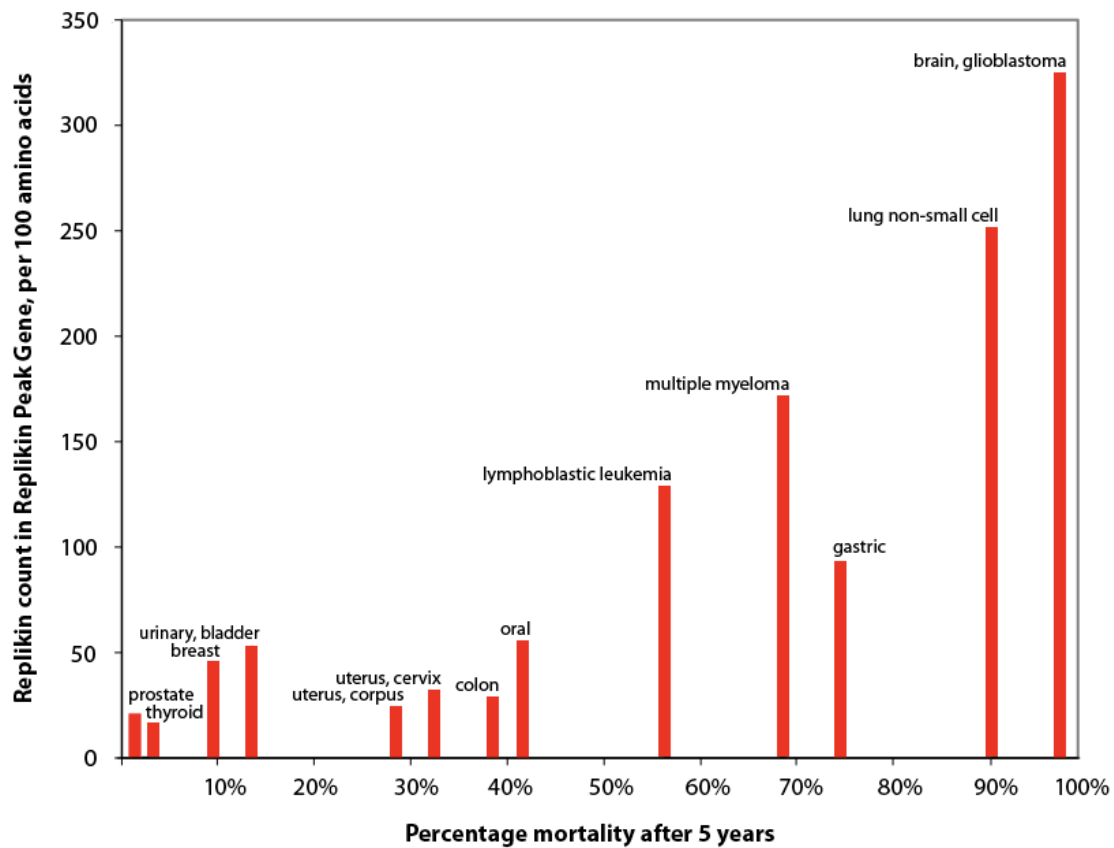


## Genome Replikin Count™ Predicts Increased Lethality of Malaria

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Figure 1- Cancer Mortality Increases With Cancer Cell Replikin Count



This communication is one of four submitted together:

Genome Replikin Count™ Predicts Increased Lethality of Resistant Tuberculosis

Genome Replikin Count™ Predicts Increased Lethality of Malaria

Genome Replikin Count™ Predicts Increased Lethality of Cancer

Genome Replikin Count™ Predicts Increased Infectivity/Lethality of Viruses

We have found that the concentration of replikins, a new class of peptides in genomic proteins, are quantitatively related to the mortality rate in human cancer of different cell types. No cancer cell genomic structure has previously been reported to be quantitatively related to mortality rate.

Figure 1 shows the relationship between the Replikin Count™ in the Replikin Peak Gene (see Methods) and the percent mortality in human cancer: the higher the Replikin Count, the higher the percent mortality after 5 years. Glioblastoma multiforme and non-small-cell lung cancer, the two cancer cell types with the greatest 5-year mortality rates in 2002 (98% and 91% respectively), are also the cell types which have the highest Replikin Counts (325 and 250 respectively).

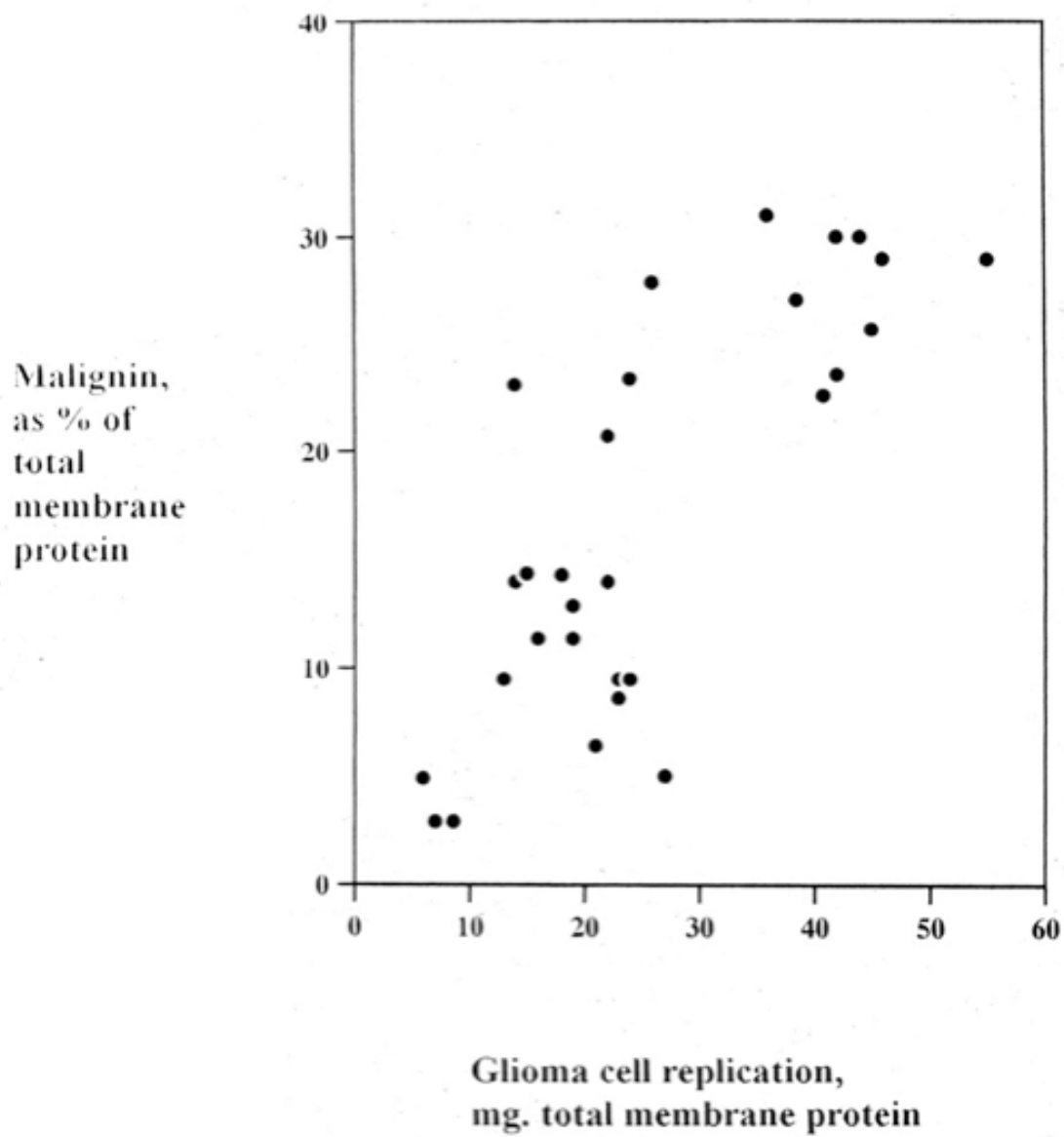
The count in Glioblastoma Multiforme is 18 times higher than the counts in prostate and thyroid cancer which have the lowest Replikin Counts (20 and 15 respectively) as well as the lowest 5-year mortality rates (each approximately 2%). The quantitative relationship of Replikin count to mortality is also seen in the figure for other common cancers, which shows some deviations from an 'ideal' curve. For example, gastric cancer has a higher mortality rate than expected from its Replikin Count (ie shifted to the right), possibly because of the known difficulty of early detection of gastric cancer. Breast and urinary bladder cancer have lower mortality rates than expected from their Replikin Counts (ie shifted to the left), possibly because of the improvement in their early detection and prompt treatment.

To obtain these results, all cancer protein sequences published on PubMed were analyzed by histological cancer cell type by ReplikinForecast™ software. The Replikin Peak Gene (RPGene), that area of the gene which contained the highest concentration of Replikins, the highest Replikin Count, was identified in each cell type. The replikins in each RPGene were counted (Replikin Count = number of replikins per 100 amino acids). The Replikin count of each RPGene then was compared in the different histological cancer cell types as shown in the Figure.

Replikin count has already been shown to be related to rapid replication, disease outbreaks, and host mortality in viruses such as influenza and HIV, bacteria, malaria, and other infectious diseases (see accompanying communications). The relationship between Replikin count and mortality in cancer parallels that found in H5N1 virus in humans and in Taura Syndrome Virus in shrimp.

We speculate that in low mortality cancers, it is possible that the immune system may be adequate to the number of invading units; but that the increased mortality rate in viruses and in cancer may reflect the overwhelming of host immune defenses by markedly increased numbers of viruses or of cancer cells produced in each case by increasing replikin concentration in the up-regulated Replikin Peak Gene. An increase in number of cancer cells per unit time produced by rapid replication has been demonstrated in the laboratory in the case of glioblastoma multiforme brain cancer cells in tissue culture\*. The quantitative relationship of Replikin Count to mortality rate has been demonstrated in the laboratory in four strains of lethal Taura Syndrome Virus in shrimp\*.

PREPRINT FROM "EVOLUTION OF LETHAL REPLIKINS",



**Figure 10**

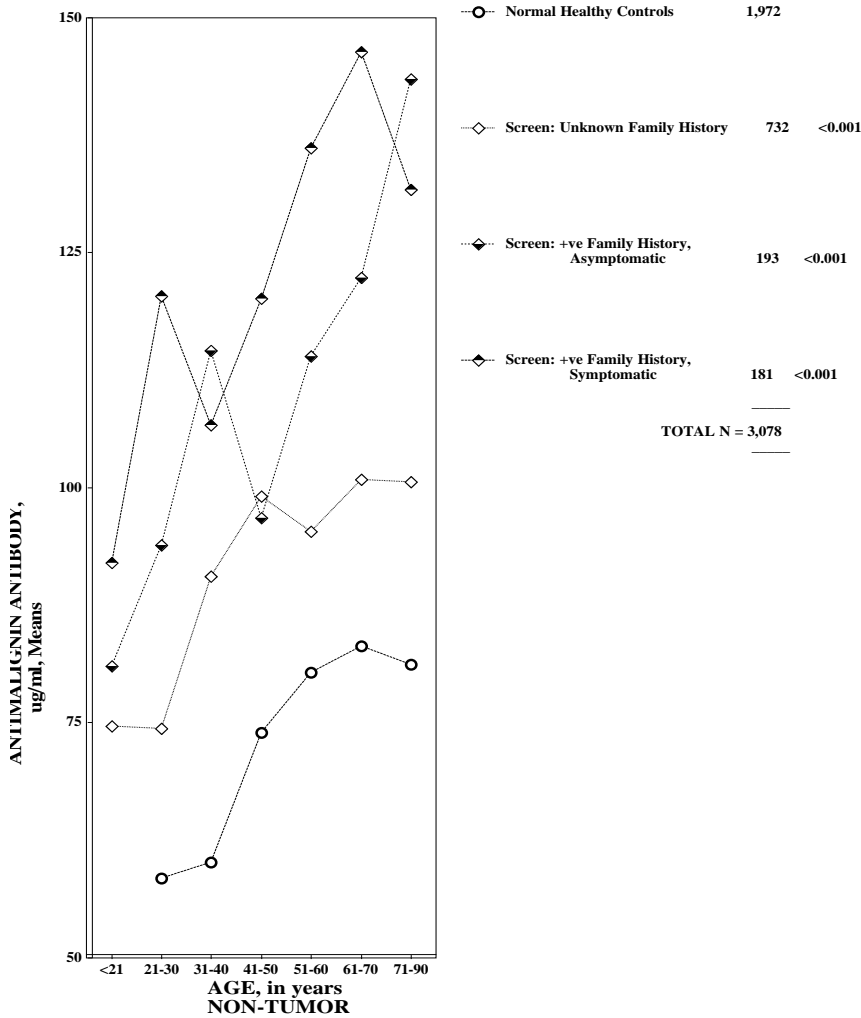
**Legend for Figure 11** –*The dates of the initial injection of the peptide as well as the booster injection are shown. The antibody response in terms of IgM is in ug/ml; in terms of IgG, the reciprocal x 100 of the serum dilution that results in an OD405 of 0.2*

**Maturation of Antimalignin Antibody in Humans**

We found that the concentration of antimalignin antibody in serum in vivo in healthy individuals has been shown to increase with age.

**Figure 12 -**

**INCREASE IN CONCENTRATION OF ANTIMALIGNIN ANTIBODY WITH AGE  
IN HEALTHY NON-TUMOR INDIVIDUALS;  
AND THE EFFECT OF A FAMILY HISTORY OF CANCER**



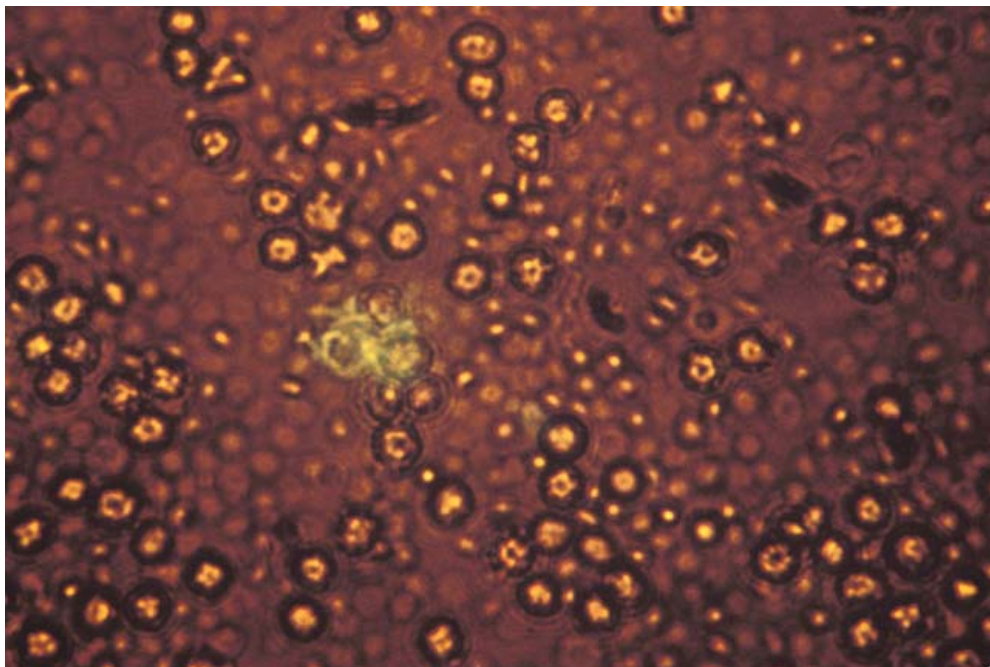
**Legend for Figure 12** – Values are mean values. *p* values are shown comparing the 'Normal Healthy Controls' to the three other groups. The concentration of the 'Unknown Family History' group falls between those of the 'Normal Healthy Controls' and the '+ve Family History' groups as expected if it contains some of each of these latter two groups. Antimalignin antibody in healthy individuals in their 20s from families with a '+ve' history is already at the level of healthy individuals in their 60s from families without any history of cancer. This suggests a possibly higher level of preparedness, or that the battle has already begun.

To our knowledge, this is the first and to date only demonstration of a developmental maturation of an antibody to cancer. These data run counter to the considerable resistance until recent years to the notion of a natural immunological defense against cancer. The data in Figure 12 clearly suggests the process of immunosurveillance because the increase with age follows a curve consistent with similar curves for 1) the increase in DNA errors with age, and 2) the incidence of cancer with increasing age.

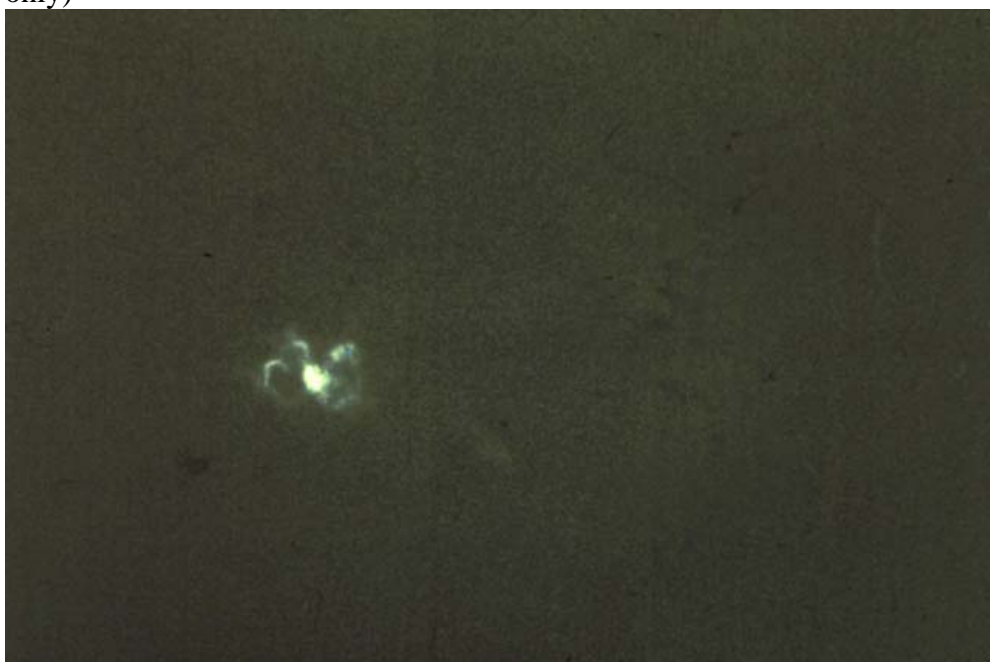
Also relevant to Figure 12 are the following: antimalignin antibody has a high specificity for a wide variety of cell types of cancer (Figures 13-23); antimalignin antibody is highly cytotoxic to cancer cells in vitro (Figure 14); antimalignin increases further in concentration in active cancer (Figure 15, 16 and 19); antimalignin is quantitatively related to survival of human cancer patients (Figure 17); antimalignin returns to normal with remission of cancer (Figure 19 and 21); antimalignin increases again on recurrence (Figure 20); and the concentration of antimalignin antibody relates quantitatively to the survival of cancer patients (Figure 18), the only such relationship which, to our knowledge has been described.

Taken together, these data support the notion of a lifelong immune defense against cancer of which one component is antimalignin antibody.

**Figure13-High specificity of antimalignin antibody for its replikin antigen**  
**Figure 13 a– Blood smear from patient with acute lymphocytic leukemia**  
(visualized with ordinary incident light and fluorescent light )



**Figure 13 b– Blood smear from patient with acute lymphatic leukemia**  
(visualized with fluorescent light only)



**Legend for Figures 13a and 13b** - *The specificity of antimalignin antibody is illustrated in Figures 13a and 13b of a blood smear stained with fluorescein labeled antimalignin antibody. Viewed under ordinary incident light as well as fluorescent light (Figure 13a), the number of red cells, white cells and platelets are seen, as well as one cell (green) labeled with fluorescein-antimalignin. When the ordinary incident light is turned off and only the fluorescent light left on (Figure 13b), only the fluorescent cell remains visible; all the normal blood cells are not stained. The specificity of the interaction of antimalignin with its antigen, the glioma replikin, is evident. The implications of this specificity are that it lies at the base of the accuracy of all diagnostic and therapeutic operations with this antigen and antibody.*

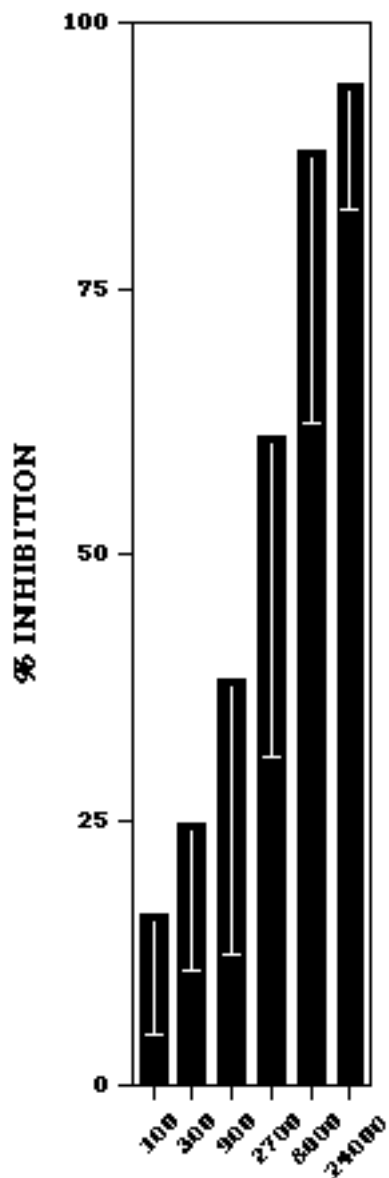
### **Antimalignin Antibody is Cytotoxic to Cancer Cells**

In vitro antimalignin antibodies have been shown to be cytotoxic to cancer cells at a concentration of picograms per cancer cell (Figure 14). Demonstrated for malignant glioblastoma cells, lymphomas, breast and lung cancer cells grown in tissue culture, antimalignin antibody is highly inhibitory to cell growth and cytotoxic at concentrations of picograms per cell (femtomolar).

Quantitative studies of other cytotoxic antibodies in cancer report activity only down to micrograms or nanograms, so that antimalignin may be among the most cytotoxic, or the most cytotoxic antibody to cancer cells described. This property has obvious relevance to the maturation of the concentration of the antibody as the risk of cancer increases, to the increase with active cancer, and to the decrease in antibody concentration on remission of cancer, all illustrated in the following figures.

### **Figure 14 – Inhibition of small cell lung carcinoma cell growth by antimalignin antibody**



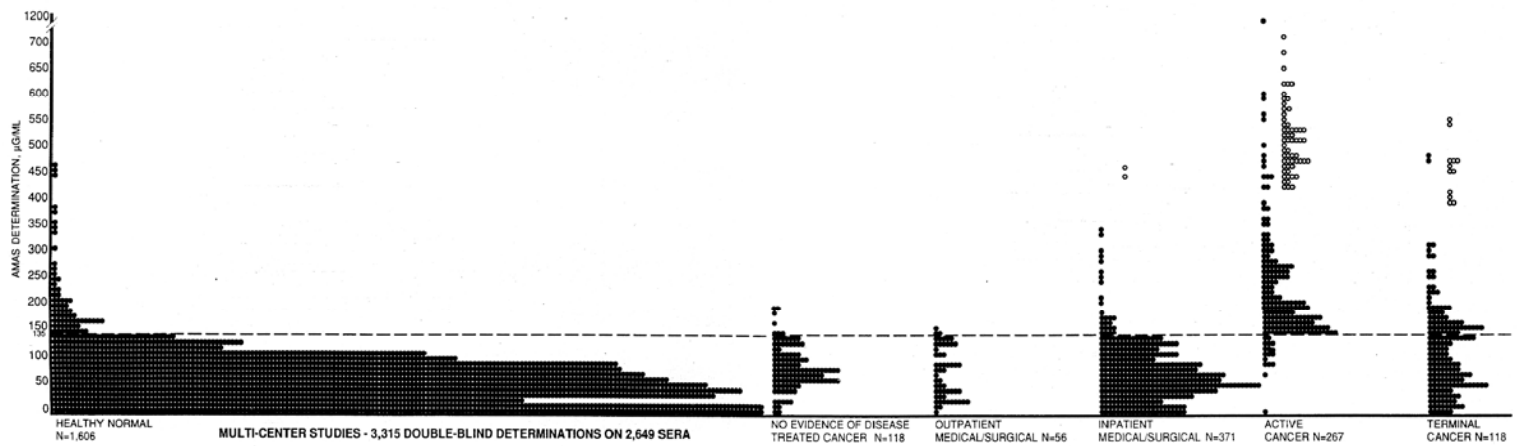


**ANTIMALIGNIN ANTIBODY,  
picograms per cell**

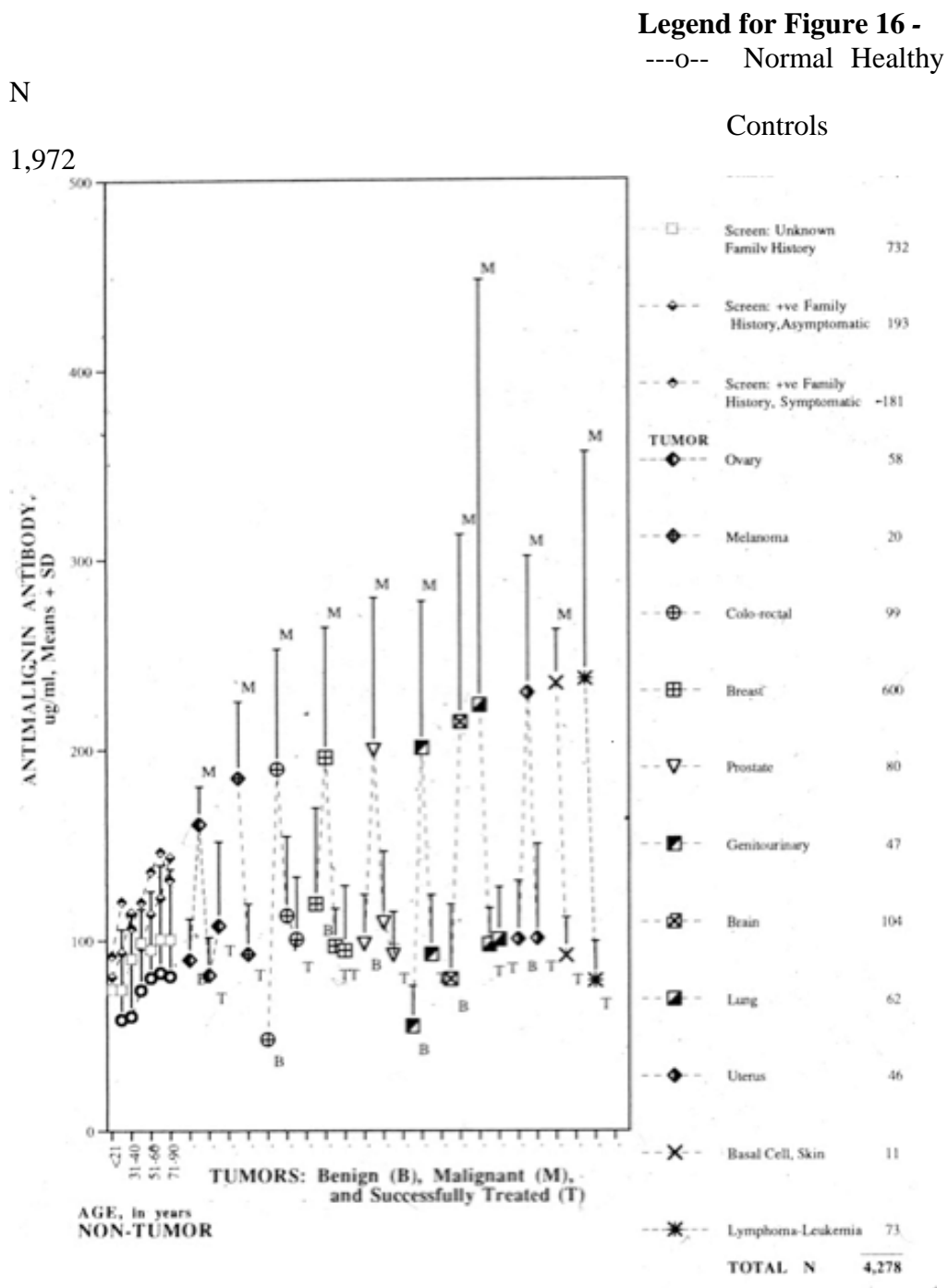
**Legend for Figure 14** - Each bar in the Figure represents the mean  $\pm$ SD for 24 wells, that is, from 3 wells for each of eight separate preparations of antimalignin at each dilution. **METHODS.** Small Cell Lung carcinoma cell line UCHNCU, grown in suspension and maintained in RPMI 1640 10% FCS (fetal calf serum) was seeded in 96 well microtitre plates (round bottom) at 104 cells per well. Serial dilutions were made of antimalignin antibody which had been purified by adsorption to immobilized malignin, original concentration 3 to 300 micrograms per ml, so that final concentration of antimalignin in RPMI FCS, diluted 1/6 to 1/1458, was 150 to 25000

*picograms per cell, and final total volume per well was 200 microlitres. Plates were incubated at 37°C in 6% CO<sub>2</sub> /air for 3 days. On day 3 cultures were pulsed with 1 uci/well tritiated thymidine (3HTdR ) for 6 hours, then cultures were harvested with automatic cell harvester on filter pads. Filters were dried for 2 hours in 37°C dry incubator, discs were placed into scintillation vials, 2 ml Optiphase scintillant added, tubes capped, cpms counted on Beckman LS 1800 beta counter and % Inhibition of cell growth calculated as Control minus Experimental/ Control x 100.*

**Figure 15 – Concentration of Antimalignin Antibody Determined Double-Blind in Cancer (breast, lung, brain, melanomas, lymphomas, leukemias, colorectal, larynx, uterus, cervix, ovary, anus, stomach, esophagus, prostate, bladder, urethra, kidney, testis, thyroid, skin, fibrosarcoma) and Non-Cancer Control Conditions in multi-center 3,315 determinations in 2,649 sera.**

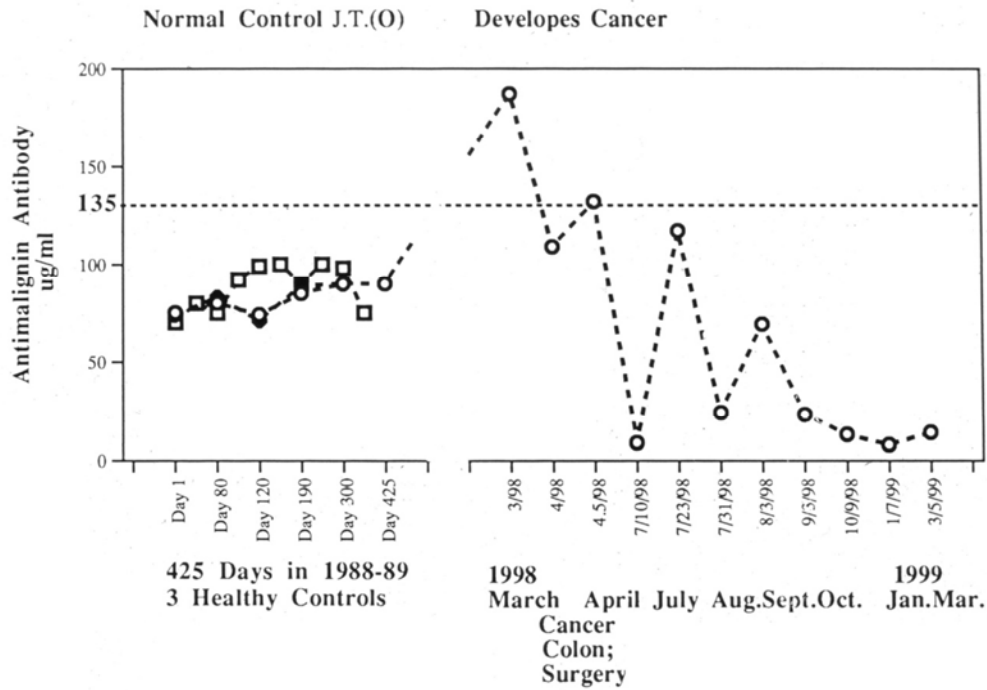


**Figure 16 - Increase in Concentration of Antimalignin Antibody in Active Cancer in Different Cell Types**



The 4,278 cases in Figure 16 were analyzed at a later date to, and include, the 2,649 cases recorded in Figure 15.

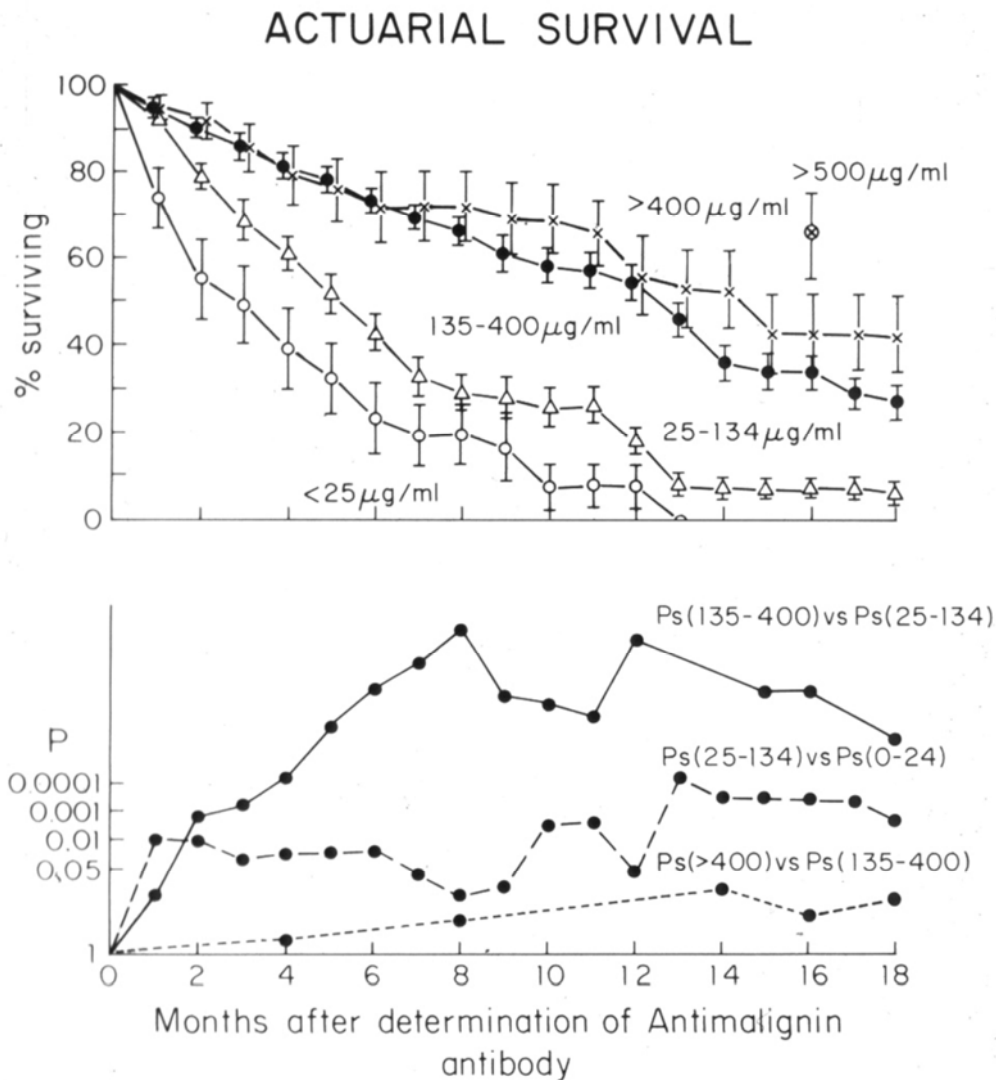
**Figure 17 - Detection of Asymptomatic Cancer with the Antimalignin Antibody in Serum (AMAS) Test**



**Legend for Figure 17** – In 1988-89, the AMAS test was performed in three healthy individuals over a period of 425 days. The constancy of the ‘normal’ values (0-134 ug/ml serum) is seen. Nine years later one of these individuals asked to have another test performed. He was asymptomatic, and only after the test gave an elevated antibody concentration (>134 ug/ml serum) did he state that he had requested the test because it was his 45<sup>th</sup> birthday and his father had died of colon cancer at the age of 45. Colonoscopy revealed three tumors, one of which was malignant on histological examination. The three tumors were removed and the subsequent normal AMAS results accompanied the state of clinical remission.

Healthy individuals from high-risk families, that is those in which there is a high incidence of cancer, are increasingly having three-monthly or annual AMAS determinations.

**Figure 18 - Quantitative Relation of the Concentration of Antimalignin Antibody to the Survival of Cancer Patients**



**Legend for Figure 18** - 511 cancer patients had one determination of antimalignin antibody and their clinical status assessed over time. Survival is seen to be quantitatively related to the concentration of antimalignin antibody at  $p$  levels shown in the lower graph.

To our knowledge, this is the only antibody whose concentration has been shown to be quantitatively related to the survival of cancer patients.

## Uses of Replikins in Clinical Diagnosis and Tracking Remission of Cancer

### Return of Elevated Antimalignin Antibody to Normal Indicates Remission of Breast Cancer: A 20 Year Study with 8,090 Determinations

*(This is the first publication of the full report of this study.)*

#### Abstract:

**Objective** - To establish whether the concentration of antimalignin antibody in serum after surgery can help to detect persistent or recurrent disease or to confirm remission of breast cancer.

**Design** - 20 year consecutive population-based random study; criterion standards: histopathology, mammography. Four independent laboratories quantitatively determined antimalignin blind on sera from three pre-biopsy and one of healthy screens trials, interspersed with specimens requisitioned by physicians.

**Setting** - U.S. and U.K hospital out-patient, in-patient, hospital and private practice physicians and surgeons ordered the test as a diagnostic aid, for monitoring for remission 1 to 30 years after surgery, or for targeted screening of high-risk individuals.

**Patients and Healthy Participants** - 8,090 consecutive blind antimalignin determinations: 1,175 benign or malignant breast disease (diagnosis unknown, 46), 1,428 non-breast benign and malignant tumors, 1,016 non-cancer medical-surgical disorders, and 4,425 screened healthy individuals.

**Results** - Normal concentrations of antimalignin (less than 135 ug/ml serum) were found in 97% of healthy controls, in 100% of those with non-tumor other breast pathology, and in 87.8 % of those with benign breast tumor. Elevated antimalignin (135 ug/ml or greater) occurred in 97.6% of active breast cancer patients. At primary diagnosis sensitivity was 98.2%, specificity 91.4%; on recurrence, sensitivity 96.2%, specificity 100%. In patients in remission 1 to 30 years after surgery, 99.7% had normal antimalignin values. In 82 breast cancer patients studied longitudinally, of those with progressive or recurrent disease 100% had elevated antimalignin, and of those in clinical remission, 100% had normal antimalignin concentrations.

**Conclusion** - The antimalignin antibody test can be useful to confirm clinical evidence of remission or to help detect primary or recurrent breast cancer.

Malignant cells may persist after treatment, but if the number of these cells is below the number detectable by microscopy, scanning methods, or tumor markers, remission may be incorrectly assumed to have been achieved and treatment stopped, only to be followed by “recurrence” a few months later. The existing tumor marker tests, which measure antigen released into the blood, eg. CEA, CA125, CA15-3 (CA27:29), are agreed to be not useful for earliest stage malignancy but become more accurate with advanced malignancy<sup>27-34</sup>. A quantitative molecular method which could provide early objective evidence of remission, or the lack thereof, would be useful.

Antimalignin is a highly specific autoantibody against a 10,000 Dalton cancer cell peptide malignin which contains the Glioma Replikin. Antimalignin is present in small amounts in normal human serum<sup>61-71</sup>, is increased in concentration in a wide variety of cell types of cancer, and is cytotoxic/cytostatic *in vitro* at picograms (femtomoles) per

cancer cell<sup>56</sup>. Because antimalignin concentration is elevated early in malignancy<sup>35-39, 42-46, 48-67</sup>, it is determined as an aid to differential diagnosis and in screening high risk patients. We now find in a study of 8,090 consecutive randomly distributed sera in which 1,175 sera were from patients with breast cancer and other breast disorders, that 1 month to 30 years after treatment, clinical remission of breast cancer is correlated with the return of elevated antimalignin to normal values.

The study of the role of brain glycoproteins in cell-cell recognition<sup>68-78</sup> led to the isolation of the 10KD cancer polypeptide antigen malignin from brain glioblastoma cell glycoprotein 10B<sup>35-39</sup>, followed by the isolation of the closely related Recognin M from MCF7 mammary carcinoma cells and Recognin L from P3J lymphoma cells<sup>41-46</sup>. When cells become transformed to malignancy, these epitopes become exposed to immunofluorescent staining<sup>36,40,47,49</sup> *in vitro* and labelling *in vivo*<sup>52</sup> by anti-recognin antibodies. This exposure of the peptide antigen epitope was found to be related to the loss of the cover of glycosylated units resulting in an aglyco peptide product<sup>78</sup>. A specific antibody was then found in human serum which has shared specificity against these three epitopes<sup>35,39,41-46</sup>. This antibody, antimalignin, could be isolated from and quantified in human serum<sup>52-67</sup>, was produced in mouse monoclonal form<sup>50</sup>, and was produced in fully human form by human lymphocytes *in vitro*<sup>56</sup>. Human antimalignin produced *in vitro* is cytotoxic/cytostatic to malignant cells *in vitro* at picograms (femtomoles) per cancer cell<sup>56,66</sup>. Antimalignin antibody in serum was found to increase in concentration with age within normal limits<sup>62</sup>, to increase more rapidly in healthy non-tumor bearing individuals from high risk cancer families<sup>62</sup>, and to be elevated two to four times the normal concentration in patients with a variety of malignancies<sup>35-67</sup>. This marked elevation has been observed to occur within days of the transformation of cells to the malignant state<sup>62</sup>. While elevation of antimalignin concentration has been shown to be accurate at the 95% level on first determination in detecting early malignancy in sera from non-terminal patients (reviewed together with other cancer biomarkers at two meetings sponsored by the National Cancer Institute in October 1993 and February 1994<sup>62</sup>), we have not reported in detail its use to objectify remission, the subject of the present study.

## Methods

**Study Population: Source of specimens.** Serum specimens were obtained from the U.S. and in the U.K. a) from four separate blind controlled trials: one of specimens from workers at a chemical plant (N=1,505), and three in which pre-biopsy antimalignin results were compared with histopathology on subsequent biopsy or surgery (N=186), and b) the balance from tests ordered by physicians - internists, general practitioners, obstetricians and gynecologists, surgeons and oncologists.

**Indications and Patient Selection.** In the controlled pre-biopsy trials, the indications for the requisition of the antimalignin test were the common indications for biopsy, that is, the presence of a lump in the breast in a suspicious mammogram. The indications used by the physicians ordering the test reflected activities and were: 1) to aid in the differential diagnosis in the presence of symptoms and/or signs, 2) to monitor treatment and recovery in known cancer, and 3) to establish baselines in the targeted screening of



asymptomatic individuals from high-risk families. Since both the physician and the patient were required to sign the Requisition Form (see below) only those patients who wished the antimalignin test performed had the test performed. There was no other known basis of selection.

Exclusions. Sera were not sent from patients with known advanced or terminal cancer, since it had been previously established<sup>51,53</sup> that due to antibody failure, antimalignin antibody concentration is reduced to the 'normal' range in these cases. 46 patients with breast disorders for whom clinical information was not available (3.8% of total breast cases) were excluded because the diagnosis (benign vs. malignant) was unknown. There were no other patient exclusions. For specimen exclusions see 'Shipment of Specimens to the Laboratory'. The number of patients who refused the test when offered it by the physician is not known.

Consent. Each serum specimen was accompanied by a Requisition Form which contained information on the date and time of collection of the specimen, the collecting laboratory location and telephone number, physician name, address, telephone and fax numbers, patient name, address, telephone number, and a "Declaration" read and signed by both patient and physician as follows: "As in all clinical laboratory tests, I understand that the antimalignin antibody test is not by itself diagnostic of the presence or absence of disease, and that its results can only be assessed as an aid to diagnosis, detection or monitoring of disease in relation to the history, medical signs and symptoms and the overall condition of the patient".

Number of Specimens. In the 20 years from January 1, 1973 through July 1, 1993, antimalignin determinations were performed on 8,090 consecutive serum specimens: 1,175 of these turned out to be from patients shown to have histopathologically established diagnoses of breast cancer or benign breast disorders (diagnosis was not available on 46 additional). These 1,175 sera were received at random interspersed among 4,425 normal control specimens, 1,428 specimens from patients with cancer and benign tumors other than breast, and 1,016 non-cancer medical-surgical disorders. The latter two groups of non-breast tumor and medical-surgical disorders, as well as additional cancer and non-cancer specimens which have had antimalignin determinations from July 1, 1993 to July 1, 1997 are to be analyzed and reported in future communications.

Age and sex distribution. Patient and healthy control age distribution was as follows: under 40 years, 18%; 41-50 years, 22%; 51-60 years, 22%; 61-70 years, 20%; 71-90 years, 18%. Of the healthy control group, 54% were females, 46% males.

Random Population-Based Sample. A random sample of a broad-based population was achieved by the following means: 1) Number of consecutive specimens: the antimalignin test was performed on 8,090 consecutive specimens 2) Time: sera were collected consecutively over a 20 year period between January 1, 1973 and July 1, 1993. 3) Geographic distribution: of the 4,425 control specimens from healthy individuals, other than 1,505 which came from individuals who worked at an Ohio chemical plant. specimens came from all over the U.S. and U.K. 4) Four independent laboratories performed the tests. 5) Diverse specialties: while the three separate pre-biopsy trials were done by hospital clinics specializing in the detection and treatment of breast cancer, the other participating physicians represented all specialties and general practice. 6) Diverse base of practice: out-patient and in-patient, individual, group and hospital based practice

are all included. This diversity of time, place and referring person resulted in sera interspersed from benign vs. malignant breast disorders, early vs. late stage malignancy, high-risk vs. low-risk families, first occurrence vs. recurrence, node positive vs node negative patients and non-cancer healthy individuals (Figures 1 and 2, and see Discussion).

**Participating Laboratories.** The four participating laboratories were: SmithKline Clinical Laboratories, Waltham, Massachusetts; Immuno-Oncology Laboratory, Baptist Hospital of Miami, Miami, Florida; Oncolab, Boston, Massachusetts; and Oncolab, London, UK. 38 of the antimalignin determinations in breast cancer performed by SmithKline Clinical Laboratories were previously published as part of a study of several cell types of cancer<sup>27</sup>. The three controlled prospective pre-biopsy breast studies were performed at: 1) Guy's Hospital, London U.K., 2) the Immuno-Oncology laboratory of Baptist Hospital, Miami, Florida<sup>53</sup> and 3) the University of Southern California School of Medicine (Oncology) at Los Angeles (Dr. A.Z. Bluming). The data from these pre-biopsy studies are included in Figure 1. Additional longitudinal clinical data will be reported separately from the Immuno-Oncology Laboratory, Baptist Hospital.

**Shipment of Specimens to the Laboratories.** Serum specimens were separated from blood collected in vacutainer tubes free of silicon (Becton Dickensen #6641) as silicon was shown to absorb the antibody. The tube used for drawing had to be the first tube used since it had been found that antibody is adsorbed by microclot in the needle. Serum separators also were prohibited because antibody was found to be adsorbed in this case as well. After clotting and centrifugation at 0 to 5°C and 3000 rpm, sera were shipped in Nalge Nunc International cryotubes (4.5ml) on dry ice (-70°C) overnight by priority air Federal Express to one of the four laboratories above for antimalignin determination within 24 hours of the blood being drawn. The date, time and laboratory location for the drawing were recorded on the Requisition Form, and if these indicated a delay in shipping the serum, the specimen was not accepted. Sera which were timely but arrived in the laboratory unfrozen with no dry ice remaining were not accepted for determination and the blood had to be redrawn. Sera were also not accepted if first stored in a freezer at -20°C since the accuracy of the test was found to be reduced in proportion to the time at this temperature.

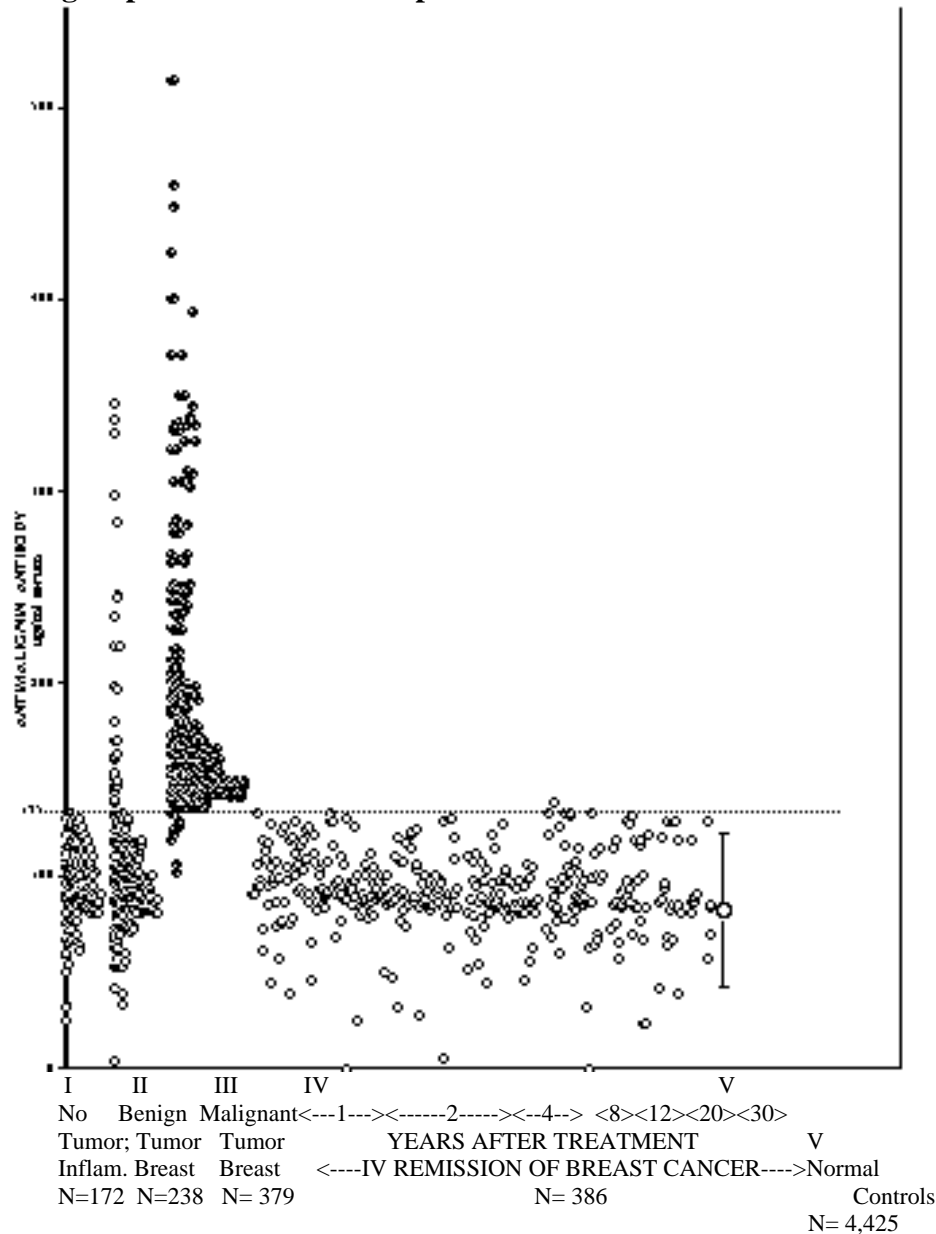
**Antimalignin Antibody Determination.** Quantitative determination of antimalignin antibody by immunoabsorption was as previously described. At each of the four laboratories, the method of determination was the same, the reagent (TARGET® reagent, Brain Research, Inc., Boston, Massachusetts) was the same, and the restrictions on the preparation and shipment of specimens (above) were the same throughout the 20 years of the study. Briefly, malignin, a 10KD peptide isolated from glioblastoma cells grown in tissue culture, of constant composition containing 89 amino acid residues is bound covalently to bromoacetylcellulose to produce TARGET reagent. TARGET reagent is shaken vigorously with 0.2 ml of patient's serum at 0 to 5°C, washed with cold saline, then shaken vigorously with 0.25M acetic acid at 37°C to elute the bound antibody, which is then quantitated as protein by absorption at 280nm and expressed as microgram per milliliter of serum. Because malignin is covalently bound to bromoacetylcellulose to form TARGET reagent, dilute acetic acid does not elute malignin, but only elutes the specific antibody, antimalignin, which has been immunoabsorbed to malignin noncovalently. All specimens were determined in duplicate. An elevated

control and a normal control antimalignin, each in duplicate, was run with each determination. As determinations were performed on batches of 20 sera at once, it was not possible to influence the outcome of the determination of an individual serum. Gross abnormalities of the run as a whole, which occurred once or twice each year, could be detected from the duplicate elevated and normal controls run with each determination and from the deviation from the usual range of values. Coefficient of variation inter-technician was 6%, and inter-laboratory 11%. Two species of antimalignin antibody exist in and have been isolated from human serum *in vivo*<sup>52</sup>, produced in mouse monoclonal form<sup>50</sup>, and produced and isolated in fully human form *in vitro*<sup>56</sup>. Both of these species of antimalignin were quantitated for each serum specimen in duplicate in four tubes for each serum determination: two which were slow-binding (2 hour reaction time), S-TAG (Slow Target-attaching-Globulin); and two which were fast-binding (10 minute reaction time)F-TAG (Fast-Target-Attaching-Globulin). Since the quantity of FTAG is included in the quantity of S-TAG, the difference S-TAG-F-TAG = Net TAG. All results given in this communication are as Net TAG, in micrograms/ml. The quantitative limits for designating normal and elevated results were as in earlier studies: 'Elevated' - Net TAG=135 ug/ml or greater; or STAG 400 ug/ml or greater; or FTAG 300 ug/ml or greater; -confirmatory repeat test recommended. 'Borderline Normal' - Net TAG=100 to134 ug/ml; - confirmatory repeat test recommended. 'Normal' - Net TAG= 0 to 99 ug/ml- can also occur in successfully treated cancer patients with 'no evidence of disease' (after up to 3 months following completion of therapy regardless of type of therapy), and in advanced or terminal patients with antibody failure ('advanced' = as with 1 cm or larger tumor, or growth outside capsule, or with metastases, or 3 years or more duration, or with secondary symptoms such as weight loss, anemia; 'terminal' = dead in one year).

Clinical Data. Clinical data was recorded by the physician on a standard form which contained date of birth, sex, social security (or National Health) number, first date of diagnosis, date of present admission, present clinical diagnosis, present signs and symptoms, histopathology-current and prior, treatment-current and prior (patient may have had surgery up to 30 years before having the test), family history of cancer or other disease (relation; organ), and exposure to cigarettes, alcohol or carcinogens. Clinical diagnosis of primary occurrence and recurrence were based on histopathology of biopsies, and when judged appropriate by the physicians, imaging studies including chest X-ray, mammograms, magnetic resonance imaging, computed tomography, bone scan, liver scan and ultrasound. The clinical data was recorded independent of the antimalignin data, then was correlated with the antimalignin data after the antimalignin test results were reported to the physicians.

## Results

**Figure 19 - Concentration of antimalignin antibody, in micrograms per ml serum, in five groups of breast disorder patients and controls**



**Legend for Figure 19** - Group I- No tumors; inflammatory and other breast disorder (N=172); Group II-Benign tumors of breast (N=238); III- Malignant tumors of breast (N=379); IV- Remission of Breast Cancer (N=386); V-Normal controls (N=4,425). In a random blind study of 1,175 patients with breast disorders and 4,425 normal healthy controls, by three laboratories, antimalignin elevation is not a function of either inflammation (Gp.I) or benign tumors (Gp.II), but only of malignant transformation and active cancer (Gp. III). Patients judged to be in clinical remission (Gp.IV) have normal concentrations of antimalignin compared to normal controls (Gp.V).

Figure 19 shows the concentration of antimalignin antibody in single determinations for individual patients performed on specimens received completely at random in terms of the diagnosis or clinical status, then after the clinical data was obtained, organized into clinical groups. Those with 'No Tumor, Other', that is other breast pathology such as inflammatory breast conditions but no tumor (Group I; N=172) all had antimalignin concentrations of less than 135 ug/ml serum. These Group I results were not significantly different from those of 'Normal Controls' (Group V; N = 4,425) shown in Figure 19. Nor did Group I results differ significantly from those of the 'Benign Tumor Breast' (Group II; N=238).

The false positive rate for 'Normal Controls' (Group V) was 3.0%, and less than 1% in those who had a second determination. The false positive rate in 'No Tumor, Other' (Group I) was 0%, for 'Remission' (Group IV) 0.3%, and for the 'Benign Tumor Breast' (Group II) was 12.2%. The obviously higher false positive rate in this last Group is examined in the Discussion.

All but one of the determinations in patients with 'Remission of Breast Cancer' (Group IV; N=386: of which 361 had been first occurrences, 25 recurrences, 26 had been node positive or with other metastases) had normal values (less than 135 ug/ml). However, of those with 'Malignant Tumor Breast' (Group III; N=379: of which 274 were first occurrences, 105 recurrences, and 106 were node positive or with other metastases) 97.6% had elevated AMAS values (135 ug/ml or more) ('false negative' rate 2.4%), and Group III was significantly elevated above the other four groups ( $p < .001$ , student t test). At primary diagnosis, sensitivity was 98.2% and specificity was 91.4%. At recurrence (relapse), sensitivity was 96.2% and specificity was 100%. After therapy, for first occurrence, sensitivity was 100% and specificity was 99.7%, and for recurrence, sensitivity was 100% and specificity was 100%.

Figures 20 and 21 show the antimalignin antibody concentrations obtained in 82 patients selected by their physicians for longitudinal study (i.e. more than one antimalignin determination) one month to 30 years after treatment of breast cancer. Fifteen of these patients clinically had either progressive disease (N=5) or recurrent disease (N=10) (Fig. 20), and sixty-seven were in clinical remission (Fig. 21). Persistent or recurrent disease which occurred in the 15 cases is seen to be associated with elevated antimalignin concentration. Clinical remission is seen in Figure 21 to be correlated with the return of antimalignin to normal values, usually within three months of treatment. These results related to the fact of remission but were independent of the type of treatment the patients in the *Remission of Breast Cancer* group (Group IV) had received (surgery 100%, chemotherapy 10.4%, radiation 5.4%), and were independent of which of the four laboratories performed the tests.

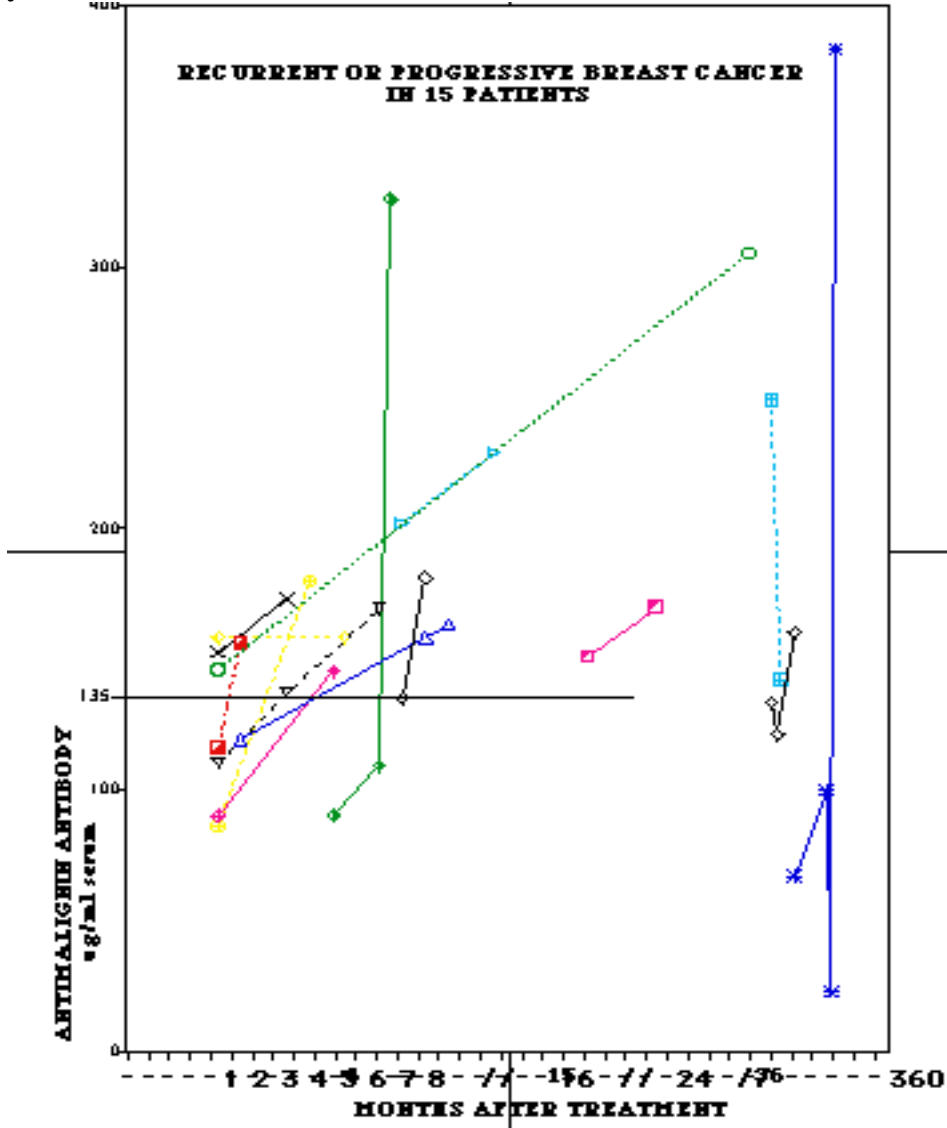
Group V data (N = 4,425) is shown here only by its mean +/-SD for comparison with the other four groups. Normal concentrations, less than 135 ug/ml; elevated, 135 ug/ml or greater), and were independent of which of the four laboratories performed the tests organized into clinical groups. Those with 'No Tumor, Other', that is other breast pathology such as inflammatory breast conditions but no tumor (Group I; N=172) all had

antimalignin concentrations of less than 135 ug/ml serum. These Group I results were not significantly different from those of 'Normal Controls' (Group V; N = 4,425) shown in Figure 19. Nor did Group I results differ significantly from those of the 'Benign Tumor Breast' (Group II; N=238) (Figure 19). The false positive rate for 'Normal Controls' (Group V) was 3.0%, and less than 1% in those who had a second determination. The false positive rate in 'No Tumor, Other' (Group I) was 0%, for 'Remission' (Group IV) 0.3%, and for the 'Benign Tumor Breast' (Group II) was 12.2%. The obviously higher false positive rate in this last Group is examined in the Discussion.

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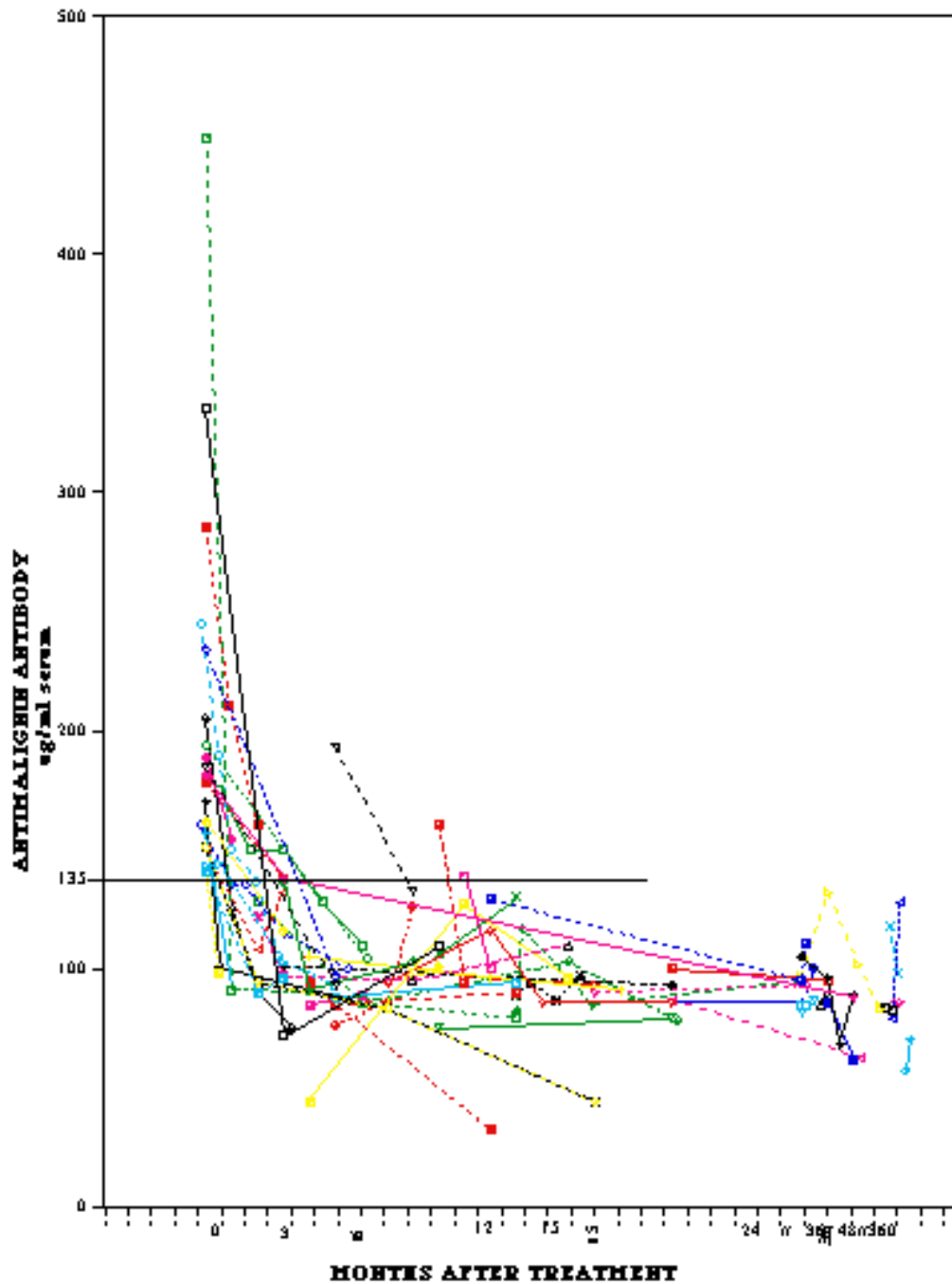
Figure 20. Recurrent or progressive breast cancer in 15 patients one month to 30 years after treatment.



*Legend for Figure 20* - The curve for each patient shows the concentration of antimalignin antibody, in micrograms per ml serum, at two or more different times after treatment. In 5 patients with progressive clinical disease after treatment, antimalignin remained above 135 ug/ml; and in 10 with recurrent disease after treatment, antimalignin had returned to normal, less than 135 ug/ml, then increased to elevated concentrations, 135 ug/ml or more.

Figure 21 -

**INDIVIDUAL LONGITUDINAL ANTIMALIGNIN ANTIBODY CONCENTRATIONS INDICATING REMISSION IN 67 PATIENTS**



*Legend for Figure 21-* The curve for each patient shows the concentration of antimalignin antibody, in micrograms per ml serum, at two or more different times after treatment. These 67 patients achieved or remained in clinical remission. After treatment antimalignin returned to and/or remained at normal concentrations, less than 135 ug/ml.

**Discussion**



## Comparison of Antimalignin with other Tumor Markers.

**Antigen vs. antibody; late vs. early.** In all other serum tumor marker tests, antigens are measured ('antigen tests'), not antibodies. In contrast, the antimalignin test measures an antibody rather than an antigen. The more commonly used antigen tests include CEA, CA19.9, CA125, PSA, and CA15.3 (CA27:29)<sup>27-29</sup>. The determination of these antigens in serum has proven less sensitive in early stages of malignancy and more sensitive as the cancer progresses and the antigens are increasingly released into the serum<sup>27-29</sup>. The antigen tests also have not been found to be useful in early stages of breast cancer. Both CA15,3 and CA27:29 have been shown to lack the required sensitivity and specificity for the routine detection of breast cancer<sup>31-34</sup>. With CA27.29(CA15.3), only 6% of patients were detected in Stage 0, Stage I or Stage II breast malignancy (95% for antimalignin). Although 63% of Stage III and 80% of Stage IV were detected with CA27:29<sup>33</sup>, the sensitivity of CA27:29 for detecting recurrence of Stage II and Stage III breast cancer was found to be 57.7%<sup>8</sup> compared to the antimalignin test's 96.2% sensitivity for recurrence (see Results above). In another study<sup>59</sup>, the antigen markers CEA, CA19.9, CA125 and CA15.3 were compared with antimalignin in a prospective blind study of patients with an abnormality on mammography who were biopsied after having the tumor marker tests. The antigen markers detected only between 0 and 16%: CEA-0%, CA19.9-5%, CA15.3-11%, CA125-16%, whereas antimalignin detected 95% of breast cancers 1mm to 10 mm in size pre-biopsy. Antimalignin is most sensitive in the early stages of breast malignancy and less sensitive as the cancer progresses to advanced and terminal stages when the antibody response is interfered with and antibody increase cannot be used as an aid to diagnosis<sup>51,53,61</sup>. Due to failure of this antibody, 'normal' antimalignin concentrations are observed in almost all cases of advanced and terminal malignancy<sup>61</sup>. Therefore the utility of the antimalignin test compliments that of the antigen tumor markers as diagnostic aids in terms of the stage of the disease: antimalignin is more useful early, antigen markers are more useful late in the disease.

**General vs. Specific.** The antigen markers are specific for one or a few cell types rather than general<sup>27-29</sup>. Antimalignin is a general transformation antibody made in response to the appearance of a cancer cell peptide during transformation to the malignant state independent of cell type<sup>52</sup>. Again antimalignin compliments the antigen tests, since for most symptoms and signs where the differential diagnosis includes cancer of one of several organs in the anatomical region, the cell-type specific antigen markers do not suffice and a general test which addresses the question cancer vs. benign disease can be useful. For example, a negative CA125, especially in Stage I cancer of the ovary, provides no assurance that the ovaries or for that matter any other pelvic organs are free of cancer, whereas a normal antimalignin can be of help in this regard since it is not organ-restricted. In a case example, an enlarged cervical node initially was not biopsied because an earlier biopsy of a lump in the breast of a 33 year old was negative. However, antimalignin concentration was elevated. Biopsy of the cervical node revealed a totally unsuspected thyroid cancer. Antimalignin determination can therefore be useful to answer the question of cancer vs. non-cancer if the organ or cell-type source is unknown. That the antimalignin antibody test is a general test for cancer is understood following the demonstration of cross-reactivity between extracted peptides from brain gliomas, breast

cancer, and lymphoma, as described under basic mechanisms, together with the finding that the active epitope responsible for this antigenicity and antibody formation is a replikin structure which shares key structural features with the other common cancer replikins, and the realization that replikins are related as a class to the phenomenon of rapid replication rather than cell or organ type.

### **A Second 'General' Cancer Test .**

While an accurate 'general' serum cancer test has not been previously described, it should be noted that the microscopic examination of morphological characteristics of rapidly replicating metastatic cells by the unaided but trained eye of the pathologist, in lymph nodes for example, has for a century provided a reliable 'general' cancer test, providing a distinction between benign and malignant cells independent of the ability to define the cell type of origin. It is therefore not surprising that changes which are visible to the naked eye should have distinguishable biochemical differences visible in immunofluorescent staining, due to the exposed peptide malignin<sup>58</sup>, and a resultant autoantibody measurable in serum, antimalignin antibody.

### **Test of Sample Randomness.**

That a random sample was achieved in this study is supported by the conformity of the relative numbers in the sample of the different types of cancer, benign and malignant breast tumors, the number in remission, and the number with recurrence<sup>30</sup>. For example, the % recurrence is in the expected range in the following two tests of the data: 1) % recurrence in single determinations in Figure 19 = number of recurrent cancer in Group III(Group III Recurrence),divided by Group III Recurrence plus number in remission in Group IV, Figure 19 = 21.4%; 2) % recurrence in longitudinal studies in Figure 20 and 21 = 18.3%. These are also in the range of the 15.7% recurrence in another study<sup>8</sup> of smaller N and shorter follow-up time.

### **False Negatives.**

In non-advanced and non-terminal cases, if false negatives are expressed as percent of malignancies missed by the antimalignin test, then the false negative rate in this study is 2.4% (see Figure 19, Group III). However, if false negatives are expressed as percent of true-plus-false negatives, and all 8,040 data (Groups I through V) are included, then the false negative rate is 0.1%. Almost all known advanced and terminal malignancy cases<sup>35</sup> were excluded from this study by the referring physicians (see Methods), yet most false negatives were associated with malignancies larger than 1 cm, of longer than three years duration, or with metastases. If not so recognized clinically, these advanced cases would have increased the false negative rate. Although there were no false negatives in the small group of 82 patients followed longitudinally up to July 1, 1993 in the present study, the larger group studied longitudinally since 1993, for which follow-up is not yet complete, shows a false negative rate of at least 3%.

### **False Positives and Intermittent Elevations.**

87.8% of the 'Benign Tumor' Group (Fig. 19, Group II) had normal antimalignin values. The elevated values observed in 12.2% of this Group do not all likely represent 'false' positives, since in this randomized sample of 8,090 specimens, false positives also would have appeared to a similar extent in three other Groups, - but did not. Thus, the false positive rate in the 'No Tumor' Group (Group I) was 0%, in the 'Remission' Group (Group IV), 0.3%, and in the Normal Control Group (Group V), 3%. The 12.2% 'false' positive rate in the 'Benign Tumor' Group therefore cannot be considered to be solely due to laboratory or other error. At least some of the elevated values in the Benign Tumor Group may reflect the presence of small clusters of malignant cells which were not detected microscopically. Indeed, for both of the only two patients of the 'Benign Tumor' group with elevated antimalignin values for whom the tissue specimen was subjected to additional sections and reexamination, small clusters of frankly malignant cells were observed. In the present study, even one elevated antimalignin is counted as positive, while in most antigen marker studies, two consecutive elevations are required before assigning a positive value<sup>8</sup>. Since the antimalignin false positive rate is from 0 to 3% in different non-tumor pathological groups and healthy controls<sup>51,53,61</sup>, the antimalignin test can be useful in combination with the antigen marker tests like PSA which have false positive rates up to 70%<sup>27-29</sup>.

Intermittent pre-clinical elevations of antimalignin, followed days or weeks later by a return to normal levels have been observed and will be discussed in a later communication. Briefly, these are 'false positives' by definition, since they occur in the absence of demonstrable signs or symptoms of cancer. However, in three studies to be published, these elevations were associated with 1) the development of clinical cancer within three years in four out of five patients who had these elevations, 2) 60% of cases of pre-hepatoma chronic Hepatitis B infection, a known precancerous condition, and 3) in rats, with the pre-hepatoma appearance of malignant cells in solvent-induced carcinoma of liver. In the light of the above evidence of elevation of antimalignin with the early appearance of malignant cells pre-tumor mass, because of the clear association of single and repeated elevated antimalignin values with early active clinical cancer<sup>9-41</sup> while the false positive rate is so low, even an asymptomatic 'false' positive antimalignin is assumed at a high level of probability to be associated with the presence of malignant cells not yet at the critical mass or site to permit clinical detection. Indeed, asymptomatic cancer detected with antimalignin elevation, histopathologically proven and treated, has been described<sup>57,59,82</sup>. Because antimalignin is cytotoxic to cancer cells at very low concentrations, it is possible, and with the evidence at hand the most likely hypothesis, that the intermittent increase in the concentration of antimalignin antibody has resulted from the pre-clinical appearance of malignant cells, followed by the return to normal antimalignin levels after their destruction. Therefore, an intermittent antimalignin elevation is not ignored by most physicians, especially in high risk patients, but is treated as a possible early warning stimulating increased alert, and more frequent patient follow-up visits.

### **Prediction of Risk vs. Present Transformation**

Unlike genetic alterations in DNA which may predict relative future risk of developing cancer rather than indicate its presence, antimalignin elevation is directly correlated with the presence of transformed, i.e., malignant cells<sup>35-67</sup>. Thus the antimalignin test is not predictive of future malignancy; if elevated, it is indicative of present malignancy, that is of the presence of malignant cells. On the other hand, the absolute concentration of antimalignin antibody at the time of active clinical cancer has been shown to have some quantitative predictive value with regard to survival; that is, the higher the concentration of antimalignin the longer the survival of cancer patients<sup>51,53</sup>. Antimalignin compliments DNA testing since if DNA results suggest higher risk, the antimalignin test can help indicate the current presence or absence of malignancy.

### **Public Health Considerations.**

In addition to reviewing the limitations imposed by the false positive and false negative rates, in signing the Consent Form (see Methods) the patient and the physician verify each time the test is ordered that they understand that the antimalignin test by itself is not diagnostic of the presence or absence of disease. Nonetheless, efforts at the early detection of cancer are stressful<sup>54</sup>. The patient and physician together need to decide whether the effort is worth the stress and the cost, not only of the antimalignin test but also of any follow-up tests of other types which may be indicated. The antimalignin test is comparable in cost to mammography. The American Cancer Society has educated the public for decades to the benefits of early detection, now stating that 95% of cancer patients' lives could be saved with early detection<sup>55</sup>. In the case of a young woman whose mother and grandmother both died of breast cancer, the choice of whether or not to pursue early detection is easier. With stress already present due to the family history, rather than adopting a 'head in the sand' approach or passively waiting for a lump or a sign on mammography to appear, such high-risk patients usually choose to employ every reasonable means to increase the chances of early detection and early treatment. The detection with the antimalignin test of completely asymptomatic cancer, confirmed by needle biopsy then treated, introduces the possibility of even earlier cancer diagnosis and treatment<sup>62</sup>. While the antimalignin test can be added to other methods which are employed for early detection, we believe that it should not be used to substitute for any other early detection procedures of proven value such as mammography.

### **Direct Evidence of An Immune Defense Against Cancer.**

There have previously been many indirect types of evidence, such as the increased incidence of certain malignancies in immunocompromised states<sup>83,84</sup>, but little if any direct evidence in patients of the participation of the immune system in a defense against cancer. The demonstration that an antibody against a specific cancer cell epitope, antimalignin, is universally present in all healthy individuals<sup>36</sup>, increases in its concentration with age within the normal range in proportion to the increase in the risk of cancer<sup>52</sup>, is present in greater concentrations in healthy individuals of families with a high frequency of cancer<sup>62</sup>, is a potent anti-cancer agent *in vitro* in the picogram (femtomole) range per cancer cell<sup>56,66</sup>, increases markedly in concentration early in malignancy<sup>35-67</sup>, can be eluted from cancer cells removed at surgery in cancer patients<sup>49</sup>,

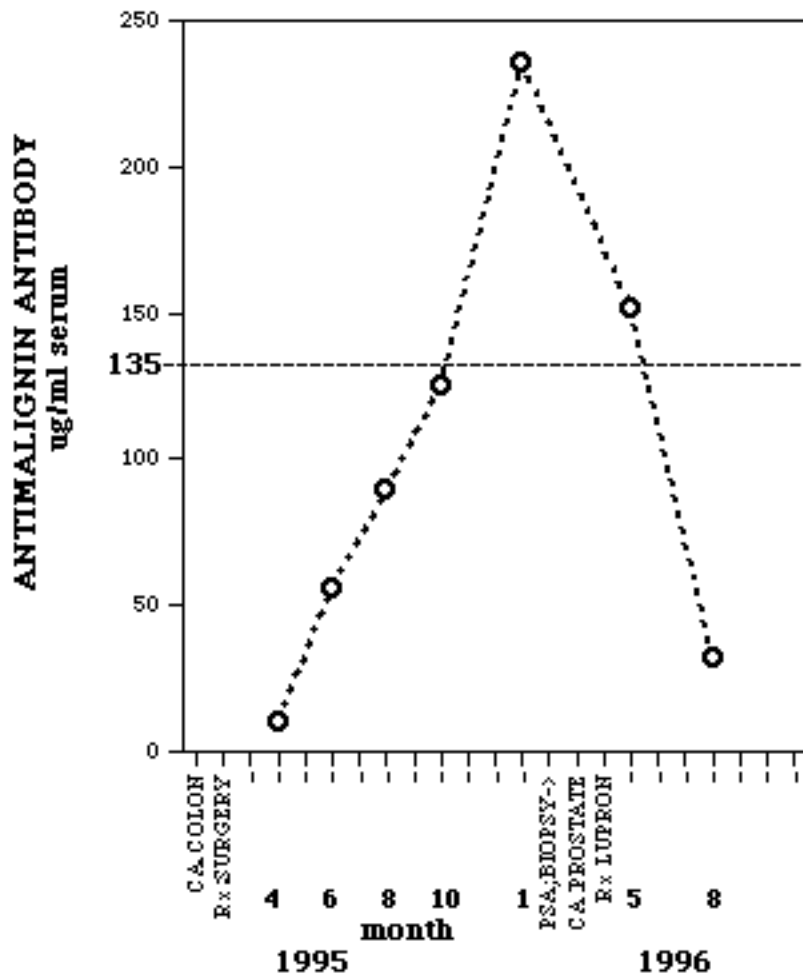
is quantitatively related to survival of cancer patients<sup>51,53</sup>, and here is shown to return to normal levels with clinical remission of breast cancer and to increase again with recurrence, - all are direct evidence consistent with a cancer immune defense function for antimalignin in humans. The possibility of augmenting this defense by active immunization with a vaccine derived from malignin, or by passive immunization with human antimalignin antibody, now both appear to be feasible<sup>52</sup>.

## **Conclusion**

Antimalignin antibody in serum concentration is here found to provide an objective, quantitative molecular index of recovery from breast cancer. As in all clinical diagnostic tests, the antimalignin test is not by itself diagnostic of the presence or absence of disease, but remission can be assumed with greater certainty when, together with other signs, the patient's elevated antimalignin has returned to and remains within the normal range.

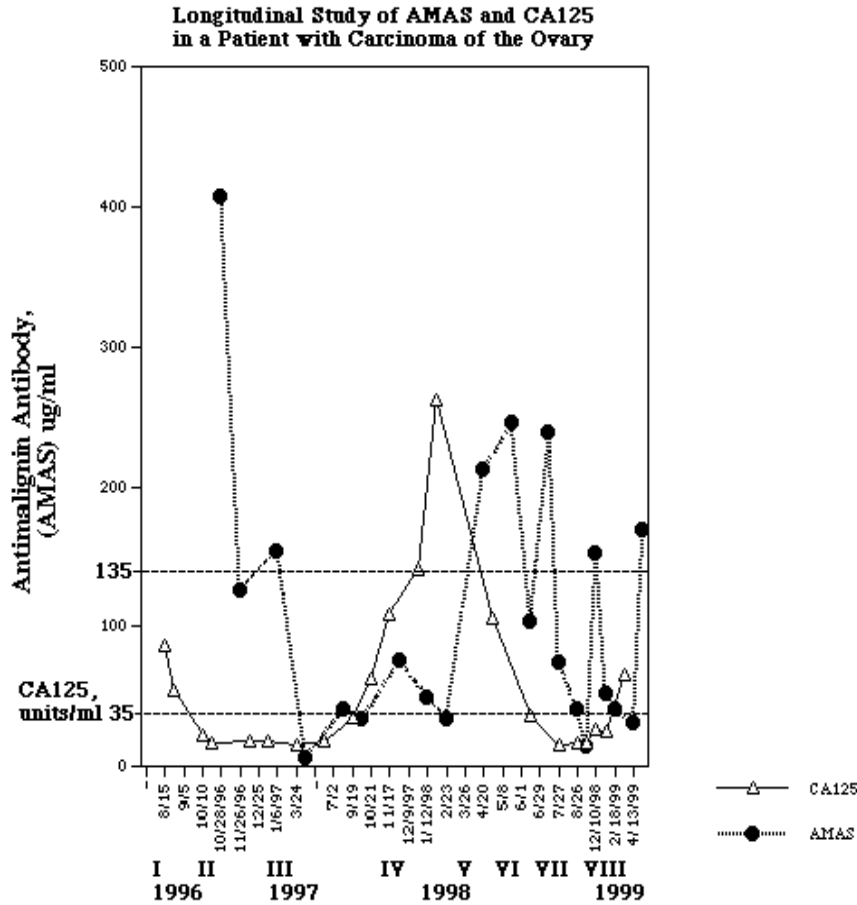
## **Figure 22**

**EARLY DETECTION OF PROSTATE  
CANCER WITH THE AMAS TEST**



*Legend for Figure 22 – Following the successful surgical treatment of cancer of the colon in this patient, the AMAS test was performed monthly to search for advance warning of a recurrence. A rising concentration of AMAS suggested recurrence but colonoscopy revealed no recurring lesion. Performing other tests to attempt to account for the rising AMAS, a PSA test was then also found to be elevated, biopsy revealed a cancer of the prostate, and after treatment with Lupron both the PSA and AMAS returned to normal.*

Figure 23 - Antimalignin Antibody in Ovarian Cancer



**Legend for**

**Figure 23** – Longitudinal studies in ovarian cancer have become more possible to conduct since effective repeated courses of chemotherapy have been introduced. In this case, comparison of AMAS performed by our laboratory, with CA125, performed independently and blind by another laboratory, is useful since the first is an antibody, the second an antigen. From stages I to II in Figure 23, a course of chemotherapy has resulted in improvement in the patient’s symptoms and a decline in CA125. However AMAS, which was also reduced, took longer to return to low normal levels, until stage III. The recurrence of the malignancy was evidenced by the return of signs and symptoms and by the increase in both CA125 and AMAS in stage IV. However, as frequently observed in other cases in which cancer advances either in terms of tumor mass or metastases, or both, as the antigen release (in this case CA125) is increased (Stage IV to V), AMAS production and/or release is inhibited. Another course of effective chemotherapy followed (Stages V to VII), reducing both the clinical signs of the disease

*and the concentration of CA125; during this period, AMAS (?liberated from antigen excess) again rises, perhaps then 'mopping up' remaining cancer cells, until in stage VII both CA125 and AMAS become normal. Clinical recurrence in stage VIII was again accompanied by a rise of AMAS in advance of the rise of CA125, which again was accompanied by 'inhibition' of the AMAS increase.*

### **Summary: replikins in cancer**

The shared antigenicity of the replikins of cancer, is seen in the examples above, whether the cell type is brain, colon, breast, ovarian, prostate, or other common cancers, all giving rise to increased antimalignin antibody.

The data in Figure 12 clearly suggests the process of immunosurveillance because the increase with age follows a curve consistent with similar curves for 1) the increase in DNA errors with age, and 2) the incidence of cancer with increasing age. Also relevant:

- 3) antimalignin antibody has a high specificity for a wide variety of cell types of cancer (Figures 13 – 23);
- 4) antimalignin antibody is highly cytotoxic to cancer cells in vitro (Figure 14);
- 5) antimalignin increases further in concentration in active cancer (Figure 15, 16 and 19);
- 6) antimalignin is quantitatively related to survival of human cancer patients (Figure 17);
- 7) antimalignin returns to normal with remission of cancer (Figure 19 and 21);
- 8) antimalignin increases again on recurrence (Figure 20); and
- 9) the concentration of antimalignin antibody relates quantitatively to the survival of cancer patients (Figure 18), the only such relationship which, to our knowledge has been described.

Taken together, these data support the notion of a lifelong immune defense in cancer of which one component is antimalignin antibody.

For cancer to become clinically apparent, two processes are required:

- 1) transformation of cells to the malignant state, and 2) rapid replication. Taken together, these results suggest that anti-replikin antibodies may be a part of a mechanism of control of both cell transformation and replication.

As in the case of the difference between dormant infectious viruses and raging epidemics, without rapid replication, malignant transformation alone frequently goes unobserved. Thus 70% of men in their 70s have been found at autopsy following unrelated accidental deaths to have malignant cells in their prostate, dormant and without symptoms. Only a small percent of these acquire the second component, rapid replication, to make the process clinically apparent and the risk much greater. Just as we have seen in the case of the influenza viruses, where low replikin counts spell dormancy and rapid replication and high replikin count indicate epidemics or pandemics, and in HIV where low replikin counts spell slower progression and higher replikin counts indicate advanced disease, the replikin counts directly observed in glioblastoma multiforme in culture and indirectly



observed through the increased antibody concentration in brain and other cancers in patients distinguish dormant from rapidly replicating killer replikins.

The detailed mechanisms of action of replikins in both malignant transformation and rapid replication are as yet unknown. The replikins provide substrates to examine both states. As shown in glioma cells, the stage in cancer at which cells have only been transformed to the immortal malignant state but remain quiescent or dormant, now can be distinguished from the more active life-threatening replicating state, which is characterized by the increased concentration of replikins. In addition, clues to the viral pathogenesis of cancer may be found for example in the fact that glioma glycoprotein 10B has a 50% reduction in carbohydrate residues when compared to the normal 10B. This reduction is associated with virus entry in the case of influenza (see sialoresponsins<sup>89</sup>), and so may be evidence of the attachment of virus for the delivery of tumor virus replikins into glial cells as a step in the transformation to the malignant state.

The fact that we are just at the beginning of our understanding of the basic mechanisms of action of the replikins does not inhibit our use of what we have learned of the structure and immunogenicity of the replikins to devise accurate synthetic vaccines against them. This will be discussed in the chapter on replikin immunity.

This communication is one of four submitted together:

Genome Replikin Count™ Predicts Increased Lethality of Resistant Tuberculosis

Genome Replikin Count™ Predicts Increased Lethality of Malaria

Genome Replikin Count™ Predicts Increased Lethality of Cancer

Genome Replikin Count™ Predicts Increased Infectivity/Lethality of Viruses

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