Hanna, eds., Plenum Press, New York, pp 55-109, 1985.
2. HUMPHREY LJ, ESTES NC, MORES PA, et al: Serum antibodies in patients with mammary disease. Cancer 34:1516-1520, 1974.
3. GUPTA RK, MORTON DL: Monoclonal antibody based ELISA to detect glycoprotein tumor-associated antigen specific immune complexes in cancer patients. J Clin Lab Analysis 6:329-336, 1992.
4. GORSKY Y, VANKY F, SULITZEANU D: Isolation from patients with breast cancer of antibodies specific for antigens associated with breast cancer and other malignant disease. Proc Natl Acad Sci, USA 73:2101, 1976.
5. MAIDMENT BW, PAPSIDERO LD, NEMOTO T, et al: Recovery of immunologically reactive antibodies and antigens from breast cancer immuno-complexes by preparative isoelectric focusing. Cancer Res 41:795, 1981.
6. EIRAS, AS, ROBINS RA, BALDWIN RW, et al: Circulating immune complexes in patients with bone tumors. Int J Cancer 25:735-739, 1980.
7. GROPP G, HAVEMAN K, SCHERFE T, et al: Incidence of circulating immune complexes in patients with cancer and their effect on antibody-dependent cytotoxicity. Oncology 37:71-76, 1980.
8. BUKH AJ, AGUADO MT, KIMOSE HH, et al: A controlled study of preoperative and postoperative levels of circulating immune complexes in cancer patients. Cancer 61:2033-2044, 1988.
9. DENT PB, LOUIS JA, McCULLOCH PB, et al: Correlation of elevated C1q binding activity and carcinoembryonic Antigen levels with clinical features and prognosis in bronchogenic carcinoma. Cancer 45:130-136, 1980.
10. HERBERMAN RB, BORDER M, LAMBERT PH, at al: A report on international comparative evaluation of possible value of assays for immune complexes for diseases of human breast cancer. Int J Cancer 27:569-576, 1981.
11. GUPTA RK, LEITCH AM, MORTON DL: Nature of antigen and antibodies in immune complexes isolated by staphylococcal protein A from plasma of melanoma patients. Cancer Immunol Immunother 16:40-47, 1983.
12. BUAMAH PK, BENT DJ, BODGER WA, et al: A profile of serum CA15-3, carcinoembryonic antigen, alkaline phosphatase, and gamma-glutamyl transferase

levels in patients with breast cancer. J Surg Oncol 53:84-87, 1993.

13. ZAMAGNI C, MARTONI A, CACCIARI N, et al:CA-549 serum levels in breast cancer monitoring. Int J Biol Markers 7:217-221, 1992.
14. GION M, RUGGERI G, MIONE R, et al: A new approach to tumour marker assessment by preoperative determination in breast and colorectal cancer. Int J Biol Markers 8:8-13, 1993.
15. ESKELINEN M, HIPPELAINEN M, CARLSSON L, et al: A decision support system for predicting a recurrence of breast cancer, a prospective study of serum tumour markers TAG 72, CA15-3 and MCA. Anticancer Res. 12:1439-1442, 1992.
16. BATES SE, LONGO DL: Tumor markers: Value and limitation in the management of cancer patients. Cancer Treat Rev 12:163-207, 1985.
17. DALY L, FERGUSON J, CRAM GP, et al: Comparison of a novel assay for breast cancer mucin to CA15-3 and carcinoembryonic antigen. J Clin Oncol 10:1057, 1992.
18. REPETTO L, ONETTO M, GARDIN G, et al: Serum CEA, CA15-3, and MCA in breast cancer patients: A clinical evaluation. Cancer Detection & Prevention 17:411-415, 1993.

Prospective Evaluation of TA90 Immune Complex Assay for Preoperative Diagnosis of Benign and Malignant Breast Lesions

Jeffry Zavotsky, Rishab K. Gupta, Meghan B. Brennan, Reynold Yee, Armando E. Giuliano, Donald L. Morton

The Joyce Eisenberg-Keefer Breast Center and the John Wayne Cancer Institute at Saint John's Health Center, Santa Monica, CA.

Supported by funding from the Ben B. and Joyce E. Eisenberg Foundation (Los Angeles, CA), the Fashion Footwear Association of New York (New York, NY), the Associates for Breast Cancer Research (Santa Monica, CA), and Army grant DAMD17-94-J-4459.

Correspondence to: Donald L. Morton, MD, John Wayne Cancer Institute, 2200 Santa Monica Boulevard, Santa Monica, California 90404, USA. Phone: (310) 829-8781. Fax: (310) 582-7185.

Running Head: TA90 Assay for Breast Lesions

Summary

Background To evaluate the utility of TA90-IC, an immune complex of 90-kD tumor-associated glycoprotein antigen and IgG antibody, compared with mammography as a screening tool for early breast cancer.

Patients and Methods Serum was drawn preoperatively from patients requiring open biopsy for mammographic abnormalities, palpable breast masses, or bloody nipple discharge. The serum was coded and tested blindly for TA90-IC by an enzyme-linked immunosorbent assay (ELISA). An optical density (OD) $\geq$ 0.41 at 405 nm was considered positive based on three standard deviations above the mean of 250 normal controls.

Results Of the 138 patients studied, 111 had an abnormal mammogram (23 with a palpable mass), and 27 had a normal mammogram (26 with a palpable mass, one with bloody nipple discharge). There was a significant difference in TA90-IC values between the 82 patients with benign lesions and the 42 patients with invasive carcinoma (0.253±0.236 and 0.436±0.209 OD, respectively, p=0.0001). Of 111 patients with an abnormal mammogram, 14 had ductal carcinoma in situ (DCIS) and 36 had an invasive carcinoma; among the subgroup of 41 patients with a positive TA90-IC, 31 had invasive carcinoma and 4 had DCIS. Of the 27 patients with a normal mammogram, 6 had invasive carcinoma that in 4 cases was accompanied by a positive TA90-IC. Of the 97 patients with a negative TA90-IC, 21 (22%) had a malignancy: 10 had DCIS and 11 had an invasive carcinoma. Fifty-two carcinomas were evaluable by size; TA90-IC was positive in 6 of 15 (40%) lesions <1 cm and in 28 of 37 (82%) lesions $\geq$1 cm (p=0.014).

Interpretation Serum TA90-IC assay appears to be a useful adjunct to mammography. It may identify patients with neoplasms missed by mammography or patients who require more frequent surveillance.

Key Words: breast neoplasm, immune assay, mammography, glycoprotein tumor-associated antigen, TA90 immune complex

Introduction

Presently, screening for breast cancer relies on physical examination and mammography. Mammography has been shown to benefit women over the age of 49. It can detect cancers at an earlier stage, resulting in an increased survival for women in this age group.[1,2] However, not all breast cancers are detected by mammography, and a false-negative rate of 16.5% has been reported.[3] Also, mammography cannot distinguish benign disease from invasive disease. Therefore, many women undergo unnecessary biopsies. CA15-3, carcinoembryonic antigen (CEA), and numerous other tumor markers have been developed to aid in the diagnosis, prognosis, and early detection of breast cancer recurrence,[4-18] but none has yet proved effective as an adjuvant to mammography in breast cancer screening.

We previously described a 90-kD glycoprotein tumor-associated antigen (TA90) associated with breast cancer, melanoma, and other solid tumors.[19-23] TA90 is present in the serum as an immune complex (IC) with anti-TA90 IgG antibody, and we developed an enzyme-linked immunosorbent assay (ELISA) to measure TA90-IC in cancer patients.[19,24] TA90-IC ELISA has a sensitivity of 77% in predicting the recurrence of melanoma.[20] This assay also shows significantly higher serum levels of TA90-IC in lung cancer patients than in normal controls (63% vs. 3.2%; $p<0.05$).[21]

In an earlier study, we retrospectively examined the incidence of TA90-IC in the serum of 106 patients with known breast cancer.[22] TA90-IC was identified in 63% of serum samples, compared with only 3% of healthy controls. CEA and CA15-3 were also analyzed in a subgroup of 68 serum samples: TA90-IC was identified in 55 (81%), CEA

in 16 (24%), and CA15-3 in 23 (34%). Thus TA90-IC was significantly more sensitive than standard tumor markers in patients with known breast cancer.

In the present study, we prospectively investigated the incidence of TA90-IC in the preoperative sera of patients with a palpable breast mass, mammographic abnormality, or bloody nipple discharge that warranted an open biopsy. TA90-IC values were then correlated with mammographic findings, standard tumor markers, and the pathology found on breast biopsy.

Patients and Methods

Patient population

After obtaining informed consent, serum samples for TA90-IC ELISA were procured preoperatively in a blinded fashion from 138 women scheduled to undergo open biopsy for an abnormal mammogram, breast mass, or bloody nipple discharge. All biopsy procedures were performed by surgical staff of the John Wayne Cancer Institute. Biopsy specimens were examined for tumor size, tumor grade, DNA ploidy, S-phase, estrogen and progesterone receptors, and HER2neu expression. Serum samples were tested for TA90-IC in a blinded fashion (without knowledge of pathology findings). Pathology findings were then correlated with TA90-IC values.

TA90-IC detection assay

Serum levels of TA90-IC were determined with TA90-IC ELISA, an antigen-specific assay that utilizes murine monoclonal antibody AD1-40F4 against TA90. The monoclonal antibody and the glycoprotein antigen were prepared as previously

described.[19] Briefly, 100 µl of the AD1-40F4 ascites was diluted to a protein concentration of 100 µg/ml and then dispensed in wells of gluteraldehyde-activated microtiter plates (Dynex Technologies, Inc., Chantilly, VA). The plates were incubated at 4°C for 16 h and then washed with phosphate-buffered saline (PBS) supplemented with 0.5% Triton X-100 (PBS-TX). The plates were then blocked with 100 µl of 1% bovine serum albumin (BSA), using 100 µl per well, in PBS-TX at 23°C for 1.0 h. Serum samples were diluted 1:60 with PBS-TX supplemented with 1% BSA, 0.5% normal mouse serum and 0.01 M ethylene diamine tetraacetic acid (EDTA). One hundred microliters of the diluted sample was placed in duplicate wells of the activated plates; plates were incubated at 37°C for 45 min and then washed with PBS-TX. One hundred microliters of alkaline phosphatase conjugated to Fab fragment of goat anti-human IgG (Sigma Chemical Company, Saint Louis, MO) at 1:500 dilution per well was added to each test well and control well of the plates; plates were then incubated at 37°C for 45 min and washed with PBS-TX. Two hundred microliters of p-nitrophenyl phosphate (1.0 mg/ml) in 10% diethanolamine buffer as substrate was added, and the plates were incubated in the dark at 23°C for 1.0 h. The absorbance was read at 405 nm. Each sample was tested twice with positive and negative controls. Each sample was blanked individually in the same microtiter plate. Each test plate also included controls for nonspecific protein binding and binding of conjugate to the immobilized murine monoclonal (capturing) antibody. The net optical densities of the control samples were used to generate a correction factor to normalize the net optical density of the test samples analyzed on that particular test plate. If the correction factor for a test plate fell outside the range of 0.8 to 1.2, the assay was considered invalid. The upper limit of

normal for TA90-IC was 0.41 (mean ± 3 SD ELISA values of over 250 normal sera determined from previous studies). Sera with a value ≥0.41 OD were considered positive for TA90-IC.

Statistical analysis

The Kruskal-Wallis test was used to examine the difference between mean TA90-IC values of sera from patients with invasive carcinomas and benign lesions. The Spearman correlation coefficient was used to analyze the relationship between TA90-IC values and tumor size, tumor grade, DNA ploidy, and S-phase. The Kappa test was used to examine the consistency of TA90 status and estrogen receptor status, progesterone receptor status, and HER2neu expression. Pearson chi-square test was used to investigate the relationship between TA90-IC status and tumor size (< 1 cm versus ≥ 1 cm).

Results

Biopsy specimens were characterized as benign, ductal carcinoma in situ (DCIS), or invasive carcinoma. The distribution of positive and negative TA90-IC values for each pathology is shown in Table 1. Mean TA90-IC was 0.254 ± 0.239 OD in the 82 patients with benign lesions, 0.315 ± 0.230 OD in the 14 patients with DCIS, and 0.436 ± 0.209 OD in the 42 patients with invasive lesions; the difference between benign and invasive groups was highly significant (p=0.0001).

Of the 138 patients studied, 42 had invasive carcinoma, 31 (74%) with a positive TA90-IC; 14 patients had DCIS, 4 (29%) with a positive TA90-IC; and 82 had benign lesions, 6 (7%) with a positive TA90-IC.

Twenty-six patients had a normal mammogram in the face of a palpable mass, and one had a normal mammogram and a bloody nipple discharge. Of these 27, none had DCIS; 6 (22%) had invasive carcinoma, 4 with a positive TA90-IC; and 21 (88%) had benign lesions, none with a positive TA90-IC. Mammography was positive in 9 patients with invasive carcinoma and negative in six patients with invasive carcinoma. Mammography was positive in 14 patients with benign lesions and negative in 21 patients with benign lesions (Table 2).

Among the 42 patients with invasive breast cancer, 27 (64%) had a positive mammogram plus a positive TA90-IC, 4 (10%) had a positive TA90-IC only, 9 (21%) had a positive mammogram only, and 2 (5%) had neither a positive mammogram nor a positive TA90-IC.

Of the 56 patients with invasive carcinoma or DCIS, 52 had lesions evaluable by size. Of the 15 patients with tumors <1 cm, 6 (40%) had a positive TA90-IC. Of the 37 patients with tumors $\geq$ 1 cm, 28 (82%) had a positive TA90-IC ($p=0.014$). Thirty-one of the 56 tumors were tested for S-phase: of the 20 patients with a positive TA90-IC, 8 (40%) had tumors with an S-phase greater than 5%; of the 11 patients with a negative TA90-IC, only 3 (27%) had tumors with an S-phase greater than 5%. However, statistical analysis using S-phase and TA90-IC as continuous variables revealed a positive correlation between TA90-IC and S-phase (Spearman correlation coefficient $=0.4011$; $p=0.0253$) (Figure 1). There was no demonstrable association between TA90-

IC value and tumor grade among 45 patients with invasive or noninvasive tumors (Spearman correlation coefficient = 0.2789, p=0.0635) or among the subgroup of 34 patients with invasive tumors (Spearman correlation coefficient=0.3040, p=0.0804).

Of the 38 tumors tested for estrogen receptors (ER) and progesterone receptors (PR), 7 were ER-negative: 6 (86%) of these were TA90-IC positive, compared with 21 (68%) of the 31 ER-positive tumors (p=0.8282). Of the 7 PR-negative tumors, 5 (71%) were TA90-IC positive, compared with 22 (70%) of the 31 PR-positive tumors (p=0.5097). Of the 34 tumors tested for HER2/*neu* expression, 8 were positive: 6 (75%) of these were TA90-IC positive, compared with 17 (65%) of the 26 HER2/*neu*-negative tumors (p=0.3056).

The serum of 36 patients with a malignancy was tested for CEA and/or CA15-3 in addition to TA90. CA15-3 was positive in 3 patients (one invasive carcinoma, 2 DCIS) of 32 patients (26 invasive carcinoma, 6 DCIS). CEA was positive in 2 patients (invasive carcinoma) of 33 patients (26 invasive carcinoma, 7 DCIS). Of the 36 patients, 24 (67%) were positive for TA90-IC, whereas only 3 (8%) were positive for CEA or CA15-3 (Table 3). One patient with DCIS had a positive CA15-3 but a negative CEA and TA90-IC. Of the 22 patients with a positive TA90-IC and invasive carcinoma, only 2 were positive for CEA or CA15-3. This confirms our earlier study demonstrating the superior sensitivity of the TA90-IC assay.[22]

Of the 42 invasive carcinomas, 17 were American Joint Committee on Cancer (AJCC) Stage I, 18 were AJCC stage IIa, 5 were AJCC stage IIb, and 2 were AJCC stage IIIa. TA90-IC values were positive in 11 (65%) stage I tumors, 14 (78%) stage IIa tumors, 5 (100%) stage IIb tumors, and 1 (50%) stage IIIa tumor (Figure 2).

Discussion

This study evaluated the utility of TA90-IC for identifying patients with early breast cancer. Results indicate that TA90-IC shows promise as an adjunct to mammographic screening. Eight-five percent of patients with a positive TA90-IC had a breast malignancy. Of the 97 patients with a negative TA90-IC, 76 (78%) had benign disease and 21 (22%) had a malignancy. Only 6 of 82 (7%) patients with a benign lesion had a positive TA90-IC, whereas 4 of 6 patients with invasive carcinoma and a normal mammogram had a positive TA90-IC (Figure 3). Of 27 patients with a normal mammogram, 6 (22%) had a malignancy; similarly, 21 of 97 (22%) TA90-IC values <0.41 OD were falsely negative. Five (45%) of the 11 patients with a positive mammogram had a malignancy. When TA90-IC and mammographic results were combined, 50 of 52 (96%) breast neoplasms were detected.

Although mammography has reduced the mortality of breast cancer by about one third in patients 50 to 69 years old, it has limitations. Not all breast cancers are demonstrable on mammography, in which case the mammogram would be falsely negative. In our study, 4 of 42 (10%) patients with invasive carcinoma had a negative mammogram. Coveney et al[3] found a 16.5% false-negative rate for mammography in 291 patients of all ages with palpable cancers. Retrospective review of false-negative mammograms showed that 30% were normal (true negatives), 20% were obvious oversights, and 50% had radiographic abnormalities that were indeterminate. A serum marker indicating whether or not a lesion was malignant might decrease the rate of false-

negative mammography. This would be particularly useful in 40-49 year old patients without a palpable mass.[25]

False positives are also a problem with mammography. Only 17% to 32% of non-palpable lesions found on mammography are malignant.[3,26-34] Therefore, a large percentage of open biopsies reveal benign disease. Because women younger than 50 years have a much higher rate of false-positive mammograms,[27,35] the cost of screening mammography is five times higher in the 40-49 year age group than the 50-69 year group.[36] The current consensus of the National Institutes of Health is that mammography cannot universally be recommended in women between 40 and 49 years old.[37] A serum marker that could distinguish between benign and malignant lesions on a mammogram might make mammography in this age group more cost-effective and beneficial.

Serum tumor markers have been described for the early detection and monitoring of metastases from a primary breast cancer.[4,5] However, many of these markers are questionable, with the possible exception of CA15-3, in regards to both diagnosis of systemic disease and response to therapy.[6,7] Tomlinson et al[13] reported 70% sensitivity, 96% specificity, and an 87% predictive value for CA15-3 in metastatic disease, but this marker does not appear to be useful in screening. Our study confirmed this finding – only 3 of 32 patients with a malignancy had a positive CA15-3. Serum markers such as TPS,[14] alpha-1-antiprotease,[15] YKL-40,[16] MCA,[17] and c-*erb*B-2[8,10-12] have shown promise in predicting survival and monitoring disease regression or recurrence, but are not suitable for screening.

Our study indicates that TA90-IC is a potentially useful screening marker in patients with early breast carcinoma. Although a higher proportion of stage IIa patients

were TA90 positive, 11 of 17 stage I patients were positive for TA90-IC. This means that TA90-IC is detectable in the blood of patients with smaller, premetastatic tumors. Although this marker is not 100% sensitive or specific, it complements mammography by identifying cancers by an independent means. In our study, we showed that TA90-IC can identify 67% of tumors not visualized on a mammogram. Of course, these tumors were palpable and did not present a diagnostic dilemma. In this group of patients, all of whom had an indication of breast biopsy, TA90-IC proved to be a useful adjunct to mammography. The role of TA90-IC in breast cancer screening will be determined by prospectively examining whether a positive serum TA90-IC value can be used with mammography for routine screening of women at risk of breast cancer.

References

1. US Preventive Services Task Force: *Guide to Clinical Preventive Services.* 2nd ed. Baltimore, Md: Williams & Wilkins, 1996

2. Kerlikowske K, Grady D, Rubin SM, Sandrock C, Ernster VL: Efficacy of screening mammgraphy: a meta-analysis. JAMA 273:149-154, 1995

3. Coveney EC, Geraghty JG, O'Laoide R, Hourihane JB, O'Higgins NJ: Reasons underlying negative mammography in patients with palpable breast cancer. Clin Radiol 49(2):123-125,1994

4. Foekens JAA, van Putten WLJ, Potengen H, de Konong HYWCM, Thirion B, Alexieva-Figuschland Kijn GM. Prognostic value of PS2 and cathepsin D in 710 human primary breast tumors: multivariate analyss. J Clin Oncol 11:899-908, 1993

5. Ng JSY, Sturgeon CM, Seth J, Paterson GM, Roulston JE, Leonard RCF: Serological markers for metastatic breast cancer. Dis Markers 11:217-223, 1993

6. Robertson JFR, Pearson D, Price MR, Selby C, Blamy RW, Howell A: Objective measurement of therapeutic response in breast cancer using tumor markers. Br J Cancer 64:757-763, 1991

7. Tondini C, Hayes DF, Gelman R, Henderson IC, Kufe DW: Comparison of CA15-3 and carcinoembryonic antigen in monitoring the clinical course of patients with metastatic breast cancer. Cancer Res 48:4107-4112, 1988

8. Leitzel K, Teramoto Y, Konrad K, Chinchilli VM, Volas G, Grossberg H, Harvey H, Demers L, Lipton A: Elevated serum c-*erb*B-2 antigen levels and decreased response to hormone therapy of breast cancer. J Clin Oncol 5:1129-1135, 1995.

9. Iacobell S, Sismondi P, Giai M, D'Egidio M, Tinari N, Amatetti C, Di Stefano P, Natoli C: Prognostic value of a novel circulating serum 90K antigen in breast cancer. Br J Cancer 69:172-176, 1994

10. Volas GH, Leitzel K, Teramoto Y, Grossberg H, Demers L, Lipton A: Serial serum c-*erb*B-2 levels in patients with breast carcinoma. Cancer 78:267-272, 1996

11. Carney WP, Hamer PJ, Petit C, Retos C, Greene R, Zabrecky JR, McKenzie S, Hayes D, Kufe D, DeLellis R, Naber S, Wolfe H: Detection and quantification of human neu oncoprotein. Tumor Marker Oncol 6:53-72, 1991

12. Yu FZ, Sugano K, Ohkura H, Mori S: Development of sandwich radioimmunogenic assay for serum c-erbB-2 oncogene product and its significance in diagnosing breast carcinoma. Rinsho Byori 39:1087-1092, 1991

13. Tomlinson IPM, Whyman A, Barrett JA, Kremer JK: Tumour marker CA15-3: Possible uses in the routine management of breast cancer. Eur J Cancer 31:899-902, 1995

14. Bremer K, Eklund G, Bjorklund B: Notable relationship between the level of tumor marker TPS in serum and survival in breast cancer. Anticancer Res 16:905-910, 1996

15. Varela AS, Saez JJBL: Utility of plasmatic levels of alpha-1-antiprotease (A1AP) as a cancer marker. Cancer Letters 89:15-21, 1995

16. Johanson JS, Cintin C, Jorgenson M, Kamby C, Price PA: Serum YKL-40: a new potential marker of prognosis and location of metastases of patients with recurrent breast cancer. Eur J Cancer 31:1436-1442, 1995

17. Muller-Brand J, Macke H: Clinical significance of the new tumor marker MCA in the follow-up of patients with mammory carcinoma. Int J Biol Markers 8:130-132, 1993

18. Murray A, Clinton O, Earl H, Price M, Moore A: Assessment of five serum marker assays in patients with advanced breast cancer treated with medroxyprogesterone acetate. Eur J Cancer 31:1605-1610, 1995

19. Gupta RK, Morton DL: Monoclonal antibody based ELISA to detect glycoprotein tumor-associated antigen specific immune complexes in cancer patients. J Clin Lab Analysis 6:329-336, 1992

20. Kelley MC, Jones RC, Gupta RK, Yee R, Stern S, Wanek L, Morton DL. Tumor-associated antigen TA-90 immune complex assay predicts subclinical metastasis and survival in patients with early-stage melanoma. Cancer, in press.

21. Gupta RK, Morton DL. Prognostic value of a 90kD subunit containing glycoprotein tumor-associated antigen specific immune complexes in lung cancer patients. Dis Markers, 12:51-61, 1994.

22. Gupta RK, Morton DL: Detection and comparison of a 90 kD glycoprotein tumor-associated antigen specific immune complexes with CEA and CA15-3 in breast cancer. Int J Oncol 7:741-747, 1995.

23. Euhus DM, Gupta RK, Morton DL: Characterization of a 90-100 kDa tumor associated antigen in the sera of melanoma patients. Int J Cancer 45:1065-1070, 1990

24. Euhus DM, Gupta RK, Morton DL: Detection of a tumor associated glycoprotein antigen in serum and urine of melanoma patients by murine monoclonal antibody (AD1-40F4) in enzyme immunoassay. J Clin Lab Analysis 3:184-190, 1989

25. Chew SB, Hughes M, Kennedy C, Gillet D, Carmalt H: Mammographically negative breast cancer at the Strathfield Breast Centre. Aust N Z J Surg 66(3):134-137, 1996

26. Griffen MM, Welling RE: Needle-localized biopsy of the breast. Surg Gynecol Obstet 170(2):145-148, 1990

27. Lein BC, Alex WR, Zebley DM, Pezzi CM: Results of needle localized breast biopsy in women under age 50. Am J Surg 171:356-359, 1996

28. Hall JA, Murphy DC, Hall BR, Hall KA: Mammographic abnormalities and the detection of carcinoma of the breast. Am J Obstet Gynecol 168(6 Pt 1):1677-1680, 1993

29. Albert MP, Sachsse E, Coe NP, Page DW, Reed WP: Correlation between mammography and the pathology of nonpalpable breast lesions. J Surg Oncol 44(1):44-46, 1990

30. O'Laoide R, Geraghty JG, Coveney EC, McDermott EW, Hourihane JB, McCabe M, O'Higgins NJ: Mammographic needle localisation of impalpable breast lesions. Ir Med Jun;(2):70-72, 1992.

31. Rissanen TJ, Makarainen HP, Mattila SI, Karttunen AI, Kiviniemi HO, Kallioinen MJ, Kaarela OI: Wire localised biopsy of breast lesions: a review of 425 cases found on screening or clinical mammography. Clin Radiol 47(1):14-22, 1993

32. Johnston JA, Clee CZ: Analysis of 308 localisation breast biopsies in a New Zealand hospital. Australas Radiol 35(2):148-151, 1991

33. Mueller X, Amery A, Lallemand RC: Biopsy of mammographically-detected breast lesions in a district hospital. Eur J Surg Oncol 19:415-419, 1993

34. Alexander HR, Candela FC, Dershaw DD, Kinne DW: Needle-localized mammographic lesions. Results and evolving treatment strategy. Arch Surg 125:1441-1444, 1990

35. Rothman LP, Harvald TB, Overgaard H: Impalpable breast lesions diagnosed by mammography: incidence of histologically confirmed malignancy. Eur J Surg 162:691-694, 1996

36. Salzmann P, Kerlikowske K, Phillips K: Cost-effectiveness of extending screening mammography guidelines to include women 40 to 49 years of age. Ann Intern Med 127:955-965, 1997

37. National Institutes of Health: Breast cancer screening for women ages 40-49. NIH Consensus Statement Jan 21-23, 1997. 15(1):1-35, 1997

LEGENDS

Figure 1. TA90 status according to S-phase of breast lesion.

Figure 2. The incidence of TA90 positivity increases linearly with AJCC stage of disease.

Figure 3. Overlap between results of mammography and TA-90 IC ELISA in 40 patients with invasive breast carcinoma. Two additional patients with invasive carcinoma had negative mammographic findings and a negative TA90-IC ELISA.

Table 1. *TA90-IC Results by Pathology*

	BENIGN	*DCIS*	*INVASIVE*	*TOTAL*
TA90 <0.41	76 (93%)	10 (71%)	11 (26%)	97 (70%)
TA90 ≥0.41	6 (7%)	4 (29%)	31 (74%)	41 (30%)
TOTAL	82	14	42	138

Table 2. *TA90 and mammographic results according to pathology of the breast lesion*

	Pathology of the Breast Lesion		
Screening test results	*Benign*	*DCIS*	*Invasive cancer*
TA90+ Mammography+	6	4	27
TA90+ Mammography-	0	0	4
TA90- Mammography+	53	10	9
TA90- Mammography-	19	0	2

Table 3. *Incidence of three serum markers (TA90, CEA, CA15-3) in sera of patients with DCIS or invasive breast cancer.*

	Histopathology of breast lesion	
Marker status	*DCIS*	*Invasive cancer*
TA90+ CEA/CA15-3+	0	2
TA90+ CEA/CA15-3-	2	20
TA90- CEA/CA15-3+	1	0
TA90- CEA/CA15-3-	4	7

Accuracy of TA90 Immune Complex Assay Compared with Mammography for the Detection of Breast Cancer

Nizar Habal, MD; Jeffry Zavotsky, MD; Rishab K.Gupta, PhD; Reynold Yee, BS; Tim Johnson, PhD; Meghan B. Brennan, RN; R. James Brenner, MD; Nora Hansen, MD, Armando E. Giuliano, MD; Donald L. Morton, MD

Author Affiliations: Joyce Eisenberg-Keefer Breast Center of the John Wayne Cancer Institute at Saint John's Health Center, Santa Monica, California (Drs. Habal, Zavotsky, Brenner, Hansen, Giuliano, Morton; Ms. Brennan); Division of Tumor Markers and Immunodiagnosis of the John Wayne Cancer Institute (Dr. Gupta, Mr. Yee), the Statistical Coordinating Unit of the John Wayne Cancer Institute (Dr. Johnson)

Corresponding Author and Reprints: Donald L. Morton, MD, John Wayne Cancer Institute, 2200 Santa Monica Boulevard, Santa Monica, California, 90404, USA. Phone: 310-829-8781; Fax: 310-582-7185; E-mail: mortond@jwci.org.

Word Count: 2541

Abstract

Context and Objective We have identified a 90-kD glycoprotein serum tumor marker in the form of immune complexes (TA90-IC) in patients with a variety of solid neoplasms, including breast cancer. This study compared the efficacy of a TA90-IC assay with that of mammography for the detection of breast cancer.

Patients and Methods One hundred and fifty-seven women (ages 26-82 years) were referred for open breast biopsy because of an abnormal mammogram (n=105) or a palpable breast mass (n=52). Fifty-six (36%) patients were younger than 50 years. Preoperative serum samples were drawn prior to biopsy and tested blindly for TA90-IC using an enzyme-linked immunosorbent assay. A positive result was defined as an optical density $\geq$ 0.410 at an absorbance of 405 nm. Mammogram reports were obtained from the patient's chart and classified as suspicious or benign. Histopathologic results of biopsy were classified as positive (infiltrating or intraductal cancer) or negative. Sixty-nine (44%) patients had breast cancer.

Results Although less sensitive than mammography (77% vs. 84%, p=0.0038), TA90-IC was more specific (90% vs. 46%, p<0.0001) and had a lower false-positive rate (10% vs. 54%, p=0.0038) in this group of patients selected for open biopsy. A combination of tests improved the accuracy of screening (defined as true positives plus true negatives divided by sample population) to 75%, compared with 64% for mammography alone.

Conclusion A combination of tests improves the accuracy of screening, and the lower false-positive rate may help reduce the number of unnecessary tests and breast biopsies.

INTRODUCTION

Breast cancer remains the most frequently diagnosed cancer in women and the second leading cause of death in women over the age of 40. A woman's lifetime risk of developing breast cancer is 12%.[1] Two screening modalities have been approved by the American Cancer Society: mammography and clinical breast examination. Nine separate randomized trials of these modalities in the United States or Europe have shown a 30% reduction in mortality for women ages 50-69 years who comply with screening guidelines.[2,3]

Although the reduction in mortality in the screened populations is a significant advance in the fight against breast cancer, the accuracy of mammography remains in question. A recent meta-analysis of data from large randomized trials showed that the sensitivity of mammography ranges from 83% to 95%, but the number of open breast biopsies performed to find one breast cancer varies from 3 to 10.[4] Furthermore, there is a growing concern about the cumulative false-positive rate (FPR) during a lifetime of yearly mammographic screening. In a recent article by Elmore et al.,[5] the 10-year cumulative FPR was estimated at 49%, with an approximately 33% risk of unnecessary invasive testing. The psychological and financial burden implied by these percentages underlines the need for more accurate screening tests.

In this study, we investigated the possible usefulness of a novel serum tumor marker as an adjunct to mammography for the detection of breast cancer. TA90 is a tumor-associated 90-kD glycoprotein that was initially described and characterized in melanoma[6] and has since been demonstrated in many other solid malignancies, including breast cancer (71%).[7] It circulates in the serum in the form of IgG-bound immune complexes, TA90-IC.[8] This study compared the accuracy of TA90-IC assay with that of mammography in patients selected for open biopsy of a palpable breast mass or a suspicious mammographic finding.

METHODS

Between 1991 and 1998, 157 women seen at the John Wayne Cancer Institute or the Jonsson Comprehensive Cancer Center of the University of California in Los Angeles (UCLA) were entered prospectively into this study. Their average age was 56.5 years (range 26 – 88 years). After signing an informed consent approved by the Institutional Review Board of Saint John's Health Center, all patients underwent open surgical biopsy for either a palpable breast mass or an abnormality labeled 'suspicious' or 'indeterminate' on mammography. No more than one week prior to the surgical procedure, serum was drawn for TA90-IC analysis; an informed consent was obtained before serum sampling.

Members of the Department of Radiology at the Saint John's Health Center reviewed all mammograms and interpreted the results as suspicious, indeterminate, or benign. For the purpose of this analysis, mammographic results labeled as suspicious or indeterminate were considered positive because they required biopsy confirmation.

TA90-IC was assayed with an antigen-specific enzyme-linked immunosorbent assay (ELISA) that uses murine monoclonal antibody AD1-40F4 against TA90. The monoclonal antibody and the glycoprotein antigen were prepared as previously described.[7] AD1-40F4 ascites (100 μl) was diluted to a protein concentration of 100 μg/ml and then dispensed in wells of microtiter plates (Dynex Technologies, Inc., Chantilly, VA). The plates were incubated at 4°C for 16 h and then washed with phosphate-buffered saline supplemented with 0.5% Triton X-100 (PBS-TX). The plates were then blocked with 1% bovine serum albumin (BSA), using 100 μl per well, in PBS-TX at 23°C for 1 h. Serum samples were diluted 1:60 with PBS-TX supplemented with 1% BSA, 0.5% normal mouse serum, and 0.01 M ethylene diamine

tetraacetic acid. One hundred microliters of the diluted samples was placed in duplicate wells of the activated plates; plates were incubated at 37°C for 45 min and then washed with PBS-TX. Alkaline phosphatase (100 µl) conjugated to Fab fragment of goat anti-human IgG (Sigma Chemical Company, Saint Louis, MO) at 1:500 dilution per well was added to each test well and control well of the plates; plates were then incubated in the dark at 23°C for 1 h. The absorbance was read at 405 nm. Each sample was tested twice with positive and negative controls. Each sample was blanked individually in the same microtiter plate. Each test plate also included controls for nonspecific protein binding and binding of conjugate to the immobilized murine monoclonal antibody. The net optical densities (ODs) of the control samples were used to generate a correction factor to normalize the net OD of the test samples analyzed on that particular test plate. If the correction factor of the test plate fell outside the range of 0.8-1.2, the assay was considered invalid. The upper limit of normal for TA90-IC was 0.410 (mean $\pm$ 3 SD ELISA values for serum specimens from > 250 self-declared healthy volunteers, determined during previous studies). Sera with ODs $\geq$ 0.410 at an absorbance of 405 nm were considered positive for TA90-IC.

Statistics

All serum samples were coded and all TA90-IC assays were performed in a blinded fashion by a separate technician at the immunodiagnostic laboratory. Results were recorded separately and provided directly to the Statistical Coordinating Unit at the John Wayne Cancer Institute (JWCI). The computer package S-Plus (version 4.0) was used to perform all the statistical analyses. The McNemar's test for correlated proportions between paired data sets was used to analyze significant differences between results of TA90-IC assay and mammography. Table 1 identifies the formulas used to calculate the sensitivity, specificity, positive predictive

value, FPR, and overall accuracy of the individual tests. A paired bootstrap (10,000 bootstrap samples) was used to assess the differences of these five statistics between mammography and TA90-IC. A permutation test for linear trend was performed to test for an increasing trend of TA90-IC with pathology.[9]

RESULTS

One hundred and fifty-seven breast biopsies were performed in the same number of patients. Benign disease was found in 88 patients (56%), ductal carcinoma in situ (DCIS) was found in 17 patients (11%), and invasive adenocarcinoma was found in 52 patients (33%). Of the 52 patients with invasive carcinoma, 23 had American Joint Committee on Cancer stage I disease, 21 had stage IIA, 3 had stage IIB, and 5 patients had stage IIIA disease (Table 2).

Serum samples for the TA90-IC assay were available for all 157 patients. Average TA90-IC values were 0.282 for benign disease (median 0.189), 0.327 for DCIS (median 0.182), and 0.460 for invasive carcinoma (median 0.462). In those patients with invasive cancers, the mean TA90-IC values were 0.534 for stage I, 0.370 for stage IIA, 0.452 for stage IIB, and 0.455 for stage III. The percentage of patients with elevated TA90-IC values increased from benign disease to DCIS to invasive cancer (Figure 1, $p<0.0001$).

Table 3 shows the pathologic findings of open biopsy according to the results of mammography and TA90-IC assay. Of the 105 patients with suspicious or indeterminate mammograms, 64 (62%) had normal levels of TA90-IC. Thirteen of these 64 patients had invasive disease, 9 had DCIS, and 42 had benign disease on pathologic examination. Forty-one patients had both a positive mammogram and an elevated TA90-IC level: 30 had invasive cancer, 6 had DCIS, and only 5 had benign disease. Therefore, in the group of patients with

positive mammograms, TA90-IC assay correctly identified 42 of the 47 benign biopsies (89%), but missed 13 of the 43 (30%) invasive cancers and 9 of the 15 (60%) DCIS cases (Table 3).

Of the 52 patients with a negative mammogram, 41 had normal TA90-IC levels: on pathologic examination, 3 had invasive cancer, 1 had DCIS, and 37 (90%) had benign disease. Of the 11 patients with elevated TA90-IC, 6 (55%) had a cancer, 1 (9%) had DCIS, and only 4 (36%) were benign on pathologic examination. Therefore, the addition of TA90-IC to a negative mammogram correctly identified 7 of the 11 cancers missed by mammography (Table 3). In total, of the 69 cases of DCIS and invasive cancers, 11 (16%) were missed by mammography and 26 (37.7%) were missed by TA90-IC assay but only 4 (5.8%) were missed by both tests.

The corresponding sensitivity, specificity, positive predictive value (PPV), FPR, and overall accuracy of the two screening tests are listed in Table 4. The patients who presented with indeterminate and/or suspicious mammograms were considered positive radiographically; the patients who presented with DCIS and/or with invasive adenocarcinoma pathologically were considered positive for these calculations. Although the sensitivity of mammography alone was higher than that of TA90-IC (84% vs. 77%, p=0.0038), all other parameters, specifically FPR (54% vs 10%, p=0.0038) were superior when TA90-IC was used as the screening test. A combination of tests, defined as an elevation of either test, yielded a superior sensitivity (94%) than each test alone, but at the expense of increasing FPR and decreasing overall accuracy.

Excluding the 20 indeterminate mammograms, which potentially could have biased the accuracy of mammography in a negative fashion, did not significantly affect the analysis (Table 4). Mammography remained slightly more sensitive than TA90-IC assay (82% vs. 61% respectively, p=0.007), but at the expense of higher FPR (46% vs. 21% respectively, p=0.007) and lower overall accuracy (66% vs. 77% respectively, p=0.0056).

Looking only at the subset of patients with benign disease, the number of positive TA90-IC tests was significantly lower than the number of positive mammograms (Table 5); this discrepancy was significant in all age groups except patients over 70 years of age. Among all 56 patients younger than 50 years of age, 6 of the 16 cases of invasive and noninvasive cancers were missed by mammography, 5 were missed by TA90-IC assay, but only one was missed by both tests (Table 3). In this age group the overall accuracy of mammography decreased to 58% but the accuracy of TA90-IC improved to 87% (Table 4).

DISCUSSION

Mammography has long been the mainstay of breast cancer screening and it remains a model for other screening tests. It is highly sensitive (83% to 95%) and specific (over 95%), relatively inexpensive, and well tolerated by patients. Also, eight randomized trials involving almost 300,000 patients in Europe and the United States have shown a 20 - 43% decrease in breast-cancer mortality attributed to mammography-based early detection.[1-4, 10]

Nevertheless, the FPR reported in the major trials is perhaps misleading. First, it is difficult to interpret the published data because the definition of a positive result varies. In the strictest sense, a positive test is based on open breast biopsy confirmation; in the broadest sense, that label applies to any mammogram that leads to an additional test.[5] Second, a review of the predictive value of abnormal mammograms (which represent 11% of all mammograms performed) showed that the yield of a cancer confirmed by open biopsy can be as low as 21-34%.[5,8] In other words, up to 67% of all breast biopsies performed as a result of an abnormal mammogram will be performed for benign disease. This problem is compounded by the fact that a positive mammogram is often followed by several tests designed to improve its diagnostic

resolution: ultrasound, fine-needle aspiration, core biopsy and more recently, magnetic resonance imaging,[11-14] all of which further inflate the costs of working up a positive mammogram.

Besides the immediate costs involved, the long-term impact of these unnecessary procedures is even more significant. The estimated cumulative FPR for a woman who undergoes yearly screening is almost 50% after 10 years. The psychological anxiety of the screened women notwithstanding, the cost of performing the additional tests needed to evaluate a false-positive result inflated the cost of screening by an estimated 33%.[5,15]

TA90-IC is elevated in many solid malignancies, including breast adenocarcinoma.[7] We recently presented our experience with the first 138 patients who were selected to undergo open breast biopsy based on an abnormal mammogram or palpable mass.[16] Seventy-four percent of patients with invasive cancers had elevated TA90-IC levels (compared with only 7% of patients with benign disease), and it was suggested that this assay may be of benefit in identifying a neoplasm missed by mammography. Also, it was noted that TA90-IC level did not correlate with estrogen and progesterone receptor status or with Her-2-Neu expression.

This complementary study shows that TA90-IC improves the false-positive rate and overall accuracy of mammography in a direct comparison, especially when indeterminate readings are considered with suspicious readings. As shown in Table 4, TA90-IC alone had a PPV over 1.5 times that of mammography alone (82% vs. 54%, respectively), without sacrificing sensitivity to a great degree (77% vs. 84%, respectively). Of the 105 women who had suspicious or indeterminate mammograms, 47 (45%) had no cancer in the biopsy specimen, and consequently underwent an unnecessary procedure. The TA90-IC test was able to identify 42 of these 47 false positives (89%). Indeed, the likelihood of either an invasive cancer or DCIS in the presence of negative mammographic and TA90-IC findings was less than 10% (4 of 41 patients.)

Conversely, the likelihood of benign disease was very low when both the TA90-IC level and the mammogram were abnormal (only 5 of 41 patients, or 12%). We propose that these patients undergo a core biopsy for confirmation followed by open surgical therapy. Patients with a positive mammogram and a normal TA90-IC are more likely to have a benign pathology identified by open biopsy (42 of 64 patients in the present study) and would benefit from less-invasive tests to diagnose the presence of a cancer, such as ultrasonography with a core biopsy of suspicious masses. Using these guidelines to improve the selection of patients for open biopsy should decrease the overall costs of screening.

The TA90-IC test correctly identified 7 of 11 (64%) cancers (invasive and in situ) missed by mammography. Note that a negative mammogram in our series connoted a palpable mass that brought the patient to biopsy. An elevated TA90-IC level in the face of a normal mammogram should prompt ultrasonography to better delineate the mass, followed if necessary by a biopsy. If, on the other hand, the ultrasonogram is normal in the face of an elevated TA90-IC level, we recommend a more intensive follow-up with repeat mammography and TA90-IC assay in 6 months.

Mammograms are less accurate in women younger than 50 years of age. Their sensitivity in this age group as reported in the randomized trials ranges from 53% to 84%.[3,4] A subset analysis of the patients younger than 50 years enrolled in this study showed a mammographic sensitivity similar to that of reported series[3,4] and equal to the sensitivity of TA90-IC assay (67%). Surprisingly, the specificity and overall accuracy of the TA90-IC test (93% and 87%, respectively) were far superior to those of mammography alone (55% and 58%, respectively) in this age group. The potential benefit in terms of minimizing unnecessary procedures is even greater in this age group: if both a positive mammogram and an elevated TA90-IC level are

required to perform a biopsy, the procedure will yield positive results in 6 of 7 patients: only one unnecessary procedure will have been performed.

Therefore, although mammography remains an excellent screening test for the early detection of breast cancer, its accuracy can be improved by the addition of a simple blood test to measure the TA90-IC level. We propose combining the two tests into the screening schedule recommended by the American Cancer Society. It should be noted that the results reported in this study are based on a single measurement of TA90-IC; it is highly probable that serial measurements on an annual basis can further increase the accuracy of detecting occult carcinomas in the screened population.

Financial Disclosure:

This work was supported by the Army grant DAMD17-94-J-4459, Associates for Breast Cancer Research (Santa Monica, California) and the Ben B. and Joyce E. Eisenberg Foundation (Los Angeles, California).

Dr. Habal is supported in part by Grants P01 CA 29605 and CA 1038 from the National Cancer Institute.

References

1 Harris J, Morrow M, Norton L. Malignant tumors of the breast. In: DeVita VT, Hellman S, Rosenberg SA, eds. *Cancer, Principles and Practice of Oncology.* 5th ed. Philadelphia: Lippincott-Raven Press; 1997, 1557-1616.

2 Harris KM, Vogel VG. Breast cancer screening. Cancer Met Rev. 1997; 16: 231-262.

3 Rosenquist CJ, Lindfors KK. Screening mammography beginning at age 40 years, a reappraisal of cost-effectiveness. Cancer. 1998; 82: 2235-2240.

4 Mushlin AI, Kouides R, Schapiro DE. Estimating the accuracy of screening mammography: a meta-analysis. Am J Prev Med. 1998; 14: 143-153.

5 Elmore JG, Barton M, Moceri V, Polk S, Arena P, Fletcher S. Ten-year risk of false positive screening mammogams and clinical breast examinations. N Engl J Med. 1998; 338: 1089-1096.

6 Euhus DM, Gupta RK, Morton DL. Characterization of 90-100 kDa tumor-associated antigen in the sera of melanoma patients. Int J Cancer. 1990; 45: 1065-1070.

7 Gupta RK, Morton DL. Monoclonal antibody-based ELISA to detect glycoprotein tumor-associated-antigen-specific immune complexes in cancer patients. J Clin Lab Anal. 1992; 6: 329-336.

8 Brown ML, Houn F, Sickles EA, Kessler LG. Screening mammography in community practice: positive predictive value of abnormal findings and yield of follow-up diagnostic procedures. AJR Am J Roentgenol. 1995; 165: 1373-1377.

9 Good PI. *Permutation Tests: A Practical Guide to Resampling Methods for Testing Hypotheses.* New York: Springer-Verlag Press, 1994.

10 Wagner JL. Cost-effectiveness of screening for common cancer. Cancer Metast Rev. 1997; 16:281-294.

11 Kramer S, Schultz-Wendtland R, Hagedorn K, Bautz W, Lang N. Magnetic resonance imaging and its role in the diagnosis of multicentric breast cancer. Anticancer Res. 1998; 18(3C): 2163-2164.

12 Yang WT, Lam WW, Cheung H, Suen M, King WW, Metrewli C. Sonographic, magnetic resonance imaging, and mammographic assessments of preoperative size of breast cancer. J Ultrasound Med. 1997; 16: 791-797.

13 Muller-Schimpfle M, Stoll P, Stern P, Kurz S, Dammann F, Claussen CD. Do mammography, sonography, and MR mammography have a diagnostic benefit compared with mammography and sonography? AJR Am J Roentgenol. 1997; 168: 1323-1329.

14 Boetes C, Mus RD, Holland R, et al. Breast tumors; comparative accuracy of MR imaging relative to mammography and US for demonstrating extent. Radiology. 1995; 197: 743-747.

15 Mandelblatt J, Freeman H, Winczewski D, et al. The costs and effects of cervical and breast cancer screening in a public hospital emergency room. Am J Public Health. 1997; 87: 1182-1189.

16 Zavotsky J, Gupta RK, Brennan MB, Yee R, Giuliano AE, Morton DL. Prospective evaluation of TA90 immune complex assay for preoperative diagnosis of benign and malignant breast lesions. Proc Am Soc Clin Oncol. 1998; 17:106a.

Table 1. Criteria Used to Evaluate Mammography and TA90-IC

		Pathologic Diagnosis	
		Positive[a]	Negative
T E S T	Positive[b]	True Positive (TP)	False Positive (FP)
	Negative	False Negative (FN)	True Negative (TN)

Sensitivity = TP / TP + FN ; **Specificity** = TN / TN + FP

False Positive Rate (FPR) = 100 – Specificity

Positive Predictive Value (PPV) = TP / TP + FP

Accuracy = TP + TN / TP + TN + FP + FN

[a] Includes invasive carcinoma or DCIS; [b] Includes suspicious or indeterminate mammogram

Table 3. Results Subdivided First by Mammography and Then by TA90-IC Level, for the Entire Group of 157 Patients and Then for the 56 Patients Below the Age of 50

		Pathologic Findings *(number of patients in each group)*		
		Cancer		Benign
		Invasive	DCIS	
All Patients				
Mammo + *(N=105)*	TA90+ *(N=41)*	30	6	5
	TA90- *(N=64)*	13	9	42
Mammo – *(N=52)*	TA90+ *(N=11)*	6	1	4
	TA90- *(N=41)*	3	1	37

Number of invasive tumors missed by a negative TA90 = 16/52
Number of DCIS tumors missed by a negative TA90 = 10/17
Number of benign tumors missed by a positive TA90 = 9/88

Patients < 50 years old				
Mammo + *(N=28)*	TA90+ *(N=7)*	5	1	1
	TA90- *(N=21)*	2	2	17
Mammo – *(N=28)*	TA90+ *(N=6)*	4	1	1
	TA90- *(N=22)*	1	0	21

Number of invasive tumors missed by a negative TA90 = 3/12
Number of DCIS tumors missed by a negative TA90 = 2/4
Number of benign tumors missed by a positive TA90 = 2/40

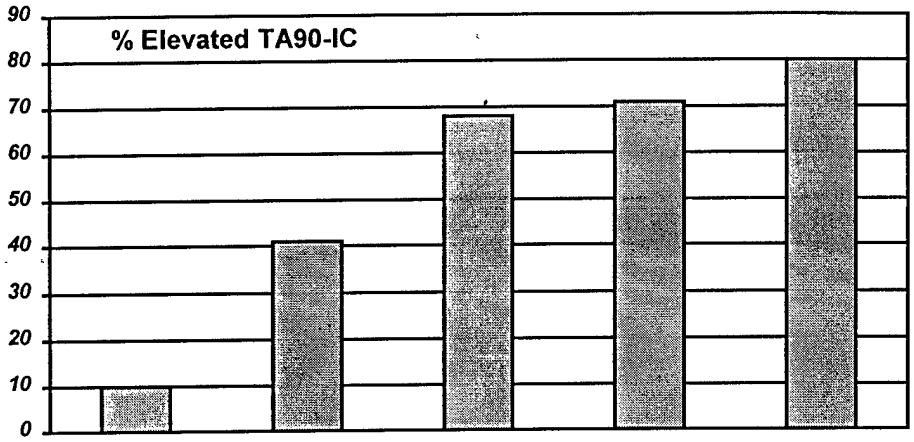
'Mammo +' includes suspicious and indeterminate mammograms combined into one group, 'Mammo –' includes negative mammograms only; 'TA90 +' includes elevated TA90-IC levels, 'TA90-' includes normal TA90-IC levels.

Table 4. Accuracy of Mammography Compared with TA90-IC Assay

	Sensitivity	Specificity	PPV	Accuracy	FPR
All Patients					
Mammogram	84%	46%	54%	62%	54%
TA90-IC	77% *	90% §	82% §	84% *	10% *
Patients < 50 years old					
Mammogram	67%	55%	34%	58%	45%
TA90-IC	67%	93% §	83%	87%	7%
Indeterminate Mammograms Excluded					
Mammogram	82%	54%	58%	66%	46%
TA90-IC	61% ‡	79% §	82% §	77% ‡	21% ‡
Test Combinations					
Either Positive	94%	41%	56%	64%	59%
Both Positive	50%	95%	89%	75%	5%

§ $p < 0.0001$ *‡ $p < 0.007$* ** $p < 0.005$*

Figure 1. Percent of patients with elevated TA90-IC levels based on pathologic analysis.



	BENIGN	*DCIS*	*Stage I*	*Stage II*	*Stage III*
% Elevated TA90-IC	10	41	68	71	80
mean TA90-IC	0.282	0.327	0.534	0.452	0.455
n	*88*	*17*	*23*	*24*	*5*

Table 2. Mammographic Findings, TA90-IC Levels, and Final Histopathologic Outcome in All 157 Patients Studied

	Pathologic Findings *(number of patients)*					
	Invasive Cancer *(by AJCC§ Stage)*				DCIS‡	Benign
Test Results	I	II	III	*All*		**Disease**
Mammogram only †						
Suspicious	18	18	2	*38*	13	34
Indeterminate	4	0	1	*5*	2	13
Benign	1	6	2	*9*	2	41
TA90-IC only †						
Elevated	15	17	4	*36*	7	9
Normal	8	7	1	*16*	10	79

§ American Joint Committee on Cancer; ‡ Ductal Carcinoma in Situ

† McNemar's test (for paired data): compares TA90-IC elevation with positive mammogram results (suspicious and indeterminates combined), $p < 0.0001$

Table 5. Analysis of False Positive TA90-IC Levels or Mammograms (Suspicious and Indeterminates Combined) in the Subset of Patients with Benign Histopathology

Age Group	n	TA90-IC		Mammogram				*p-value* *
		Elevated		Suspicious		Indeterminate	Combined	
		n	*%*	*n*	*%*	*n*	*%*	*%*	
25-39	13	1	7.7	2	15.4	5	38.5	53.8	0.0313
40-49	27	1	3.7	5	18.5	6	22.2	40.7	0.0063
50-59	25	5	20.0	15	60.0	0	0.0	60.0	0.0063
60-69	14	1	7.1	8	57.1	1	7.1	64.3	0.0215
70+	9	1	11.1	4	44.4	1	11.1	55.6	0.2188
Total	**88**	**9**	**10.2**	**34**	**38.6**	**13**	**14.8**	**53.4**	**0.0001**

* McNemar's test for paired data comparing TA90-IC elevation with positive mammographic results (combined suspicious and indeterminate readings)

From The Department of Defense
Breast Cancer Research Program Meeting
Era of Hope
Proceedings, Vol. 1 : 181-82 , 1997

DETECTION OF OCCULT BREAST CANCER BY A NOVEL IMMUNOLOGIC APPROACH

Rishab K. Gupta, Reynold Yee, Zacarias C. Leopoldo and Donald L. Morton

John Wayne Cancer Institute at Saint Johns Health Center
2200 Santa Monica Blvd., Santa Monica, CA 90404-2301

We documented the presence of a 90kD glycoprotein tumor-associated antigen (TAA) which is immunogenic in breast cancer patients. Therefore, it is logical to assume that circulating immune complexes (IC) should form *in vivo* each time a humoral immune response is made to the antigen, and the source of the antigen is present in the patient. After purification and characterization, the 90kD TAA was used to develop a MuMoAb of IgM isotype. Blocking studies in ELISA revealed that the epitope on the 90kD TAA recognized by the MuMoAb was different from those recognized by human antibodies. Also the MuMoAb did not react with any of the normal human serum proteins including IgG and IgM. No immunologic reactivity in Western blot between 90kD TAA and a number of murine monoclonal antibodies developed to other antigens was observed. The MuMoAb was immobilized onto a solid matrix and used to capture the 90kD TAA-specific IC. Capturing of the antigen-specific IC was assessed using goat anti-human IgG conjugated to alkaline phosphatase. Feasibility of this technique was confirmed by the detection of *in vitro* generated immune complexes between purified 90kD TAA and the baboon polyclonal IgG antibodies to this antigen. The baboon polyclonal antibodies were mixed with the purified 90kD TAA in different protein proportions. After incubation at 37C for 30 min, the mixtures were tested to determine if a positive signal was generated in the MuMoAb capture assay. The ELISA signal was consistently higher over a wide range of baboon anti-TAA IgG to antigen protein concentration ratios (3:1 to 90:1) than either of the two immune reactants (antigen or antibody) alone or pre-immune baboon IgG at 405_{nm}. These results denoted that the MuMoAb captured baboon anti-TAA IgG via the TAA-specific IC only, i.e., when the IgG antibody was in the form of TAA-specific IC.

Keywords: **Glycoprotein, Tumor-associated antigen, Immune complex, ELISA, Tumor marker, CEA, CA15-3**

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD-17-94-J-4459.

Reproducibility studies in ten replicates revealed an inter-assay variation range of 0.806 to 1.311 ELISA value, with a mean of 1.010, standard deviation (SD) of 0.139, and coefficient of variation (CV) of 13.7%; and an intra-assay variation range of 0.849 to 1.214 with a mean of 1.007, SD of 0.105 and CV of 10.4%. To determine the incidence and level of 90kD TAA-specific IC, serum samples from self proclaimed healthy age matched normals (107) and breast cancer patients (106) were analyzed. The procurement of sera from breast cancer patients was random, i.e., criteria with respect to pre- or post-surgery, evidence or no evidence of disease, etc., were not used in selecting the serum samples. Comparative analysis of the data from normal and breast cancer groups revealed that the normal group had a significantly ($p < 0.05$) lower normalized ELISA value (0.212 ± 0.088) than the breast cancer group (0.570 ± 0.438). Using a cut off level of 0.410 (mean plus 3 SD of normals), a significantly ($p < 0.05$) greater proportion of breast cancer patients (67/106, 63%) was above this value than the normal group (3/107, 2.8%). From a group of 128 breast cancer patients that were positive for the 90kD TAA-specific IC pre-operatively, serum samples were obtained retrospectively 2 to 12 weeks after surgical resection of all of the accessible tumor. The serum samples (256 total) obtained before and after surgery were analyzed by the 90kD TAA-specific IC assay. Of the 128 patients, 76 (59%) became negative after surgery for the marker, suggesting either a complete resection of the tumor or a reduction in tumor burden to a level below the threshhold level that could result in the serum to become 90kD TAA-specific IC positive. Despite surgical resection of the accessible tumor, there were 52 patients who remained positive for the 90kD TAA-specific IC. Longitudinal clinical follow-up of these patients revealed that 34 of 52 (65%) post-operatively positive patients developed recurrent disease within five years, as opposed to only 9 of 76 (12%) of the post-operatively negative patients ($p< 0.05$). Thus, it is apparent that in the group of patients in whom the 90kD TAA-specific IC remained positive, all of the disease was not resected and these patients perhaps harbored micrometastases. This translates to the fact that the 90kD TAA-specific IC marker can effectively identify patients with poor prognosis. Many patients who remained disease free for greater than 5 years were consistently negative for the TAA-specific IC, and patients who had developed recurrent disease either remained positive after removal of tumor or became positive for the 90kD TAA-specific IC before developing clinically detectable disease. Analyses of serum samples from 68 breast cancer patients with evidence of disease for 90kD TAA-specific IC, carcinoembryonic antigen (CEA) and CA15-3 revealed that 55 (81%) were positive for the 90kD TAA-specific IC, 16 (24%) for CEA, and 23 (34%) for CA15-3. Despite higher incidence of the TAA-specific IC than CEA or CA15-3, it was observed that some serum samples that were positive for CEA or CA15-3 were not necessarily positive for the 90kD TAA-specific IC, and that there was no significant association between the 90kD TAA-specific IC and CEA or CA15-3. However, when either of the three or all of the three positive markers were taken into consideration, the incidence of positivity increased from 81% to 91%. Thus, use of the 90kD TAA specific-IC marker in conjunction with CEA and /or CA15-3 may prove to be more sensitive than when used alone for immunodiagnosis and immunoprognosis. Furthermore, this novel approach to a tumor marker assessment clearly denotes a positive correlation between post-operative presence of 90kD TAA-specific IC and subclinical residual or recurrent disease in breast cancer patients.

A84

PRESENCE OF A 90KD GLYCOPROTEIN TUMOR-ASSOCIATED ANTIGEN SPECIFIC IMMUNE COMPLEXES IN CIRCULATION OF MELANOMA PATIENTS SIGNIFY POOR PROGNOSIS.

Rishab K. Gupta and Donald L. Morton, John Wayne Cancer Institute

2200 Santa Monica Blvd., Santa Monica, CA 90404, USA.

Immune complexes (IC) have been observed in peripheral circulation of cancer patients; however, their clinical application has been limited. This is because currently available IC detection assays are not antigen specific. Here, we describe applicability of an antigen-specific IC detection assay. An autoimmunogenic glycoprotein tumor associated antigen of a molecular mass of 90kD (TA90) detected in human solid tumors was used to develop a murine monoclonal antibody (MAb) of IgM isotype. The MAb recognized an epitope that is different from those recognized by human polyclonal antibodies, and it did not react with normal human serum proteins. The MAb was immobilized to a solid matrix and used to capture TA90-IC formed in vivo by reacting the tests sample with the MAb-solid matrix. Capturing of the TA90-IC via interaction of the TA90 epitope with the immobilized MAb was assessed using alkaline phosphatase conjugated goat anti-human IgG. This assay discriminated between normal and cancer sera. At an ELISA value of >0.410 OD_{405} (mean plus 3 SD of normal) significantly lower proportion of normals (3.2% (8/250)) compared to cancer patients was (56,3% (364/647)) positive. Subsequently sequential serum samples obtained at regular intervals from 130 stage I melanoma patients (105 with recurrence within 10 years after removal of primary and 25 with no recurrence for 10 years) were analyzed, and a patient with 2 or more consecutively positive samples was considered positive, and of 25 non-recurrent disease patients 20 (80%) were negative for the TA90-IC ($p < 0.001$). Mean time before recurrence of the clinically detectable disease at which the test became positive was 23 ± 28 months in this group of patients. Similar results were obtained in melanoma patients with advanced stages who were rendered disease free surgically. The results of this novel approach to a tumor marker assessment clearly denote a positive correlation between post-operative presence of TA90-IC and subclinical residual or recurrent disease in melanoma patients. (Supported in part by grants from National Cancer Institute (CA12582 and CA29605), John Wayne Cancer Institute Auxiliary, Roy E. Coats Research Laboratories, Sonya Valley Ghidossi Vaccine Laboratory, Wrather Family Foundation, Steel Foundation and the Jack Green Fund).

A85

AMELOGENIN AS MARKER FOR BONE MARROW ENGRAFTMENT: SHORT-TERM AND LONG-TERM STUDIES

T. Pugatsch, R.Or, S.Slavin and J. Kapelushnik

BMT, Hadassah University Hospital, Jerusalem, Israel

The degree of chimerism, i.e. full donor chimerism (DC) or mixed chimerism (MC), in recipients of allogeneic bone marrow transplants (BMT) will to a strong extent affect the post-transplant treatment. Amplification by the polymerase chain reaction (PCR) of part of the amelogenin gene (AMG) located on both the X- and Y-chromosome, allows detection of minute numbers of male and female cells in BM and peripheral leukocytes of transplant

J. Tumor Marker Oncology 12: 77 (1997)

*407

PROSPECTIVE EVALUATION OF TA90 IMMUNE COMPLEX ASSAY IN THE PREOPERATIVE DIAGNOSIS OF BENIGN AND MALIGNANT BREAST LESIONS. *J. Zavotsky, R.K. Gupta, M.B. Brennan, R. Yee, A.E. Giuliano and D.L. Morton. John Wayne Cancer Institute at Saint John's Health Center, Santa Monica CA.*

There is a need for alternative means of breast cancer screening because up to 75% of suspicious mammographic lesions are benign on biopsy and up to 20% of malignant lesions are missed on mammography. We prospectively evaluated a 90-kD glycoprotein tumor-associated antigen (TA90) in the preoperative serum of patients with breast lesions warranting open biopsy. The serum was coded and tested in a blinded fashion for TA90 immune complexes by an ELISA. An optical density (OD) ≥ 0.41 at 405 nm was considered positive based upon three standard deviations above the mean of 250 normal controls. Of 138 study patients, 111 had a mammographic abnormality (23 with a palpable mass), and 27 had a normal mammogram with a palpable mass or bloody nipple discharge. Eighty-two had benign lesions, 14 had ductal carcinoma in situ (DCIS), and 42 had invasive carcinoma (IC). There was a significant ($p = 0.0001$) difference in TA90 values for benign lesions (0.254 ± 0.239 OD) and IC (0.436 ± 0.209 OD). Of the 111 patients with an abnormal mammogram, 50 (45%) had DCIS (N = 14), or IC (N = 36). Of the 41 patients with a positive TA90, 35 (85%) had DCIS (N = 4) or IC (N = 31). Of the 27 patients with a normal mammogram, 6 (22%) had IC. Of the 97 patients with a negative TA90, 21 (22%) had DCIS (N = 10) or IC (N = 11). Of note, the TA90 level was positive in 4 of 6 IC patients who had a normal mammogram. Fifty-two carcinomas were evaluable by size. TA90 was positive in 6 of 15 (40%) lesions <1 cm, and in 25 of 37 (68%) lesions ≥1 cm. We conclude that the serum TA90 assay may be a useful adjunct to mammography, since it may identify neoplasms missed by mammography.

*408

SIMULTANEOUS IMMUNOHISTOCHEMICAL (IHC) TUMOR CELLS DETECTION IN AXILLARY LYMPH NODES AND BONE MARROW ASPIRATES IN NODE NEGATIVE (NO) BREAST CANCER (BC). *B. Gerber, A. Krause, E. Rohde, D. Richter and K. Friese. Dept. Ob/Gyn, University of Rostock, Rostock, Germany.*

Studies of axillary lymph nodes and bone marrow aspirates from patients (pts.) with node negative BC with the same immunohistochemical method have not yet been reported. Lymph nodes and bone marrow aspirates from 176 pts. with pT1–2 N0 M0 BC were retrospectively examined for tumor cells using a cytokeratin antibody and ABC-technique. The IHC findings were correlated with conventional histologic findings and other prognostic factors. The mean follow up time was 55 ± 16 months. Totally in 55 of 176 (31.2%) pts. staged by conventional methods as pT1–2 N0 M0 BC tumor cells were detected in lymph nodes (N1a-IHC) and/or bone marrow aspirates (M1-IHC).

	Lymph nodes	
Bone marrow	N0-IHC (n = 155)	N1a-IHC (n = 21)
M0 (n = 128)	121 (68.7%)	7 (4.0%)
M1-IHC (n = 48)	34 (19.3%)	14 (8.0%)

Disease free survival and overall survival showed a prognostic disadvantage for women with tumor cell detection in any site and number compared to women without any tumor cells ($p < .05$). Differences between IHC-positive and IHC-negative pts. were found in tumor size, grading, vessel invasion, ER, S-phase and Cathepsin D. By multivariate analysis tumor size and grading, but not the tumor cells detection, were confirmed as independend prognostic factors in N0 BC. We conclude that in 31% of BC pts. who have been staged as pT1–2 N0 M0 by conventional methods tumor cells are detectable in axillary lymph nodes and/or bone marrow aspirates. A prognostic disadvantage of tumor cell detection has been proven, but it does not represent an independent prognostic factor and does not facilitate treatment decisions.

*409

THE PRESENCE OF CLONOGENIC BREAST CANCER CELLS IN PERIPHERAL BLOOD STEM CELL (PBSC) PRODUCTS CORRELATES WITH AN EXTREMELY POOR PROGNOSIS FOR PATIENTS WITH STAGE IV DISEASE. *T.J. Moss, T. Umiel, R.M. Herzig, B. Cooper, M.J. Kennedy, R. Meagher, R.A. Petri, A. Pecora, E. Copelan, H.M. Lazarus. BIS Laboratories, Reseda, CA, Hoxworth Blood Center, University of Cincinnati Medical Center, Cincinnati, OH, Ireland Cancer Center, Cleveland, OH, Johns Hopkins Oncology Center, Baltimore, MD, Ohio State University, Columbus, OH, and Hackensack Medical Center, Hackensack, NJ.*

Autologous bone marrow transplantation (ABMT) is accepted therapy for patients with stage IV breast cancer. Currently, there is considerable controversy whether PBSC collections contaminated with small numbers of breast cancer cells can contribute to relapse of disease after ABMT. Since tumor stem cells are hypothesized to be responsible for relapse of disease, we wished to determine if clonogenic breast cancer cells in PBSC correlated with a poor clinical outcome. A clonogenic soft agar assay was performed on 224 PBSC samples drawn from 130 patients with stage IV breast cancer. The clonogenic assay utilized 0.3 percent agarose, Isocove's medium with fetal bovine serum and growth factors. Mononuclear cells isolated from PBSC were plated in agarose and incubated in a humidified chamber 37°C for 10–14 days and tumor colonies were then counted. Verification that colonies were breast cancer in origin was achieved by immunofluorescent staining. Thirty-eight of 130 patients had tumor colonies in PBSC products. Thirty-seven of these 38 patients (97%) relapsed after ABMT therapy. Using log rank Kaplan-Meier survival curve analysis, the disease-free survival (DFS) for patients with tumor colonies was significantly reduced as compared to patients without colonies in their PBSC products ($p < 0.0001$). We conclude that the presence of clonogenic breast cancer cells in PBSC products portends an extremely poor prognosis. Alternate therapy should be considered for this group of patients, such as purging of the PBSC product or post-ABMT therapy.

*410

COMPARISON OF MAMMOGRAPHY (MM) AND ULTRASONOGRAPHY (US), Tc^{99m}TETROFOSMIN SCINTIMAMMOGRAPHY (SMM) AND GADOLINIUM-ENHANCED MRI IN THE ASSESSMENT OF RESIDUAL TUMOR IN PATIENTS (pts) WITH LOCALLY ADVANCED BREAST CANCER (LABC) TREATED WITH DOXORUBICIN (DOX) AND PACLITAXEL (PTX) AS NEOADJUVANT CHEMOTHERAPY. *J.L. Passos-Coelho, A. Moreira, M.L. Orvalho, T.C. Ferreira, M.R. Vieira, S. André, A. Gaspar, C. Santos-Costa, J. Menezes-Sousa, A. Fernandes, J. Oliveira. Instituto Português de Oncologia and Centro de Ressonância Magnética de Caselas, Lisboa, Portugal.*

We evaluated the clinical and pathological response rate in pts with LABC treated with neoadjuvant DOX and PTX, a very active combination in metastatic breast cancer. Since 3/97, 15 women received four cycles of DOX (60 mg/m² iv over 5 min on day 1) and PTX (200 mg/m² iv over 3h on day 2) every 3 weeks before mastectomy. Pts underwent MM and US, SMM and MRI before and after chemotherapy to assess response to treatment and the extent of preoperative residual tumor. There were 10 partial (PR) and 3 complete (CR) clinical responses but no pathological CRs; however, in 5 pts the area of residual viable tumor in the mastectomy specimen was less than 1 cm². In 3 of these 5 pts the breast mass was no longer detectable in the preoperative MM and US. SMM evaluation showed a decrease in Tc^{99m}Tetrofosmin uptake between the first and the second exam in 13 of the 14 initially tracer-positive pts; 1 pt had a false-negative SMM at diagnosis. In 2 pts with less than 1 cm² of residual viable tumor at surgery, the preoperative SMM had become normal. All pts had tumor detectable by Gadolinium-enhancement in the preoperative MRI, including the 5 pts with less than 1 cm² of viable tumor in the surgical specimen. MRI estimated accurately the size of residual tumor in mastectomy specimens in each pt ($r^2 = 0.88$, $p < 0.0001$). Furthermore, the areas of viable tumor within the resected tumor mass were correctly identified in the preoperative MRI. In conclusion, no pathological CRs were obtained in pts with LABC treated with DOX plus PTX. MRI could accurately estimate response to treatment and extent of residual disease in pts with LABC. This may be helpful to plan conservative surgery in these pts.

Use Only 1997 Abstract Forms.
DEADLINE FOR RECEIPT IS JUNE 2, 1997.

ABSTRACT
DO NOT FOLD
Type abstract inside blue space below. See sample abstract on page 4.

FOR OFFICE USE ONLY	
Date Rec'd	
CK CC C O NP	
Fee Rec'd	
Abst. #	Prog. #

20TH ANNUAL SAN ANTONIO BREAST CANCER SYMPOSIUM DECEMBER 3-6, 1997 AT MARRIOTT RIVERCENTER HOTEL

1. PRESENTATION PREFERENCE (*Check one only*):
   ☐ Poster preferred
   ☐ Slide preferred
   ☒ Poster or slide acceptable
   Program Committee will make final decision.
2. SUBJECT CATEGORY
   From the list on the reverse side, find the number of the most appropriate category for your abstract and insert. 34
3. AUDIOTAPING
   ☒ By checking this box, I hereby grant permission to Breast Cancer Symposium to record my presentation.
4. INSTRUCTIONS
   Specific instructions are provided on the reverse side of this form. Be sure to note the No-Show penalty on page 3.
5. INQUIRIES
   Questions regarding preparation and submission of abstracts should be directed to the Symposium Coordinator, Lois Dunnington. See reverse side.
6. PAYMENT
   Pay by check or money order (must be in US Dollars and drawn on US bank), or provide credit card information below.
7. CHECKLIST FOR SUBMISSION
   ☒ Original abstract form
   ☒ Original Disclosure form
   ☒ Payment ($25 each abstract)

DETECTION OF A GLYCOPROTEIN TUMOR-ASSOCIATED ANTIGEN SPECIFIC IMMUNE COMPLEXES IN BREAST CANCER PATIENTS.

Gupta RK*, Giuliano AE, Yee R, Leopoldo Z, and Morton DL, John Wayne Cancer Institute at Saint John's Health Center, Santa Monica, California, 90404

An autoimmunogenic 90kD glycoprotein tumor-associated antigen (TA90) shared by a variety of human cancers including breast carcinoma was isolated and characterized. Also, a murine monoclonal antibody (MuMoAb), AD1-40F4, of IgM isotype was developed using purified antigen as the immunogen. The epitope recognized by the MuMoAb was different from those recognized by human polyclonal antibodies. The MuMoAb did not react with any of the human serum proteins but recognized a 90kD subunit of the glycoprotein antigen. Because human antibody can bind to the antigen and form immune complexes (IC) in circulation, we examined the incidence and clinical significance of TA90-IC in sera of breast cancer patients. MuMoAb was immobilized to a solid matrix and used to capture TA90-IC. The MuMoAb-based ELISA revealed that the incidence of TA90-IC in sera of randomly selected breast cancer patients [63%(67/106)] was significantly higher ($p<0.05$) than that in normal controls [3%(3/107)]. Analyses of serum samples from 68 breast cancer patients with evidence of disease for carcinoembryonic antigen (CEA), CA15-3 and TA90-IC revealed that the incidence of these markers was 24%, 34% and 81% respectively. Despite higher incidence of TA90-IC than CEA or CA15-3, it was observed that some serum samples which were positive for CEA or CA15-3 were not necessarily positive for TA90-IC. There was no significant association between the TA90-IC and CEA or CA15-3. However, when either or all of the three positive markers were taken into consideration, the incidence of positivity increased from 81% to 91%. Thus, use of TA90-IC in conjunction with CEA and/or CA15-3 may prove to be more sensitive than when used alone for immunodiagnosis and immunoprognosis of breast cancer. [Supported by U.S. Army Medical Research and Materiel Command under DAMD-17-94-J-4459, Jack Green Fund, Associates for Breast Cancer Studies of John Wayne Cancer Institute, Ben B. and Joyce E. Eisenberg Foundation (Los Angeles), and Fashion·Footware Association of New York.]

THIS IS NOT A MEETING REGISTRATION FORM. You must register separately on the form which will accompany the final program.

ABSTRACT TO BE PRESENTED BY:

Family (Last) Name GUPTA First Name Rishab MI K. Degree Ph.D.
Institution John Wayne Cancer Institute
Complete Mailing Address 2200 Santa Monica Blvd.
City Santa Monica State CA Postal/ZIP Code 90404
COUNTRY U.S.A.

A14 CLINICAL SIGNIFICANCE OF A 90KD GLYCOPROTEIN TUMOR-ASSOCIATED ANTIGEN SPECIFIC IMMUNE COMPLEXES IN SERA OF BREAST CANCER PATIENTS

Rishab K. Gupta, Armando E. Giuliano, Mark C. Kelley, Reynold Yee, Zacarias Leopoldo, Donald L. Morton. John Wayne Cancer Institute at Saint John's Hospital and Health Center, 2200 Santa Monica Blvd., Santa Monica, CA 90404, USA

We undertook these investigations to determine the incidence and clinical significance of a 90kD glycoprotein tumor-associated antigen (TAA)-specific immune complexes (IC) in sera of breast cancer patients. Sera were randomly selected from 106 patients that had histopatho-logically proven breast cancer, and from 107 apparently healthy females. The serum samples were analyzed for the presence of the 90kD glycoprotein TAA-specific IC by a murine monoclonal antibody based ELISA. The murine monoclonal antibody, AD1-40F4, was developed to an autoimmunogenic glycoprotein TAA. This murine monoclonal antibody recognized an epitope that is different from those recognized by human polyclonal antibodies. The immobilized murine monoclonal antibody captured the *in vivo* formed IC that were present in a test sample. The captured IC were recognised by enzyme conjugated goat anti-human IgG. The incidence of 90kD glycoprotein TAA-specifc IC in sera of breast cancer patients was significantly higher than in healthy females. Of the 106 breast cancer patients 67 (63%) were positive for the TAA specific-IC, as indicated by the normalized ELISA value above 0.410 OD (mean plus 3SD of normals) at 405nm. On the contrary, only 3 (2.8%) of 107 apparently healthy controls had positive ELISA value ($p>0.05$ by Fisher's test). Analysis of post-operative serum samples of breast cancer patients and correlation of results with clinical course revealed that 79% (34/43) sera from patients with recurrent disease were positive. This incidence was significantly higher ($p<0.05$ by Chi square) than those patients who did not recur within 5 years (21%, 18/85). The results of this novel approach to a tumor marker assessment clearly denote a positive correlation between post-operative presence of TAA-specific IC and subclinical residual or recurrent disease in breast cancer patients. (Supported in part by the US Department of Army grant DAMD17-94-J4459, John Wayne Cancer Institute Auxiliary, Associates for Breast Cancer Studies, Roy E. Coats Research Laboratories and the Jack Green Fund).

Antigen-specific immune complexes as human tumor markers, breast cancer, glycoprotein tumor-associated antigen, autoimmunogenic

J. TUMOR MARKER ONCOLOGY 11: 44, 1996

March, 1999

CURRICULUM VITAE AND BIBLIOGRAPHY

NAME: **RISHAB KUMAR GUPTA**

PRESENT TITLE: Vice-President for Educational Affairs,
Director of Immunodiagnosis and Director of Safety

ADDRESS: John Wayne Cancer Institute
2200 Santa Monica Blvd.
Santa Monica, California 90404, U.S.A.
PII Redacted Phone: Office (310) 449-5265; FAX (310) 449-5273

[redacted] [redacted] [redacted]

EDUCATION:

1960-1963	B.Sc. (Hons); G.B. Pant University, Pantnagar, India
1963-1965	M.Sc. (Microbiology); G.B. Pant University, Pantnagar, India
1965-1968	M.S. (Biochemistry); Rutgers University, New Brunswick, N.J., U.S.A
1965-1968	Ph.D. (Microbiology); Rutgers University, New Brunswick, N.J., U.S.A.

SPECIAL TRAINING:

1965	Special summer course in microbiology, School of Basic Sciences, India
1967	Summer trainee at the University of Cincinnati, Department of Microbiology, Cincinnati, Ohio, U.S.A.
1967	Trainee at the Battle Memorial Institute, Richmond, Washington, U.S.A.
1968-1970	Post-doctoral fellowship at UCLA Medical School, Department of Microbiology and Immunology, U.S.A.
1970-1971	Post-doctoral fellowship at Yale University Medical School, Department of Microbiology, U.S.A.
1971-1972	Post-doctoral fellowship at Jules Stein Eye Institute, UCLA School of Medicine, U.S.A.
1973	Annual Survey Course in Immunology of the American Association of Immunologists, Woodshole, MA
1976	Protein Separation by SDS-PAGE, Scripps Research Foundation, La Jolla, Ca
1985	Molecular Immunology Course, University of California, San Francisco, Ca

ACADEMIC APPOINTMENTS:

1991-present	Director of Immunodiagnosis and Vice-president of Education, John Wayne Cancer Institute, Santa Monica, Ca
1991-present	Professor emeritus, Division of Oncology, Department of Surgery, UCLA School of Medicine, Los Angeles, Ca
1985-1991	Professor, Division of Oncology, Department of Surgery, UCLA School of Medicine, Los Angeles, Ca
1981-85	Associate Professor, Division of Oncology, Department of Surgery, UCLA School of Medicine
1979-81	Assistant Professor, Division of Oncology, Department of Surgery, UCLA School of Medicine
1975-82	Microbiologist (WOC), V.A. Medical Center, Sepulveda, CA
1975-79	Associate Research Oncologist, Department of Surgery, UCLA School of Medicine, Los Angeles, CA
1972-75	Assistant Research Oncologist, Department of Surgery, UCLA School of Medicine, Los Angeles, CA
1971-72	Postgraduate Research Microbiologist, Ophthalmic Microbiology, Jules Stein Eye Institute, UCLA
1970-71	Postdoctoral Fellow, Department of Microbiology, School of Medicine, Yale University, New Haven, CT
1968-70	Lecturer, Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, CA
1965-68	Research Associate, Department of Biochemistry and Microbiology, Rutgers University, New Brunswick

RESEARCH INTERESTS AND SPECIALTIES:

Tumor Immunology, Immunochemistry, Microbial Physiology, Membrane Transport, Hybridoma techniques, Monoclonal Antibodies, Enzyme Immunoassay (ELISA), Radioimmunoassay, Molecular biology techniques (purification and enrichment of mRNA, preparation of cDNA, gene cloning, PCR, in situ hybridization, T cell receptors and growth factors.

ACADEMIC AWARDS AND HONORS:

1963	University Merit Certificate from G.B. Pant University, India
1963-1965	USAID Traineeship Award
1965-1968	NSF Fellowship at Rutgers
1967	ASM President's Fellowship Award
1969	Sigma Xi-UCLA Chapter
1970-1971	Post-doctoral Fellowship (NRSA) at Yale University
1973	Selected for the Annual Survey Course of AAI
1978	Co-chairman, Tumor Antigens session at the AACR annual meeting
1979	Invited participant in Workshop on Human Tumor Antigens, NIH.
1980	4th Asian Cancer Congress, Bombay, India, invited participant.
1980	Workshop on Tumor Markers, Clinical Ligand Assay Society, Los Angeles, invited Faculty
1981	Invited Participant in Workshop on Melanoma Monoclonal Antibodies, NIH.
1981	Workshop on ELISA, Cordia Laboratories, Culver City, invited Faculty.
1982	Invited Faculty for Workshop on Practical Aspects of ELISA, Cordia Laboratories.
1983	Invited Faculty for Workshop on Melanoma Immunology, European Soc for Dermatological Res.
1984	Invited participant, 26th Annual Science Writer's Seminar of American Cancer Society.
1985	Invited Speaker at Central Toxicological Research Institute, Lucknow.
1986	Participating Faculty at Hybridoma Techniques Workshop, California State University, Fullerton, Ca
1987	Invited Speaker at the Indian Society of Biological Chemists.
1987	Invited Speaker at the Argentina Society of Immunology and Society of Clinical Invest.
1987	Invited speaker, "Immune Response to Human Melanoma" - Cancer Research Institute.
1988	Ad Hoc Review Member, Clinical Sciences Study Section, National Institute of Health, Bethesda, MD.
1988-1989	Study Section Member, Clinical Sciences, National Institute of Health, Bethesda, MD.
1989	Radioimmunoassay Workshop Faculty, California Polytechnic, Pamona, Ca.
1989-1991	Study Section Member, Immunology, Virology and Pathology, National Institute of Health, Bethesda, MD.
1990-1991	Research Grant Reviewer, Medical Research Council of Canada.
1992-1993	National Reviewer Reserve, NIH, Bethesda, MD.
1994-1996	State of Nebraska, Department of Health Resarch Grant Reviewer.
1993-1997	Study Section Member, Immunology, Virology and Pathology, National Institute of Health, Bethesda, MD.
1997-1998	National Reviewer Reserve, NIH, Bethesda, MD.
1998-Present	Study Section Member, Immunobiology, National Institute of Health, Bethesda, MD.

TEACHING PARTICIPATION:

Immunology, Immunochemistry, Medical Mycology, General Virology, General Mycology, Biochemistry, General Microbiology, Physiology of Fungi, Medical Mycology Seminars, In-depth laboratory Investigations in Tumor Immunology, Special Research Topics in Immunology, Interactive Teaching for second year medical students.

SCHOLARLY SOCIETY MEMBERSHIP:

American Association for Cancer Research; American Association of Immunologists; American Society of Clinical Oncology; American Society for Microbiology; Sigma Xi; Clinical Ligand Assay Society; Society for Oncodevelopmental Biology and Medicine; Clinical Immunology Society; American Academy of Microbiology; International Academy of Tumor Marker Oncology.

COMMITTEE ASSIGNMENTS:

1978-present	UCLA Mentor program.
1979	Member of Round Table Symposium Commitee of the American Academy of Microbiology.
1979-1983	Member of Research Advisory Committee for Advanced Science Training Program.
1980-1982	Member of the Interviewing Committee for the Advanced Science Training Program.
1985-1988	Control and Oversee the Divisional Business office.
1985-1991	Ad hoc member of Promotion Committees Appointed by the Academic Senate.
1991-present	Member, Human Subject Protection Committee at Saint John's Hospital and Health Center.
1991-present	Director, JWCI Safety Committee.
1996-present	Institutional Research Oversight Committee (IROC).

CERTIFICATION:

Specialist Microbiologist in Medical and Public Health Laboratory

EDITORIAL POSITIONS:

1976-	Reviewer, European Journal of Cancer
1978-	Reviewer, Journal of the National Cancer Institute
1979-	Reviewer, Clinical Chemistry
1982-	Reviewer, Journal of Clinical Immunology
1982-	Reviewer, Journal of Neurosurgery
1983-	Reviewer, Developmental and Comparative Immunology
1984-	Reviewer, Cancer Research
1984-	Associate editor, Journal of Clinical Laboratory Analysis
1986-	Member - Editorial Board, Oncology & Biotechnology News
1986-	Reviewer, Journal of Investigative Dermatology
1987-	External reviewer for grant applications of VA Central Office
1988-	Reviewer, Journal of Biological Response Modifiers
1990-93	Member - Editorial Board, Contemporary Oncology
1994-	Reviewer - Cancer
1995-	Reviewer - Proceedings of the National Academy of Sciences

RESEARCH SUPPORT:

July 1, 1973 to June 30, 1974; Cancer Research Coordinating Committee of the University of California; "Isolation and Purification of Tumor-Associated Antigens from Malignant Melanoma Tumors: Determination of their Reactivity with Homologous Sera"; $6,480; **R.K. Gupta - Principal Investigator.**

July 1, 1973 to June 30, 1974; California Institute for Cancer Research; "Purification of Specific Antibodies from Sera of Melanoma Patients"; $6,900; **R.K. Gupta - Principal Investigator.**

July 1, 1974 to June 30, 1975; Cancer Research Coordinating Committee of the University of California; "Isolation and Purification of Tumor-Associated Antigens from Malignant Melanoma Tumors: Determination of their Reactivity with Homologous sera"; $10,000; **R.K. Gupta - Principal Investigator.**

July 1, 1974 to June 30, 1975; California Institute for Cancer Research, "Isolation and purification of Tumor-Associated Antigens"; $9,458; **R.K. Gupta - Principal Investigator.**

April 1, 1972 to June 30, 1981; NIH PO1 CA 12582; "Immunology and Immunotherapy of Cancer"; Principal Investigator - Dr. D.L. Morton; **R.K. Gupta - Project Director** on "Purification and Characterization of Tumor Antigens."; Total award for the project component; $992,000.

April 1, 1981 to March 30, 1984; NIH/NCI RO1 CA 30019; "Purification of Tumor Antigens of Defined Specificities"; $231,435; **R.K. Gupta - Principal Investigator.**

July 1, 1981 to June 30, 1982; Cancer Research Coordinating Committee; "Evaluation of Efficacy, Toxicity, and Immune Mechanisms of Extracorporeal Immunoabsorption with Staphylococcus aureus, Cowan I, and Purified Protein A in Producing Tumor Necrosis in Canine Mammary Carcinoma"; $10,000; **R.K. Gupta - Principal Investigator.**

July 1, 1981 to June 30, 1986; NIH PO1 CA 12582; "Surgery, Immunology and Immunotherapy of Human Cancer"; Project II - "Characterization of Circulating Immune Complexes and Urinary Antigen of Cancer Patients"; $354,472; Dr. D.L. Morton - Principal Investigator; **R.K. Gupta - Director of Project II.**

July 1, 1981 to June 30, 1983; VA Medical Service 821-103; "Prognostic Significance of Circulating Immune Complexes in Human Lung Carcinoma"; $357,000; D.L. Morton - Principal Investigator; **R.K. Gupta - Co-Investigator.**

April 1, 1981 to June 30, 1986; NIH P01 CA 29605; "New Approaches to Surgical Oncology"; Project II - "New Approaches to Detection of Subclinical Disease in Stage I and II Melanoma"; $1,023,285; Dr. D.L.Morton - Principal Investigator ; **R.K. Gupta - Project Director.**

July 1, 1983 to Dec 31, 1988; NIH CA 09010; "Institutional Training Grant and Surgical Oncology"; $885,000; D.L. Morton Principal Investigator; **R.K. Gupta - Co-director.**

March 1, 1984 - Feb 28, 1990; NIH R01 CA 30019; "Purification of Tumor Antigens of Defined Specificities"; $538,115; **R.K. Gupta - Principal Investigator.**

April 1, 1987 - Dec 31, 1990; NIH P01 CA 12582; "Surgery, Immunology and Immunotherapy of Human Cancer" D.L. Morton Principal Investigator; Project II - "Characterization of Circulating Immune Complexes and Urinary Tumor Antigens of Cancer Patients";$262,000; **R.K. Gupta - Director of Project II.**

April 1, 1987 - Dec 31, 1992; NIH P01 CA 29605; "New Approaches to Surgical Oncology"; D.L. Morton - Principal Investigator, "New Approaches to Detection of Subclinical Disease in Clinical Stage I and II Melanoma"; $450,000; **R.K. Gupta - Director of Project II.**

July 1, 1989 to June 30, 1994; NIH T32 CA 09010; "Institutional Training Grant in Surgical Oncology"; $1,022,533 total award; $207,804 from 7/1/89 to 6/30/90; D.L. Morton - PI; **R.K. Gupta – Co director.**

November 8, 1988 - Alex Henig and First Network Savings Bank; "Equipment Purchase" grant; $15,000, **R.K. Gupta** - Investigator.

March 1 1989 to June 30, 1990; John Wayne Cancer Clinic Auxiliary; "Assessment of Clinical Significance of a 90kD Glycoprotein Tumor-Associated Antigen"; $250,000; D.L. Morton and **R.K. Gupta** - Investigators.

July 1, 1990 - June 30, 1991; John Wayne Cancer Clinic Auxiliary; "Assessment of Clinical Significance of a 90kD Glycoprotein Tumor-Associated Antigen"; $209,000; D.L. Morton and **R.K. Gupta** - Investigators.

July 1, 1990 to June 30, 1994; State of California, 1RT 77; "Significance of Immune Complexes in Lung Carcinoma"; $360,445; **R.K. Gupta** - Principal Investigator.

July 1, 1992 to Sept 29, 1997; NIH PO1 CA12582; Surgery, Immunology and Immunotherapy of Human Cancer; D. L. Morton - PI; **R.K. Gupta - Co-investigator**; 5% time commitment.

April 1, 1993 to May 31, 1998; NIH PO1 CA29605; New Approaches to Surgical Oncology; D. L. Morton - PI; New Surgical Approaches for the Management of Malignant Melanoma and Other Solid Neoplasms; $633,144 per year; **R.K. Gupta - Co-investigator of Project III.**

Oct 1, 1994 to Sept 30, 1998; U.S. Department of Army, Medical Research Aquisition Activity; Grant # DAMD17-94-J-4459; "A New Immunologic Method for Detection of Occult Breast Cancer"; $800,000; **R.K. Gupta - Principal Investigator.**

July 1, 1995 to June 30, 2000; NIH T32 CA09689-01A2; "Institutional Training Grant in Surgical Oncology"; Total direct cost for 5 yrs $794,945; D.L. Morton - Director, **R.K. Gupta - Co-director.**

Sept 1, 1996 to Aug 31, 2001; NIH PO1 CA12582; Surgery, Immunology and Immunotherapy of Human Cancer; D. L. Morton - PI; **R.K. Gupta - Co-investigator** on Project III - Clinical Investigation of Active Specific Immunotherapy of Malignant Melanoma; 10% time commitment; Direct cost for 5 yrs $885,575 to analyze serum samples.

Sept 1, 1996 to Aug 31, 2001; NIH PO1 CA12582; Surgery, Immunology and Immunotherapy of Human Cancer; D. L. Morton - PI; **R.K. Gupta - Co-leadr** with G. Gammon on Core C - "Serum, Lymphocyte and Tissue Collection and Analysis"; 15% time commitment; Direct cost for 5 yrs - $1,730,824.

June 1, 1998 to May 31, 2003; NIH PO1 CA29605; New Approaches to Surgical Oncology; D. L. Morton - PI; **R.K. Gupta - Co-investigator** on Project III - New Surgical Approaches for the Management of Malignant Melanoma; 15% time commitment; Total direct cost for 5 yrs $1,065,525 to analyze serum samples.

June 1, 1998 to May 31, 2003; NIH PO1 CA29605; New Approaches to Surgical Oncology; D. L. Morton - PI; **R.K. Gupta – Core B Leader** "Research Support"; 15% time effort; Total direct cost for 5 yrs $976,343.

PUBLICATIONS

I. ORIGINAL ARTICLES IN JOURNALS AND MONOGRAMS:

1. **Gupta RK**, Narayan R and Gollokota KG: Differentiation between heat resistance and octyl alcohol resistance of Bacillus cereus. Biochem Biophy Res Comm 38:20- 30, 1970.

2. **Gupta RK** and Pramer D: Amino acid transport by the filamentous fungus Arthorobotrys conoides. J Bacteriol 103:120-130, 1970.

3. **Gupta RK** and Pramer D: Metabolism of valine by filamentous fungus Arthorobotrys conoides. J Bacteriol 103:131-139, 1970.

4. Stone R and **Gupta RK**: Aerobic and anaerobic landfill stabilization process. J Sanit Eng Div, Amer Soc Civil Eng 96:1399-1414, 1970.

5. **Gupta RK** and Narayan R: Octyl alcohol resistance of Bacillus cereus. Factors influencing dipicolinic acid synthesis and sporulation in bacilli. IN: U.P. Agri Univ Res Bull. Gollakota KG (ed.) 2:257-263, 1970.

6. **Gupta RK** and Howard DH: Comparative physiological studies of the yeast and mycelial forms of Histoplasma capsulatum: Uptake and incorporation of L-leucine. J Bacteriol 105:690-700, 1971.

7. Howard DH, Otto V and **Gupta RK**: Lymphocyte mediated cellular immunity in histoplasmosis. J Infec Immunity 4:605-610, 1971.

8. Howard DH and **Gupta RK**: Lysis of zoopathogenic fungi by streptomycetes. Can J Microbiol 17:521-523, 1971.

9. Irie K, **Gupta RK** and Morton DL: Preparation of single cell suspension from surgical specimens of human tumors. Jap Assoc Immunol 4:903-906, 1974.

10. **Gupta RK**, Irie K and Morton DL: Ultra-micro complement fixation technique. Jap Assoc Immunol 4:939-950, 1974.

11. **Gupta RK** and Morton DL: Suggestive evidence for in vivo binding of specific anti-tumor antibodies of human melanomas. Cancer Res 35:58-62, 1975.

12. **Gupta RK** and Morton DL: Presence of human tumor-associated antigens in urine of cancer patients. Surg Forum 26:158-160, 1975.

13. Morton DL, Golub SH, Sulit HL, **Gupta RK**, Eilber FR, Holmes EC and Sparks FC: Immunologic and clinical responses to active immunotherapy of a malignant melanoma. IN: Fundamental Aspects of Neoplasia. Gotteleib, Plescia and Bishop (eds.) Springer-Verlag Inc., New York, pp. 181-201, 1975.

14. deKernion JB, Golub SH, **Gupta RK**, Silverstein MJ and Morton DL: Successful transurethral intralesional BCG therapy of a bladder melanoma. Cancer 36:1662- 1667, 1975.

15. Roth JA, Silverstein MJ, **Gupta RK** and Morton DL: Restoration of Immunocompetence by lymphocyte transfusion. J Surg Oncol 7:63-66, 1975.

16. Sulit HL, Golub SH, Irie RF, **Gupta RK**, Grooms GA and Morton DL: Fetal calf serum-grown human tumor cells: Influences on the tests for lymphocytotoxicity, serum blocking and arming effect. Int J Cancer 17:461-468, 1976.

17. Grimm EA, Silver HKB, Roth JA, Chee DO, **Gupta RK** and Morton DL: Detection of tumor-associated antigen in human melanoma cell line supernatants. Int J Cancer 17:559-564, 1976.

18. **Gupta RK**, Irie RF, Morton DL: Antigens on human tumor cells assayed by complement fixation with allogeneic sera. Cancer Res 38:2573-2580, 1978.

19. **Gupta RK**, Golub SH, Rangel DM and Morton DL: Relationship between anticomplementary activity and inhibition of PHA-induced lymphocyte blastogenesis by melanoma sera. Proc ASM 2nd Annual Meeting of Diagnostic Immunology, 1978.

20. Tang SL, Howard DH and **Gupta RK**: Uptake of metabolites by germinating blastospores of Histoplasma capsulatum. Sabouraudia 16:271-278, 1978.

21. **Gupta RK**, Golub SH, Rangel DM and Morton DL: Inhibition of mitogen induced lymphocyte proliferation correlated to anticomplementary activity in sera from melanoma patients. Cancer Immunol Immunother 5:221-228, 1979.

22. **Gupta RK**, Golub SH and Morton DL: Correlation between tumor burden and anti-complementary activity in sera from cancer patients. Cancer Immunol Immunother 6:63-71, 1979.

23. **Gupta RK** and Morton DL: Detection of cancer associated antigen(s) in urine of sarcoma patients. J Surg Oncol 11:65-74, 1979.

24. deKernion JB, Ramming KP and **Gupta RK**: The detection and clinical significance of antibodies to tumor associated antigens in patients with renal cell carcinoma. J Urology 122:300-305, 1979.

25. **Gupta RK**, Irie RF, Chee DO, Kern DH and Morton DL: Demonstration of two distinct antigens in spent tissue culture medium of a human malignant melanoma cell line. J Natl Cancer Inst 63:347-356, 1979.

26. **Gupta RK**, Silver HKB, Reisfeld RA and Morton DL: Isolation and immunochemical characterization of antibodies from cancer patients sera reactive against human melanoma cell membranes by affinity chromatography. Cancer Res 39:1683-1695, 1979.

27. **Gupta RK** and Morton DL: Double-antibody method and the Protein-A bearing Staphylococcus aureus cells method compared for separating bound and free antigen in radioimmunoassay. Clin Chem 25:752-756, 1979.

28. **Gupta RK**, Theofilopolous AN, Dixon FJ and Morton DL: Circulating immune complexes as possible cause for anticomplementary activity in humans with malignant melanoma. Cancer Immunol Immunother 6:211-221, 1979.

29. Roth JA, Chee DO, **Gupta RK** and Morton DL: Lymphocyte stimulation to tumor associated antigen. II.

Comparison of extracts from fresh human tumors with tissue culture cell lines. Cancer Immunol Immunother 7:25-29, 1979.

30. Huth JF, **Gupta RK** and Morton DL: Sequential analysis of urinary antigen(s) in patients with sarcoma. Surg Forum 30:150-152, 1979.

31. **Gupta RK**, Silver HKB and Morton DL: Production and Chracterization of xenogeneic antisera to tumor associated antigens. J Surg Oncol 13:75-89, 1980.

32. Chee DO, **Gupta RK** and Morton DL: Presence of a carcinoma associated antigen in the spent chemically defined medium of a human colon carcinoma cell line. J Surg Oncol 13:45-51, 1980.

33. **Gupta RK** and Morton DL: Radioimmunoassay for the analysis of tumor associated antigens with allogeneic antibody. IN: Serologic Analysis of Human Tumor Antigens. S.A. Rosenberg (ed.) Academic Press, pp. 645-650, 1980.

34. Rote NS, **Gupta RK** and Morton DL: Tumor associated antigen detected by autologous sera in urine of patients with solid neoplasms. J Surg Res 29:18-22, 1980.

35. Chee DO and **Gupta RK**: Chemical carcinogenesis and tumor immunology. IN: Genetic Differences and Chemical Carcinogenesis. CRC Monogram, pp. 151-184, 1980.

36. Rote NS, **Gupta RK** and Morton DL: Determination of incidence and partial characterization of tumor-associated antigens found in the urine of patients bearing solid tumors. Int J Cancer 26:203-210, 1980.

37. Higuchi M, **Gupta RK**, Irie RF and Morton DL: Natural humoral immunity in patients with malignant disease. J Clin Lab Immunol 4:141-143, 1980.

38. Renk CM, **Gupta RK** and Morton DL: Immunosuppressive factors from human breast carcinoma cell lines that affect inhibition of lymphocyte proliferation. Cancer Immunol Immunother 9:55-62, 1980.

39. **Gupta RK**: Antigenic complexity in human malignant tumors detected by allogeneic antibody. IN: Serologic Analysis of Human Tumor Antigens. S.A. Rosenberg (ed.) Academic Press, pp. 339-380, 1980.

40. **Gupta RK** and Morton DL: Detection of tumor antigens by complement fixation using allogeneic antibody. IN: Serologic Analysis of Human Tumor Antigens. S.A. Rosenberg (ed.) Academic Press, pp. 611-619, 1980.

41. **Gupta RK** and Morton DL: Possible clinical significance of circulating immune complexes in melanoma patients. IN: Fundamental Mechanisms in Human Cancer Immunology. p. 305-320, 1981.

42. **Gupta RK** and Morton DL: Clinical significance and nature of circulating immune complexes in melanoma patients. IN: Contemporary Topics in Immunobiology. F.A. Salinas (ed.) Plenum Press, New York, 15:1-53, 1984.

43. Renk CM, **Gupta RK** and Morton DL: Inhibition of normal allogeneic lymphocyte mitogenesis by a factor from human tumor cells in culture. Cancer Immunol Immunother 11:7-16, 1981.

44. Fox JN, Sheikh KM, **Gupta RK**, Rea TH, Morton DL and Levan NE: Melanoma associated antigens in benign melanocytic disorders. Dermatol 20:368-373, 1981.

45. Huth JF, **Gupta RK** and Morton DL: Development of an enzyme immunoassay to detect and quantitiate tumor-associated antigens in the urine of sarcoma patients. Cancer 47:2856-2861, 1981.

46. Huth JF, **Gupta RK** and Morton DL: Purification of antigen(s) from urine of a sarcoma patient by affinity chromatography. J Surg Oncol 18:237-247, 1981.

47. Huth JF, **Gupta RK** and Morton DL: Assessment of the in vivo effectiveness of tumoricidal chemotherapy and radiation therapy by serial analysis of tumor-associated urinary antigen titers in sarcoma patients. Cancer Treat Rep 65:1037-1042, 1981.

48. Huth JF, **Gupta RK** and Morton DL: The relationship of tumor-associated urinary antigens to disease recurrence in melanoma patients. Surg Forum, 32:417-419, 1981.

49. Huth JF, **Gupta RK** and Morton DL: Relationship between circulating immune complexes and urinary antigens in human malignant malignancy. Cancer 48:1150-1157, 1982.

50. Roth JA, Grimm EA, **Gupta RK** and Ames RS: Immunoregulatory factors derived from human tumors. I. Immunologic and biochemical characterization of factors that suppress lymphocyte proliferative and cytotoxic responses in vitro. J Immunol 128:1955-1962, 1982.

51. Bentwich Z, Fahey J, **Gupta RK**, Golub S, Chia D and Barnett E: Comparison of assays for circulating immune complexes in human diseases. IN: Progress in Rheumatology. I. Machtey (ed.) John Wright-PSG Inc, Boston, pp. 23-31, 1982.

52. **Gupta RK** and Morton DL: Clinical significance of tumor-associated antigens and antitumor antibodies in human malignant melanoma. IN: Melanoma Antigens and Antibodies. R.A. Reisfeld (ed.) Plenum, New York, pp. 139-172, 1982.

53. **Gupta RK** and Howard DH: Metabolite transport. IN: Fungi Pathogenic for Humans and Animals, Vol. 1. D.H. Howard (ed.) Marcel Dekker Inc. New York, pp. 547- 591, 1982.

54. Finck S, **Gupta RK** and Morton DL: Excretion of tumor-associated antigen(s) in the urine of patients with colon carcinoma. J Surg Oncol 21:84-89, 1982.

55. Renk CM, **Gupta RK**, Irie RF, Morton DL. Comparison of the techniques used for monitoring humoral immunity in cancer patients. J Surg Oncol 19:155-161, 1982.

56. **Gupta RK**, Huth JF, Golub SH: Application of cultured human myeloid cells (K562) for detection of immune complexes in human sera. Immunol Commun 11:401-419,1982.

57. Ahn SS, **Gupta RK**, Morton DL: Predictive value of circulating immune complexes for tumor recurrence in stage I malignant melanoma. Surg Forum 33:415-417, 1982.

58. Leitch AM, **Gupta RK**, Morton DL: Immunoabsorption with protein A-bearing Staphylococcus aureus for treatment of metastatic cancer. Surg Forum 33:412-414, 1982.

59. Huth JF, **Gupta RK**, Eilber FR, Morton DL: Predictive value of urinary antigen titers for disease recurrence in sarcoma patients. Surg Forum 33:417-419, 1982.

60. **Gupta RK** and Morton DL: Immunochemical characterization of fetal antigen isolated from spent medium of a human melanoma cell line. J Natl Cancer Inst 70:993-1004, 1983.

61. **Gupta RK**, Leitch AM and Morton DL: Detection of tumor-associated antigen in eluates from protein A columns used for ex vivo immunoadsoption of plasma from melanoma patients by radioimmunoassay. Clin Exp Immunol 53:589-599, 1983.

62. Chee DO, **Gupta RK** and Morton DL: Humoral immune responses of melanoma patients to two different tumor-associated antigens. J Surg Oncol 23:288-235, 1983.

63. **Gupta RK**, Leitch AM and Morton DL: Nature of antigens and antibodies in immune complexes isolated by staphylococcal protein A from plasma of melanoma patients. Cancer Immunol Immunother 6:40-47, 1983.

64. **Gupta RK**, Huth JF, Korn EL and Morton DL: Prognostic significance of urinary antigen analysis by enzyme-linked immunosorbent assay in melanoma patients. Diagnostic Immunol 1:303-309, 1983.

65. **Gupta RK**, Morton DL: Tumor Antigens. IN: Immunobiology of Cancer, Transplantation and Pregnancy, PK Ray (ed), Pergamon Press, New York, pp. 113-147, 1983.

66. **Gupta RK** and Morton DL: Studies of a melanoma tumor-associated antigen detected in spent culture medium of a human melanoma cell line by allogeneic antibody. I. Purification and development of a radioimmunoassay. J Natl Cancer Inst 72:67-74, 1984.

67. **Gupta RK** and Morton DL: Studies of a melanoma tumor-associated antigen detected in spent culture medium of a human melanoma cell line by allogeneic antibody. II. Immunological characterization. J Natl Cancer Inst 72:75-82, 1984.

68. **Gupta RK** and Morton DL: Studies of a melanoma tumor-associated antigen detected in spent culture medium of a human melanoma cell line by allogeneic antibody. III. Physicochemical properties. J Natl Cancer Inst 72:83-92, 1984.

69. Huth JF, **Gupta RK**, Eilber FR, Morton DL: A prospective postoperative evaluation of urinary tumor associated antigen in sarcoma patients: Correlation with disease recurrence. Cancer 53:1307-1310, 1984.

70. Morton DL, **Gupta RK**, Huth JF: New horizons in surgical oncology: Malignant melanoma. Basic Mechanisms and Clinical Treatment of Tumor Metastasis. Academic Press, New York, pp 567-571, 1985.

71. Cheng WC, **Gupta RK**, Morton DL: Characterization of antigenic components of immune complexes isolated from a melanoma patient. Immunol Invest 14:367-378, 1985.

72. Wong JH, **Gupta RK**, Morton DL: Serial determinations of tumor-associated antigen and antibody complexes in stage I melanoma patients. Arch Surg 121:1342-1345, 1986.

73. Hoon DBS, Wen D-R, Stene M, **Gupta RK**, Cochran AJ: Leukocyte migration inhibition by interleukin-2. Clin Exp Immunol. 66:566-573, 1986.

74. Hetzitheofilou C, Campbell MA, **Gupta RK**, Kern DH, Morton DL: A radioimmunometric assay for detection and characterization of lung cancer associated antibodies in sera of lung cancer patients. J Surg Res. 42:85-91, 1987.

75. Chee DO, **Gupta RK**, Gosselin EJ, Witman GB: Detection of tumor-associated antigen in cancer patients' sera by F-11 monoclonal antibody. Protids of Protein Chem, 1987.

76. **Gupta RK**: Circulating immune complexes in malignant melanoma. Disease Markers 6:81-96, 1988.

77. Wong JH, **Gupta RK**, Morton DL: Recovery of a cell surface fetal antigen and antibody in Stage I melanoma patients. Cancer Immunol Immunother 27142-146, 1988.

78. Wong JH, **Gupta RK**, Morton DL: Demonstration of a well characterized tumor associated antigen on melanoma cell surface. J Surg Oncol 38:147-150, 1988.

79. **Gupta RK**: Detection and characterization of melanoma tumor associated antigens. Medicina 48:695-708, 1988.

80. Euhus DM, **Gupta RK**, Morton DL: Induction of antibodies to a tumor-associated antigen by immunization with a whole melanoma cell vaccine. Cancer Immunol Immunother 29:247-254, 1989.

81. Euhus DM, **Gupta RK**, Morton DL: Detection of a tumor associated glycoprotein antigen in serum and urine of melanoma patients by murine monoclonal antibody (AD1-40F4) in enzyme immunoassay. J Clin Lab Anal 3:184-190, 1989.

82. Morton, DL, Foshag LJ, Nizze JA, **Gupta RK**, Famatiga E, Hoon DBS, Irie RF: Active specific immunotherapy in malignant melanoma. Sem Surg Oncol 5:420-425, 1989.

83. Wong JH, Xu SH, Saxton RE, **Gupta RK**, Morton DL: Detection and isolation of antigen-specific immune complexes from sera of melanoma patients. Int J Cancer 45:445-449, 1990.

84. Wong JH, Xu SH, **Gupta RK**, Morton DL: Tumor associated antigen immune complexes. Arch Surg 125:187-191, 1990.

85. Euhus DM, **Gupta RK**, Morton DL: Characterization of a 90-100kDa tumor associated antigen in sera of melanoma patients. Int J Cancer 45:1065-1070, 1990.

86. Wong JH, Xu SH, Saxton RE, **Gupta RK**, Morton DL: Immunochemical characterization of a tumor-associated antigen defined by a monoclonal antibody. J Surg Res 48:539-546, 1990.

87. Euhus DM, **Gupta RK**, Morton DL: Association between allo-immunoreactive and xeno-immunoreactive subunits of a glycoprotein tumor associated antigen. Cancer Immunol Immunother 32:214-220, 1990.

88. Lindemann RA, Singh KP, Shau H, **Gupta RK**. The effects of staphylococcal protein A on human lymphokine-activated killer cell induction. Cancer Immunol Immunother 33:97-102, 1991.

89. Singh KP, Shau H, **Gupta RK**, Kopald K, Ray PK. Protein A potentiates lymphokine-activated killer cell induction in normal and melanoma patient lymphocytes. Immunopharmacol Immunotoxicol 14:73-103,

1992.

90. Hunt KK, Shibata M, **Gupta RK**, Morton DL. Complement-dependent lysis of tumor cells by a baboon IgM antibody to a tumor-associated antigen. Cancer Immunol Immunother 34:377-82, 1992.

91. Morton DL, Foshag LL, Hoon DBS, Nizze JA, Wanek L, Chang C, Davtyan DG, **Gupta RK**, Elashoff R, Irie RF: Prolongation of survival in metastatic melanoma after active specific immunotherapy with a new polyvalent melanoma vaccine. Annal Surg 216:463-482, 1992.

92. Morton DL, Hoon DBS, **Gupta RK**, Nizze A, Foshag LJ, Irie RF: Active specific immunotherapy of malignant melanoma. In *Cutaneous Melanoma,* Second Edition, Balch CM, Houghton AN, Milton GW, Sober AJ and Seng-jaw, eds. JB Lippincott Co., Philadelphia, pp 544-546, 1992.

93. **Gupta RK**, Morton DL: Monoclonal antibody based ELISA to detect glycoprotein tumor-associated antigen specific immune complexes in cancer patients. J Clin Lab Analysis 6:329-336, 1992.

94. Shau H, **Gupta RK**, Golub SH: Induction of a natural killer enhancing factor (NKEF) from human erythroid cells. Cell Immunol 147:1-11, 1993.

95. Morton DL, Hoon DBS, Nizze A, Foshag LJ, Famatiga E, Wanek LA, Chang C, Irie RF, **Gupta RK**, Elashoff R: Polyvalent melanoma vaccine improves survival of patients with metastatic melanoma. In Bystryn J-C, Ferrone S, Livingston P (eds.): *Specific Immunotherapy of Cancer with Vaccine.* Ann NY Acad Sci 690:120-135, 1993.

96. Morton DL, Nizze JA, Hoon DBS, Foshag LJ, Famatiga E., **Gupta RK**, Ramming KP: Improved survival of metastatic melanoma patients receiving active specific immunotherapy with a polyvalent whole-cell vaccine. In *Adjuvant Therapy for Cancer VII,* Slamon SE (ed.) JB Lippincott Co. Philadelphia, pp398-404, 1993.

97. Singh KP, **Gupta RK**, Shau H, Ray PK: Effect of ASTA-Z (immaphosphamide) on human lymphokine activated killer cell induction. Immunopharmacol Immunotoxicol 15:525-538, 1993.

98. **Gupta RK**, Morton DL: Prognostic value of a 90kD subunit containing glycoprotein tumor-associated antigen specific immune complexes in lung cancer patients. Disease Markers 12:51-61, 1994.

99. **Gupta RK**, Morton DL: Glycoprotein tumor-associated antigen specific immune complexes in sera of breast cancer patients. In *XVI International Cancer Congress*, Monduzzi Editore, Bologna, Italy, pp 1333-1337, 1994.

100. **Gupta RK**, Morton DL: Detection and comparision of a 90kD glycoprotein tumor associated antigen specific immune complexes with CEA and CA15-3 in breast cancer. Int J Oncol 7:741-747, 1995.

101. Jones RC, **Gupta RK**, Morton DL: Antitumor vaccines. Year Book of Science & Technology. New York: McGraw-Hill, pp 354-357,1995.

102. Jones RC, Kelley M, **Gupta RK**, Nizze JA, Yee R, Leopoldo Z, Qi K, Stern S, Morton DL: Immune response to polyvalent melanoma cell vaccine in AJCC stage III melanoma: An immunologic survival model. Ann Surg Oncol 3:437-445, 1996.

103. Hsueh EC, **Gupta RK**, Qi K, Yee R, Leopoldo ZC, Morton DL: Serum assay predicts survival response to adjuvant vaccine therapy for resected stage IV melanoma. Cancer J Sci Am. 3:364-370, 1997.

104. Kelley MC, Jones RC, **Gupta RK**, Yee R, Stern S, Wanek L, Morton DL: Tumor-associated antigen TA-90 immune complex assay predicts subclinical metastases and survival in early-stage melanoma. Cancer 83:1355-1361, 1998.

105. **Gupta RK**, Yee R, Leopoldo ZC, Morton DL: Detection of occult breast cancer by a novel immunologic approach. Era of Hope - Department of Defence. Vol. I, pp 181-182, 1997.

106. Hsueh EC, **Gupta RK**, Glass EC, Yee R, Qi K, Morton DL: Positron emission tomography plus serum immune complex assay for detection of occult metastatic melanoma. J Am Coll Surg *187*:191-198, 1998.

107. Hsueh EC, **Gupta RK**, Qi K, Morton DL: Correlation of specific immune response with survival in patients receiving polyvalent melanoma cell vaccine. J Clin Oncol. 16:2913-2920, 1998.

108. Hsueh EC, Famatiga E, **Gupta RK**, Qi K, Morton DL: Enhancement of complement-dependent cytotoxicity by polyvalent melanoma cell vaccine (CancerVax): Correlation with survival. Ann Surg Oncol. 5:595-602, 1998.

109. Morton DL, Ollila DW, Hsueh EC, Essner R, **Gupta RK**: Cytoreductive surgery and adjuvant immunotherapy: A new management paradigm for metastatic melanoma. CA-Cancer J Clin, *49*:101-116, 1998.

110. **Gupta RK**, Morton DL: Association between a 90kD glycoprotein tumor-associated antigen specific immune complexes and breast cancer. (*Submitted*).

111. Zavotsky J, **Gupta RK**, Brennan MB, Yee R, Giuliano AE, Morton DL: Prospective evaluation of TA90 immune complexe assay for preoperative diagnosis of benign and malignant breast lesions. (*In preparation)*

112. Habal N, **Gupta RK**, Zavotsky J, Yee R, Stern S, Brennan M, Hansen N, Giuliano AE, Morton DL: Accuracy of TA-90-IC compred with mammography for the detection of breast cancer. (*In preparation*)

113. Habal N, **Gupta RK**, Bilchik A, Qi K, Morton DL: TA-90-IC, a new marker for colon cancer. (*In preparation*)

114. Hsueh EC, **Gupta RK**, Yee R, Leopoldo ZC, Qi K, Morton DL: Does endogenous immune response determine the outcome of surgical therapy for metastatic melanoma. Ann Surg Oncol (*Submitted*)

II. INVITED ARTICLES/SPEAKER:

1. **Gupta RK**, Silver HKB, Reisfeld RA, Morton DL: Isolation and characterization of antitumor antibodies by affinity chromatography. International Medical News Group, 1978.

2. **Gupta RK**, Morton DL: Urinary excretion of tumor antigens in sarcoma patients. Urology Observer, 1979.

3. **Gupta RK**, Silver HKB, Reisfeld RA, Morton DL: Isolation and immunochemical characterization of antibodies from the sera of cancer patients which are reactive against human melanoma cell membranes by

affinity chromatography. Year Book of Cancer, pp. 283-287, 1980.

4. Finck SJ, **Gupta RK**, Giuliano AE, Morton DL: Excretion of tumor-assoicated antigen(s) in the urine of patients with colon carcinoma. Current Surgery 40:305-306, 1983.

5. **Gupta RK**, Giuliano AE, Kelley MC, Yee R, Leopoldo Z, Morton DL: Clinical significance of a 90kD glycoprotein tumor-associated antigen specific immune complexes in sera of breast cancer patients. Invited speaker at the International Academy of Tumor Marker Oncology, Singapore, June - 1996.

6. **Gupta RK**, Morton DL: Presence of a 90kD glycoprotein tumor-associated antigen specific immune complexes in circulation of melanoma patients signify poor prognosis. Invited speaker at the International Academy of Tumor Marker Oncology, Jeruselam, Israel, June - 1997.

7. **Gupta RK**, Giuliano AE, Yee R, Morton DL: Detection of breast cancer by a novel immunologic technique using the TA90 specific immune complexes. Satellite Symposium on Tumor Immunology, Chittaranjan National Cancer Institute, Calcutta, India, Invited guest speaker. October 28-30, 1998 (Abstract *Submitted*).

8. **Gupta RK**: A novel approach to detect immunogenic tumor-associated antigen in sera of cancer patients. K.G. Medical College, Lucknow, India, October 20, 1998. (*Invited lecturer*).

9. **Gupta RK**, Hsueh EC, Qi K, Morton DL: Immune response to 90kD glycoprotein tumor antigen in patients receiving melanoma cell vaccine. Invited guest speaker at the 10th International Immunology Congress - 1998, New Delhi, India, October 31 - November 7, 1998.

10. **Gupta RK**: Giuliano AE, Yee R, Morton DL: A new immunologic approach involving antigen (TA90) specific immune complexes to detect occult breast cancer. Invited guest speaker at the Mini Symposium on Mechanisms of Tumor Invasion, Metastasis and Host-tumor interactions at the Cancer Research Institute, Tata Memorial Centre, Mumbai, India, November 9-11, 1998.

11. **Gupta RK**, Yee R, Morton DL: Glycoprotein tumor-associated antigen (TA-90) specific immune complexes in ovarian cancer. Invites speaker at the International Academy of Tumor Markers Oncology, Lugano, Switzerland, June 17, 1998.

12. **Gupta RK**, Hsueh EC, Qi K, Yee R, Morton DL: TA-90 a tumor marker for monitoring immune response to immunotherapy in melanoma patients. Invited speaker at the 16th international Conference on Human Tumor Markers, Budapest, Huhngary, June 13-16, 1999.

ABSTRACTS:

1. **Gupta RK** and Pramer D: Amino acid transport in filamentous fungi. Bacteriol Proc 68:124, 1968.

2. **Gupta RK** and Pramer D: Transport and metabolism of valine in Arthorobotrys conoides. Theobold Smith Society, New Jrsey, 1968.

3. Howard DH, Otto V and **Gupta RK**: Cellular immunity to histoplasmosis. Bacteriol Proc 71:117, 1971.

4. Howard DH and **Gupta RK**: The intercellular fate of Histoplasma capsulatum. Bacteriol Proc 72:133, 1972.

5. **Gupta RK**: Elution of antibodies from human malignant melanomas. Proc Amer Assoc Cancer Res 15:87, 1974.

6. **Gupta RK**: Common cancer-associated antigen(s) in human neoplasms. Proc Amer Soc Clin Oncol 11:262, 1975.

7. **Gupta RK**, Yee R, Irie RF and Morton DL: Immunologic differentiation between human tumor-associated antigen(s) and heterologous antigens. Proc Amer Assoc Cancer Res 16:146, 1975.

8. **Gupta RK** and Morton DL: Tumor associated antigens in urine of cancer patients. Proc Amer Assoc Cancer Res 17:92, 1976.

9. Naness J, Sheikh K, **Gupta RK**, Rea T, Frion G, Morton DL and Levan N: Antibodies to melanoma associated antigens in benign melanocytic disorders. Proc Clin Res, p. 266A, 1976.

10. **Gupta RK** and Morton DL: Distribution of antibodies to common tumor associated antigens in cancer and non-cancer sera. Proc Amer Assoc Cancer Res 18:184, 1977.

11. Rangel DM, **Gupta RK**, Golub SH and Morton DL: Demonstration of tumor associated antigenic activity in immunosuppressive melanoma sera. Proc Amer Assoc Cancer Res 18:194, 1977.

12. Ramming KP, **Gupta RK** and deKernion JB: Detection of antibodies to tumor associated antigens in patients with hypernephroma. Proc Amer Soc Clin Oncol 18:313, 1977.

13. **Gupta RK**, Silver HKB, Reisfeld RA and Morton DL: Isolation and characterization of anti-tumor antibodies from cancer patients sera by affinity chromatography. Proc Amer Soc Clin Oncol 19:360, 1978.

14. **Gupta RK**, Silver HKB, Morton DL and Penderleith IG: Reactivity of human tumor extracts to xenogeneic antisera raised against human melanoma extract. Proc Amer Soc Clin Oncol 19:360, 1978.

15. **Gupta RK** and Morton DL: Tumor related immune complexes in sera from melanoma patients. Fed Proc 37:595, 1978.

16. Kern DH, **Gupta RK**, Hencock WK and Holmes EC: An inhibition of binding assay for the detection of soluble melanoma associated antigen. Proc Amer Assoc Cancer Res 19:134, 1978.

17. Rote NS, **Gupta RK** and Morton DL: Detection of tumor-associated antigen in the urine of sarcoma patients. Proc Amer Assoc Cancer Res 19:134, 1978.

18. Chee DO, **Gupta RK** and Morton DL: Carcinoma associated antigen(s) in spent medium of HT-29 colon carcinoma grown in serum free medium. Proc Amer Assoc Cancer Res 19:135, 1978.

19. **Gupta RK** and Morton DL: Protein A bearing Stapylococcus aureus (Cowan I) as imunosorbant in a radioimmunoassay. Clinical Radio Assay Society, Fourth Annual Meeting, 1978.

20. **Gupta RK**, Irie, Chee DO, Kern DH and Morton DL: Antigens in spent serum-free medium of a human melanoma cell line. Bacteriol Proc 78:54, 1978.

21. Chee DO, **Gupta RK** and Morton DL: Humoral response to BCG and allogeneic melanoma cells in stage II postoperative melanoma patients. Tissue Culture Assoc, p. 370, 1978.

22. **Gupta RK**, Morton DL and Golub SH: Detection of immune complex-like material in human sera by binding to cultured myeloid (K562) cells. Fed Proc 38:1358, 1979.

23. Ramming KP, **Gupta RK**, deKernion JB: Induction of antibody to human renal cell cancer in vivo by injections of xenogeneic immune RNA. Proc Amer Assoc Cancer Res 20:215, 1979.

24. **Gupta RK** and Morton DL: Quantitation of allogeneic antibody to tumor-associated antigen by radioimmunoassay, Proc Amer Assoc Cancer Res 20:216, 1979.

25. Huth JF, **Gupta RK** and Morton DL: Urinary antigen titers in sarcoma patients. Proc Amer Assoc Cancer Res 20:217, 1979.

26. **Gupta RK**, Golub SH, Hammond WG and Morton DL: Correlation between incidence of serum anticomplementary activity and tumor burden. Proc Amer Soc Clin Oncol 20:331, 1979.

27. **Gupta RK** and Morton DL: Specificity by radioimmunoassay of tumor-associated antigen isolataed from spent culture medium of a melanoma cell line. Fed Proc 39:1144, 1980.

28. Huth JF, **Gupta RK** and Morton DL: Competitive inhibition enzyme immunoassay for detection of melanoma-associated urinary antigen. Proc Amer Soc Clin Oncol 21:370, 1980.

29. Goodnight JE Jr, Irie RF, Saxton RE, **Gupta RK**, Ramming KP and Morton DL: Humoral immune response to bone and soft tissue sarcomas. Proc Amer Soc Clin Oncol 21:370, 1980.

30. **Gupta RK**, Huth JF, Golub SH and Morton DL: Quantitation of immune complexes in cancer sera. Proc Amer Assoc Cancer Res 21:222, 1980.

31. Roth JA, Grimm EA, **Gupta RK**, Morton DL and Eilber FR: Purification of an immunoregulatory factor in extracts of lung carcinoma. Proc Amer Assoc Cancer Res 21:222, 1980.

32. Hatzitheofilou C, Kern DH, **Gupta RK**, Morton DL and Storm FK: Definition of antigens associated with human lung carcinoma. Proc Amer Assoc Cancer Res 21:223, 1980.

33. Roth JA, Grimm EA, **Gupta RK** and Morton DL: Characterization and purification of an immunosuppressive factor in extracts of lung carcinoma. Fourth International Congress of Immunology, Paris, 1980.

34. Roth JA, Grimm EA, **Gupta RK**, Sugerbaker PH and Ames R: Supression of lymphocyte proliferative and cytotoxic responses by human tumor-associated immunoregulatory factors. 14th International Leukocyte Culture Conference, Heidelberg, 1981.

35. **Gupta RK**, Cheng LY, Leitch AM and Morton DL: Characterization of tumor-associated antigen isolated from spent culture medium of a human melanoma cell line. Fed Proc 40:981, 1981.

36. Roth JA, Grimm EA, **Gupta RK**, Ames R, Trehan E: Presence of immunoregulatory factors in extracts

from several histologically distinct human malignancies. Fed Proc 40:1089, 1980.

37. Finck S, **Gupta RK** and Morton DL: Serum LDH in stage II melanoma patients. Proc Amer Soc Clin Oncol 22:344, 1981.

38. Cheng LY, **Gupta RK**, Huth JF and Morton DL: Characterization of urinary antigen detected in melanoma patients by autologous and allogeneic antibody. Proc Amer Assoc Cancer Res 22:292, 1981.

39. Leitch AM, **Gupta RK**, Eilber FR and Morton DL: Correlation of the presence of melanoma-associated antigen in urine and serum by competitive enzyme immunoassay. Proc Amer Assoc Cancer Res 22:296, 1981.

40. Finck S, **Gupta RK**, Storm FK and Morton DL: Tumor-associated antigens in the urine of patients with colon carcinoma: A preliminary study. Proc Amer Assoc Cancer Res 22:299, 1981.

41. **Gupta RK**, Huth JF and Morton DL: Characterization of antigen component of immune complexes isolated from sera of melanoma patients. Proc Amer Assoc Cancer Res 22:293, 1981.

42. **Gupta RK** and Morton DL: Detection and quantitation of oncofetal antigen and its chemical characterization. Proc Oncodevel Biol Med 1:47, 1981.

43. Finck S, Giuliano AE, **Gupta RK** and Morton DL: Tumor-associated antigens in the urine of carcinoma patients. Proc Amer Assoc Cancer Res 23:272, 1982.

44. Leitch AM, **Gupta RK** and Morton DL: Isolation of tumor-associated antigen (TAA) from serum of melanoma patients. Proc Amer Soc Clin Oncol 1:42, 1982.

45. **Gupta RK** and Morton DL: Use of ^{125}I-labelled tumor-associated antigen to characterize immune complexes circulating in melanoma patients. 13th International Cancer Congress, pp. 309, 1982.

46. **Gupta RK** and Morton DL: Nature of circulating immune complexes in melanoma patients. Fed Proc 41:323, 1982.

47. **Gupta RK**, Leitch AM, Morton DL: Characterization immune complexes isolated by staphylococcal protein A from melanoma patients. Fed Proc 42:403, 1983.

48. **Gupta RK**, Leitch AM, Morton DL: Tumor-associated antigen in eluates of protein A columns used for ex vivo immunoadsoption of melanoma patients. Proc Amer Assoc Cancer Res 24:201, 1983.

49. **Gupta RK**, Huth JF, Korn EL, Morton DL: Urinary antigen analysis by enzyme-linked immunosorbent assay: Prognostic significance for melanoma patients. Proc Amer Soc Clin Oncol 2:49, 1983.

50. **Gupta RK**, Morton DL: Physicochemical characterzation of a melanoma tumor-associated antigen. International Immunology Congress, Kyoto, Japan, 1983.

51. Cheng WC, **Gupta RK**, Morton DL: Isolation of tumor-associated antigens from circulating immune complexes of melanoma serum. Proc Amer Assoc Cancer Res 25:242, 1984.

52. **Gupta RK**, Huth JF, Morton DL: Immunologic similarity between tumor-associated antigens detected in

urine of melanoma patients and those expressed by melanoma cells. Proc Amer Soc Clin Oncol 3:9, 1984.

53. **Gupta RK**, Morton DL: Dynamics of tumor-associated antigen, antibody, and immune complexes circulating in melanoma patients. Proc Amer Assoc Cancer Res 26:284, 1985.

54. **Gupta RK**, Morton DL: Detection of tumor-associated antigen, antibody and immune complexes in sera from melanoma patients. Proc Amer Soc Clin Oncol 4:16, 1985.

55. Neslund GG, **Gupta RK**: Partial purification and characterization of melanoma-associated urinary antigen. Fed Proc 4:795, 1985.

56. **Gupta RK**, Morton DL: Immunochemical characterization of tumor-associated antigens excreted into urine of melanoma patients. Proc Am Assoc Cancer Res 27:354, 1986.

57. **Gupta RK**, Euhus DM, Morton DL: Detection of tumor-associated antigen in urine of melanoma patients by allogeneic antibody. Fed Proc. 48:1059, 1987.

58. **Gupta RK**, Morton DL: In vitro production of antibodies to a melanoma tumor-associated antigen by Epstein Barr virus transformed human lymphoblastoid cells. Proc Am Assoc Cancer Res. 28:349, 1987.

59. Wong JH, **Gupta RK**, Storm FK, Morton DL: Demonstration of a cell surface fetal antigen in circulating immune complexes of melanoma patients. Proc Am Assoc Cancer Res. 28:357, 1987.

60. Morton DL, Hoon DBS, Niku DS, **Gupta RK**: Suppressor T-cell activity against human melanoma tumor-associated antigen (M-TAA) antibody production. Proc Am Assoc Cancer Res. 28:374, 1987.

61. **Gupta RK**, Wong JH, Morton DL: Increase in antibody level to a tumor-associated antigen in melanoma patients undergoing immunotherapy with a tumor cell vaccine. Proc Am Soc Clin Oncol. 6:249, 1987.

62. Wong JH, **Gupta RK**, Eilber FR, Morton DL: Demonstration of the presence of a melanoma tumor-associated antigen on the cell surface. Proc Am Soc Clin Oncol. 6:242, 1987.

63. **Gupta RK**, Wong JH, Morton DL: Clinical relevance of a tumor-associated antigen in malignant melanoma. J Steroid Chem. 28:1255, 1987.

64. Morton DL, Nizze JA, **Gupta, RK**, Famatiga E, Hoon DBS, Irie RF: Active specific immunotherapy of malignant melanoma. IN: *Current Status of Cancer Control and Immunology*, Kim JP, Kim BS, Park J-G (eds), Seoul, pp 152-161, 1987.

65. Morton DL, Hoon DBS, **Gupta RK**, Nizze JA, Irie RF: Humoral immune response in melanoma patients receiving active specific immunotherapy of melanoma tumor cell vaccine (MCV) alone or combined with low dose cyclophosphamide (CYP). J Leuk Biol 40:357, 1987.

66. Euhus DM, **Gupta RK**, Wong JH, Ramming KP, Morton DL: Effect of melanoma cell vaccine on level of antibody to a tumor associated antigen in melanoma patients, Proc Amer Assoc Cancer Res 28:349, 1987.

67. **Gupta RK**, Wong JH, Euhus DM, Morton DL: Detection of tumor antigen specific immune complexes in sera of melanoma patients and its relationship with disease recurrence. FASEB 2:1470, 1988.

68. Morton DL, Nizze JA, Hoon DBS, Famatiga E, **Gupta RK** Irie RF: A trial of immunomodulation in patients receiving active specific immunotherapy with melanoma cell vaccine (MCV). International Symposium on Molecular Probes, Technology and Medical Applications, 1988.

69. Euhus DM, Langsford C, **Gupta RK**, Wong JH, Morton DL: Measurement of a glycoprotein tumor associated antigen (TAA) using antibodies of different isotypes from a melanoma patient. Proc FASEB J 2:A1836, 1988.

70. Euhus DM, **Gupta RK**, Wong JH, Morton DL: Demonstration and isolation of a glycoprotein tumor associated antigen from sera of melanoma patients. Proc Am Soc Clin Oncol 7:44, 1988.

71. Euhus DM, **Gupta RK**, Wong JH, Ramming KP, Morton DL: Murine monoclonal antibody to a glycoprotein tumor associated antigen in sera and urine of melanoma patients. Proc Am Assoc Cancer Res 29:393, 1988.

72. **Gupta RK**, Wong JH, Euhus DM, Morton DL: Immunoprognostic significance of tumor associated antigen specific immune complexes in melanoma patients. Proc Am Assoc Cancer Res, 29:390, 1988.

73. Wong JH, **Gupta RK**, Huth JH, Euhus DM, Morton DL: Augmentation of anti-fetal antigen antibody levels in melanoma patients undergoing active specific immunotherapy with a tumor cell vaccine. Proc Am Soc Clin Oncol 7:248, 1988.

74. Wong JH, **Gupta RK**, Euhus DM, Morton DL: 69.5 kD glycoprotein fetal antigen tumor marker in melanoma. Proc Am Soc Clin Oncol 7:252, 1988.

75. **Gupta RK**, Wong JH, Morton DL: Correlation of humor immune response to a melanoma tumor associated antigen with survival of melanoma patients receiving active specific immunotherapy. Proc Am Assoc Cancer Res 30:344, 1989.

76. Wong JH, Xu SH, **Gupta RK**, Morton DL: IgG monoclonal antibody to detect and isolate antigen specific immune complexes from sera of melanoma patients. Proc Am Assoc Cancer Res 30:355, 1989.

77. Kopald KH, Shibata M, **Gupta RK**, Guiliano AE, Morton DL: Complement dependent cytotoxicity against tumor cell lines by a purified baboon IgM antibody to a tumor associated antigen. Proc Amer Assoc Cancer Res 30:346, 1989.

78. Bauer R, Kopald K, **Gupta RK**, Euhus D, Morton D: Detection of tumor antigen containing immune complexes in sera of cancer patients. Proc Amer Assoc Cancer Res 30:358, 1989.

79. Kopald K, Bauer R, **Gupta RK**, Euhus D, Morton D: Immunoprognostic significance of a tumor associated antigen excreted into the urine of melanoma patients. Proc Amer Soc Clin Oncol 8:290, 1989.

80. Morton DL, Hoon DBS, **Gupta RK**, Nizze AJ, Famatiga E, Foshag LJ, Furutani S, Irie RF: Treatment of malignant melanoma by active specific immunotherapy in combination with biological response modifiers. IN: *New Horizons of Tumor Immunotherapy*. Torisu M, Yshida T (eds), Elsevier Science Publishers B.V., pp 665-683, 1989.

81. Morton DL, Hoon DBS, Foshag LJ, Nizze A, Famatiga E, **Gupta RK**, Irie RF: Clinical results of a trial

of active specific immunotherapy with melanoma cell vaccine and modulation in metastatic melanoma. Venice, Italy, 1989.

82. Morton DL, Nizze A, Famatiga E, Hoon DBS, **Gupta RK**, Irie RF: Clinical results of a trial of active specific immunotherapy and immunomodulation in metastatic melanoma. Proc Amer Assoc Cancer Res 30:383, 1989.

83. Shibata, M, **Gupta RK**, Morton DL: Assessment of histamine H_2 receptors on human peripheral blood lymphocytes in a competitive radioligand binding assay. Proc Amer Assoc Cancer Res 30:386, 1989.

84. Wong JH, Xu SH, **Gupta RK**, Morton DL: Correlation of a tumor associated antigen specific immune complexes in sera of melanoma patients with recurrence disease. Proc Amer Assoc Cancer Res 9:278, 1990.

85. Bauer R, **Gupta RK**, Kopald K, Morton D: Improved immunoassay to detect antigen specific immune complexes in sera of cancer patients. Proc Amer Assoc Cancer Res 31:263, 1990.

86. Kopald K, **Gupta RK**, Bauer R, Morton D: Diurnal variation in the excretion of tumor associated antigen into the urine of cancer patients. Proc Amer Assoc Cancer Res 31:261, 1990.

87. Morton DL, Nizze A, Foshag LJ, Hoon DS, Famatiga E, **Gupta RK**, Irie, R: Long-term survival in metatastic melanoma after active specific immunotherapy with melanoma cell vaccine and immunomodulation. Proc Amer Assoc Cancer Res 31:281, 1990.

88. Gutierrez D, Hoon DS, Foshag LJ, **Gupta RK**, Morton DL: Enhancement of melanoma-associated antigen antibodies by melanoma patients' lymphocytes in vitro by interleukins 2, 4, 6 or 7. Proc Amer Assoc Cancer Res 31:241, 1990.

89. Morton DL, Hoon D, Nizze A, Foshag L, **Gupta R**,Irie RF: Active specific immunotherapy with melanoma cell vaccine and immunomodulation in patients with metastatic melanoma. Proc Amer Soc Clin Oncol 10:295, 1991.

90. Prasad R, **Gupta RK**, Morton DL: Clinical significance of tumor associated immune complexes in sarcoma patients. Proc Amer Soc Clin Oncol 10:349, 1991.

91. **Gupta RK**, Bauer RL, Morton DL: Clinical significance of a 90kDa glycoprotein tumor-associated antigen containing immune complexes in melanoma patients with Stage I disease. Proc Amer Soc Clin Oncol 10:37, 1991.

92. **Gupta RK**, Morton DL: Tumor associated antigen specific immune complexes in sera of patients with breast carcinoma as a prognostic indicator of recurrence. Proc Amer Soc Clin Oncol 12:113, 1993.

93. Morton D, Nizze A, Hoon D, Foshag L, Famatiga E, **Gupta R**, Irie R, Ramming K: Improved survival of advanced stage IV melanoma following active immunotherapy: Correlation with immune response to melanoma vaccine. Proc Amer Soc Clin Oncol 12:391, 1993.

94. Singer FR, **Gupta RK**, Morton DL: Antibodies to sarcoma associated antigens are present in sera of patients with Paget's disease of bone. Program of the 16th Annual Meeting of the American Society for Bone and Mineral Research, pp S229, 1994.

95. Jones RC, Guenther JM, **Gupta RK**, Morton DL: Adverse effect of IgG antibody response on the survival of patients receiving melanoma cell vaccine. 48th Annual Cancer Symposium of the Society of Surgical Oncology, 1995.

96. Jones RC, **Gupta RK**, Morton DL: Clinical significance of a glycoprotein tumor-associated antigen-specific immune complex in patients with ocular melanoma. Proc Am Assoc Cancer Res 36:640, 1995.

97. **Gupta RK**, Morton DL: Detection of a 90kD glycoprotein tumor-associated antigen specific immune complexes in sera of patients with breast cancer. Presented at the XXIII Meeting of the International Society for Oncodevelopmental Biology and Medicine, Montreal, Canada, 1995.

98. **Gupta RK**, Brosman SA, Morton DL: Tumor-antigen specific immune complexes in sera of prostate cancer patients. Presented at the 5th International Congress on Hormone and Cancer, Quebec, Canada, 1996.

99. **Gupta RK,** Nizze JA, Yee R, Kelley M, Leopoldo Z, Stiles A, Dickey A, O'Neill K, Halpin, A, Morton, DL: Prospective evaluation of 90kDa glycoprotein tumor-associated antigen in melanoma patients. Proc Am Soc Clin Oncol 15:93, 1996.

100. **Gupta RK,** Leopoldo Z, Yee R, Morton DL: Isolation and purification of a 90kDa subunit from a glycoprotein complex of a urinary tumor-associated antigen. Proc Am Assoc Cancer Res 37:465, 1996.

101. Jone RC, Kelley M, **Gupta RK**, Morton DL: IgG subclass response to melanoma cell vaccine: The gamma 4 connection. Proc Am Assoc Cancer Res 37:477, 1996.

102. Jones R, Kelley M, **Gupta R**, Nizze A, Yee R, Leopoldo Z, Qi K, Stern S, Morton DL: Response to melanoma cell vaccine in AJCC stage III melanoma: An immunologic survival model. Soc Surg Oncol 92, 1996.

103. Kelley M, Jones R, **Gupta R**, Wanek L, Morton D: Tumor-associated antigen TA90 predicts recurrence, lymph node status, and survival in stage I and II melanoma. Soc Surg Oncol 92, 1996.

104. **Gupta RK**, Morton DL: A novel approach to detect tumor-antigen specific immune complexes in sera of cancer patients. Clin Ligand Assay Soc. 1996.

105. **Gupta RK**, Morton DL: A new method to detect tumor antigen specific immune complexes in lung cancer patients. Clin Immunol Soc. 1996.

106. **Gupta RK**, Brosman SA, Yee R. Leopoldo Z, Morton DL: Presence of a glycoprotein tumor-antigen specific immune complexes in prostate cancer. The FASEB Journal 10:A1467, 1996.

107. **Gupta RK**, Giuliano AE, Kelley MC, Yee R, Leopoldo Z, Morton DL: Clinical significance of a 90kD glycoprotein tumor-associated antigen specific immune complexes in sera of breast cancer patients. Internatl Conf on Human Tumor Markers. 11:44, 1996.

108. Hsueh EC, **Gupta RK**, Glass EC, Yee R, Morton DL: Positive correlation of TA90 immune complex assay with positron emission tomography in detection of metastatic melanoma. 50th Annual Meeting of the Society of Surgical Oncology, 1997.

109. Hsueh EC, **Gupta RK**, Yee R, Leopoldo ZC, Morton DL: Immune response elicited by melanoma cell vaccine determine prognosis after cytoreductive surgery and adjuvant immunotherapy for stage IV melanoma. 50th Annual Meeting of the Society of Surgical Oncology, 1997.

110. **Gupta RK**, Hsueh EC, Yee R, Leopoldo ZC, Morton DL: Detection of 90kD glycoprotein tumor-associated antigen specific immune complexes in patients with ovarian cancer. Proc Am Assoc Cancer Res 38:30, 1997.

111. Hsueh EC, **Gupta RK**, Qi K, Yee R, Leopoldo ZC, Morton DL: Serum assay predicts survival response to adjuvant vaccine therapy for resected stage IV melanoma. Am Radium Soc, 1997.

112. **Gupta RK**, Hsueh EC, Brosman SA, Yee R, Leopoldo ZC, Morton DL: An ELISA to detect tumor-associated antigen specific immune complexes in sera of cancer patients. Am Soc Microbiol 97: May, 1997.

113. **Gupta RK**, Brosman SA, Hsueh EC, Yee R, Leopoldo ZC, Morton DL. Detection of 90kD glycoprotein tumor-associated antigen specific immune complexes and comparison with prostate specific antigen in patients with prostate cancer. Proc Am Soc Clin Oncol, 16:334a, 1997.

114. Hsueh E, **Gupta R**, Yee R, Leopoldo Z, Qi K, Morton D. Immune response to melanoma-associated antigens determines prognosis after cytoreductive surgery and adjuvant immunotherapy for stage IV melanoma. Presented at the Society of Surgical Oncology, Chicago, 1997.

115. **Gupta RK**, Giuliano AE, Yee R, Leopoldo ZC, Morton DL: Detection of a glycoprotein tumor-associated antigen specific immune complexes in breast cancer patients. Presented at the 20th Annual San Antonio Breast Cancer Symposium. Breast Cancer Treatment Repost. Dec. 3-6, 1997.

117. Hsueh EC, **Gupta RK**, Yee R, Leopoldo Z, Qi K, Morton DL. Serum TA90 immune complex correlates with recurrence following adjuvant immunotherapy for regional metastatic melanoma. Proc Am Assoc Cancer Res, 39:440, 1998.

118. Chu K, **Gupta RK**, Bilchik A, Yee R, Leopoldo Z, Morton D: Survival and the humoral immune response to TA90 in patients receiving active specific immunotherapy with CancerVax for metastatic cancers. Presented at the Society of Surgical Oncology, San Diego, March 25-29, 1998.

119. Hsueh EC, Famatiga E, **Gupta RK**, Qi K, Morton DL: Enhancement of complement-dependent cytotoxicity by melanoma cell vaccine: correlation with recurrence. Presented at the Society of Surgical Oncology , San Diego, CA, March 25-29, 1998.

120. Hsueh EC, **Gupta RK**, Gammon G, Foshag LJ, Essner R, Qi K, Morton DL: Correlation of specific immune responses with survival in AJCC stage IV melanoma patients receiving polyvalent melanoma cell vaccine. Proc Am Soc Clin Oncol, 17:435a, 1998.

121. Zavotsky J, **Gupta RK**, Brennan MB, Yee R, Giuliano AE, Morton DL. Prospective evaluation of TA90 immune complex assay in the preoperative diagnosis of benign and malignant breast lesions. Accepted for poster presentation at the American Society for Clinical Oncology, 17:106a, 1998.

122. **Gupta RK**, Yee R, Morton DL: Glycoprotein tumor-associated antigen (TA-90) specific immune

complexes in ovarian cancer. J. Tumor Marker Oncol. 13:40, 1998.

123. **Gupta RK**, Giuliano AE, Yee R, Morton DL: Detection of breast cancer by a novel immunologic technique using the TA90 specific immune complexes. Satellite Symposium on Tumor Immunology, Calcutta, India, 1998, (abstract *Submitted*).

124. **Gupta RK**, Hsueh EC, Qi K, Morton DL: Immune response to 90kD glycoprotein tumor antigen in patients receiving melanoma cell vaccine. Invited speaker at the 10th International Immunology Congress - 1998, New Delhi, India, 1998. (Abstarct *Submitted*).

125. **Gupta RK**: Giuliano AE, Yee R, Morton DL: A new immunologic approach involving antigen (TA90) specific immune complexes to detect occult breast cancer. Mini Symposium on Mechanisms of Tumor Invasion, Metastasis and Host-tumor interactions. Mumbai, India, 1998. (Extended abstract *Submitted)*.

Gupta RK, Giuliano AE, Yee R, Leopoldo Z, Morton DL: Purification and cahracterization of an immunogenic 90kD glycoprotein tumor-associated antigen detected in breast cancer. 21st Anuual San Antonio Breast Cancer Symposium - 1998 (*Submitted*).

Habal N, **Gupta R**, Yee R, Stern S, Brennan M, Brenner R, Hansen N, Giuliano A, Morton D: Accuracy of TA-90 immune complex in the detection of breast carcinoma. Proc Am Assoc Cancer Res 40:470, 1999.

DiFronzo LA, **Gupta RK**, Wanek LA, Stern SL, Yee R, Leopoldo Z, Morton DL: TA-90 immune complex predicts recurrence and survival in patients with stage II melanoma treated with polyvalent melanoma cell vaccine. Proc Am Assoc Cancer Res 40:424, 1999.