A New Immunoassay for Quantification of a Novel Cancer Antigen in Serum and Immunostaining of Carcinoma Tissues and Cultured Cells Revealing the Antigenic Cellular Location.

Emily Christine McDuffee

East Tennessee State University

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A New Immunoassay for Quantification of a Novel Cancer Antigen in Serum and Immunostaining of Carcinoma Tissues and Cultured Cells Revealing the Antigenic Cellular Location

A dissertation
presented to
the faculty of the Department of Anatomy and Cell Biology
East Tennessee State University

In partial fulfillment
of the requirements for the degree
of doctorate in Biomedical Sciences

by
Emily McDuffee
December 2002

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Keywords: ELISA, cancer, chicken antibodies, tumor marker
ABSTRACT

A New Immunoassay for Quantification of a Novel Cancer Antigen in Serum and Immunostaining of Carcinoma Tissues and Cultured Cells Revealing the Antigenic Cellular Location

by

Emily McDuffee

The purpose of this study is 1) to examine the presence of the antigen in serum by employing a newly developed ELISA immunoassay that quantifies the total antigen and bound antigen (antigen-antibody complex) using polyclonal chicken antibodies directed against an IgM-binding epitope of the new antigen, and 2) to determine the location of the antigen in carcinoma and normal cells. Sera from healthy volunteers (n = 147) and cancer patients (n = 26) were compared for both bound and total antigen concentrations using the new ELISA. Healthy volunteers were subdivided into three groups: those with a personal history of cancer (n = 13), those with no personal or family history of cancer, (n = 36) and those with a family history of cancer (n = 97). Ovarian, breast, colon carcinoma tissues and their normal counterparts and cultured ovarian and prostatic carcinoma cells were subjected to immunofluorescence using IgY antibodies and goat anti-chicken fluorescent secondary antibodies. Basic imaging was performed on tissue sections while confocal microscopy was performed on cultured cells. Furthermore, immunohisto-chemical staining using an anti-chicken HRP-conjugated secondary antibody was performed on 16 normal ovarian tissues, 53 ovarian adenocarcinomas, and 3 borderline ovarian tumors. Statistical analysis revealed significant differences in cancer patients’ bound and total antigen levels compared to that of healthy volunteers (p < 0.005). Bound and total antigen levels of cancer patients were also significantly higher than those of the healthy volunteers with no personal or family history of cancer and
those with a family history of cancer (p < 0.01). However, no significant difference existed between the bound (p > 0.120) and total antigen levels (p > 0.076) of cancer patients and patients with a personal cancer history. Immunohistochemical staining of ovarian tissues revealed a significant difference in the lumenal staining of the carcinomas compared to that of the normal ovarian tissues. Furthermore, fluorescence imaging revealed that the antigen is localized to the cell membranes of the carcinoma cells but is absent from the normal tissues. Confocal microscopy further emphasized the antigen’s association with the membrane and also revealed some filamentous cortical staining.
DEDICATION

This work is dedicated to my ever loving and supportive family. To my parents, Larry and Linda McDuffee, I thank you for your patience, encouragement, support, but most of all, your unfailing love. To my sister and brothers, thank you for your love and for reminding me to keep things in perspective. I would also like to thank all of my friends who have supported me during the past few years for being the arms that pushed me when I needed encouragement and the shoulders that I could cry on when life was extremely difficult. I could not have done this without any of you.
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Finally, it is with sincere humility that I extend my deepest thanks to my committee who have contributed their time, shared their wisdom, challenged my thinking, and showed great patience during the course of this project. Thank you, Drs. Paul Monaco, Mark Airhart, Scott Champney, Richard Skalko, Jerry Thornthwaite, and Rob Wondergem.

I especially wish to acknowledge my mentor, Dr. Jerry Thornthwaite, for providing me the opportunity to conduct my research under his instruction. Without his constant encouragement and support, this work would not have been possible. Thank you, Jerry, for all that you have done.

Thank you to the Institutional Review Board (RPN#: 99-9-02) of Freed-Hardeman University for their approval of this study.

The entire CARE test, including all reagents, is under current review as a U.S. and foreign patent application.
LIST OF ABBREVIATIONS

Ab – antibody
ADCC – antibody-dependent cell-mediated cytotoxicity
AFP – α-fetoprotein
Ag – antigen
AOTF – acoustooptical tunable filter
ASCO – American Society of Clinical Oncologists
ATCC – American Type Culture Collection

BLAST – Basic Local Alignment Search Tool
BPH – benign prostatic hyperplasia
BSA – bovine serum albumin

CA – cancer antigen
CA 15-3 – cancer antigen 15-3
CA 19-9 – cancer antigen 19-9
CA 125 – cancer antigen 125
CA 27.29 – cancer antigen 27.29
CARE – CAncer REcognition
CBI – Commonwealth Biotechnologies Incorporated
CDRS – complementary-determining regions
CEA – carcinoembryonic antigen
CH – constant heavy domain
CHTN – Cooperative Human Tissue Network
CL Domain – constant light domain

ΔOD – delta OD – difference in OD of sample and OD of plate blank
DMEM – Dulbecco’s Modified Eagle’s Medium
DRE – digital rectal exam

EDTA – ethylenediaminetetraacetic acid
ELISA – enzyme-linked immunosorbent assay
ER – estrogen receptor

FIGO – Federation of Gynaecology and Obstetrics
FN – false negative
FP – false positive
FPLC – fast performance liquid chromatography

HAMA – human anti-mouse antibody
HBSS – Hank’s balanced salt solution
HCC – hepatocellular carcinoma
HRP – horseradish peroxidase
HUVEC – human umbilical vascular endothelial cells
HV – healthy volunteers
IRB – institutional review board

MAbs – monoclonal antibodies
MRC – mean relative concentration
MWM – molecular weight markers

NCBI – National Center for Biotechnology
NET OD – net optical density
NPV – negative predictive value

OD – optical density

PBE – PBS, EDTA, sodium azide
PBS – phosphate buffered saline
PPD – p-phenylenediamine
PPV – positive predictive value
PR – progesterone receptor
PSA – prostate specific antigen
PTB – phosphate buffer, Tween 20, BSA buffer
PTWB – plate washing buffer

SD – standard deviation
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPL - sample
STD – standard
SUE – surrounding uninvolved epithelium

TMB - tetramethylbenzidine
TN – true negative
TP – true positive
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CHAPTER 1
GENERAL INTRODUCTION

Introduction

Despite all of the research that has been done to prevent cancer, it is second only to heart disease as the leading cause of death in the US (American Cancer Society 2002). About 1.3 million new cancer cases are expected to be diagnosed in the US in 2002 (American Cancer Society 2002). Approximately 555,500 Americans will die of cancer in 2002, which is more than 1500 people per day (American Cancer Society 2002). Despite great emphasis on screening and prevention, the incidence of certain tumors continues to rise, and the mortality rates for certain cancers have changed little in the past decade (Figure 1) (Abbott 2002). Data displayed in Figure 1 may actually be an underestimation according to some researchers. Some argue that cancer mortality should not only include deaths from cancer but also deaths caused by the treatment for cancer (Hopkins Tanne 2002).

The key to cancer survival and treatment is early detection of the disease. Detecting cancer in its early stages is not an easy task due to the “silent” period of tumor growth when those affected are asymptomatic. Cancer is the result of a normal cell undergoing a malignant transformation resulting in uncontrolled growth, differentiation, and invasiveness due to loss of contact inhibition. Cell divisions result in one transformed cell becoming two abnormal cells that then become four abnormal cells, etc. This type of division is called doubling time. For some fast-growing cancer, the doubling time may be one to four weeks; slower-growing cancers may double in a few months (Dollinger et al. 1997). By the time a tumor is the size of a pinhead, it may contain over one million cells yet still be undetectable. For a tumor to be visualized by X-ray, it must be about 1 cm in diameter and will contain about one billion cells (Dollinger et al. 1997). It is likely at this stage that a person with cancer will be able to feel a lump or will be
suffering from symptoms such as pain or bleeding. Nevertheless, even at this stage, a person may be asymptomatic.

**Figure 1**

*Mortality Rates For Certain Cancers*[^Abbott2002]


Figure 1 shows deaths of females and males for 1990 and 2000 from lung, breast (females), prostate (males), and colon & rectal cancers in the United States.

*Slow progress: US mortality rates for the major cancer killers have changed little in 10 years.*

*Adapted from Abbott 2002.*

Figure 1 shows deaths of females and males for 1990 and 2000 from lung, breast (females), prostate (males), and colon & rectal cancers in the United States.
Detecting the cancer early allows for a person to be potentially cured. The problem is that the war in early detection of cancer is being lost, and cancer morbidity and mortality are changing very little, if not increasing. One area of clinical research devoted to early cancer detection is in the area of tumor markers.

Cancer cells produce enzymes, serum proteins, metabolites, and hormones that may become elevated during tumor growth. In addition, they must express different cell surface components than normal cells because cancer cells are able to migrate and invade surrounding tissues. These cell surface components and the cancer cell products can be used as tumor markers. Tumor markers are defined as substances that are “present in or produced by a tumor, or the host, that can be used for differentiating neoplastic from normal tissue based on measurements of blood or secretions” (Way and Kessler 1996). Furthermore, tumor marker assays may be able to detect tiny cancers not visible by X-ray or MRI (Dollinger et al. 1997).

The measurement of tumor markers is less invasive and expensive than other clinical tests. The use of serological measurements of certain tumor markers have increased significantly in the past few years. They show some value in clinical practice for aiding in the diagnosis of cancer, monitoring cancer progression or regression, and in monitoring recurrence of disease. Despite the number of markers and the research devoted in this area, there are currently no markers that are approved by the American Society of Clinical Oncologists (ASCO) for the screening of cancer, because they lack the necessary sensitivity and specificity.

*Sensitivity and specificity*. The ability of a tumor marker to be used in a clinical setting depends on its sensitivity and specificity. These two parameters are thoroughly investigated by the U.S. Food and Drug Administration (Aziz and Maxim 1993). When developing a test, the test result must be compared to the true diagnosis. In the case of cancer, the true diagnosis is typically based upon pathology. Those that are disease positive have cancer while those that are disease negative do not. The proportion of the
patients who are disease positive and who are also test positive is known as the sensitivity (Bland 1997). True positives are the patients in a population with a positive test result for a tumor marker who have cancer (Bland 1997; Wu and Nakamura 1997). False positives are the patients who have a positive test result but do not have cancer (Bland 1997; Wu and Nakamura 1997). Sensitivity is calculated from the sum of the number of patients who are both disease positive and test positive divided by the number who are disease positive (Table 1). The proportion of patients who are disease negative and who are also test negative is known as the specificity (Bland 1997). True negatives are the patients who have a negative test result and do not have cancer (Bland 1997; Wu and Nakamura 1997). False negatives are the patients who have a negative test result but do have cancer (Bland 1997; Wu and Nakamura 1997). Specificity is calculated from the sum of the patients who are both disease negative and test negative divided by the number who are disease negative (Table 1).

**Table 1**

**Definitions Of Statistical Criteria For Evaluating Tumor Marker Assays**

<table>
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<tr>
<th>Test Results</th>
<th>Cancer Present</th>
<th>Cancer Absent</th>
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<tr>
<td>Positive</td>
<td>TP</td>
<td>FP</td>
</tr>
<tr>
<td>Negative</td>
<td>FN</td>
<td>TN</td>
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\[
\text{Sensitivity} = \frac{TP}{(TP + FN)} \times 100
\]

\[
\text{Specificity} = \frac{TN}{(FP + TN)} \times 100
\]

*Source: Adapted from Rimer et al. 2001.

Table 1 defines statistical criteria involved in evaluating tumor marker assays. TP (true positives) are those patients who have cancer and have a positive test result. FP (false positives) are those patients who have a positive test result but do not have cancer. FN (false negatives) are those patients who have a negative test result but do have cancer. TN (true negatives) are those patients who have a negative test result and do not have cancer.
Sensitivity and specificity of a test depend upon the study design, cut-off levels, population studied, comparisons to normal populations and populations with benign disease, grade of tumor, and combination of tumor markers with other diagnostic tests (Way and Kessler 1996). The sensitivity and specificity of the test can be changed by determining the cut-off level. Given that high values are indicative of disease, if the cut-off point is elevated, the sensitivity will be decreased, but there will be fewer false-positives causing the specificity to be increased (Bland 1997). In contrast, if the cut-off point is lowered, the sensitivity will be increased, but there will be an increase in the false-positives causing a decrease of specificity (Bland 1997). It is evident that there will be a trade-off between specificity and sensitivity depending upon where the cut-off point is set. Therefore, no tumor markers to date have ever reached 100% specificity and 100% sensitivity. Some common tumor markers are alpha-fetoprotein (AFP), CA 15-3, CA 19-9, CA 125, carcinoembryonic antigen (CEA), and prostate specific antigen (PSA). These tumor markers are briefly discussed below.

Alpha-fetoprotein (AFP) is a glycoprotein produced in large amounts by the liver and the yolk sac of the fetus. AFP becomes replaced by albumin postpartum, and in the normal adult AFP can be found in circulation in concentrations of no more than 10ng/ml (Way and Kessler 1996). Cancerous cells tend to revert to a phenotype found in fetal life, and, therefore, would express AFP. Increased levels of AFP can be found associated with hepatocellular carcinoma (HCC) lending it a valuable marker for HCC. AFP is the gold standard tumor marker used for HCC. Approximately 50% of patients with primary liver cancer have elevated serum AFP levels of >1000 ng/ml (Wu and Nakamura 1997). AFP is also elevated in pregnancy and benign liver disease such as cirrhosis and hepatitis (Way and Kessler 1996). One study of 188 patients showed the cut-off for AFP to be set at 100 ng/ml lending a 55% sensitivity and a 99% specificity (Tsai et al. 1997). Another study of 256 patients showed a 65% sensitivity and a 100% specificity with a cut-off of 120 ng/ml (Tsai et al. 1995).
CA 15-3 is an epitope of a mucin glycoprotein used as the gold standard for circulating tumor markers in breast cancer (Duffy 1999). It is found elevated in 70-80% of patients with metastatic breast cancer using a cut-off of >35 U/ml (Wu and Nakamura 1997). This marker has also been found to be elevated in a number of benign conditions such as hepatitis, cirrhosis, tuberculosis, benign breast disease, and lupus (Wu and Nakamura 1997; Duffy 1999) and in non-breast malignancies such as colorectal cancer, lung cancer and ovarian and pancreatic malignancies (Duffy 1999). Furthermore, despite different cut-off levels, preoperative CA 15-3 levels are rarely elevated in primary breast carcinoma patients (Duffy 1999). CA 15-3 is a more sensitive and specific marker for monitoring patients with metastatic breast cancer than that of carcinoembryonic antigen (CEA) (Wu and Nakamura 1997). One study of 103 patients without breast metastasis showed that during follow-up, 20% of the patients had a recurrence of cancer with an overall CA 15-3 sensitivity of 61% and specificity of 91% (Lumachi et al. 1999). Another study of 70 metastatic breast cancer patients showed CA 15-3 sensitivity to be 79% (Lauro et al. 1999). One study including 53 patients with metastases showed that CA 15-3 sensitivity was 81% in those metastatic patients (Guadagni et al. 2001). Eskelinen et al., reported CA 15-3 sensitivity at 27% and a specificity of 90% in a study of 624 patients (1997).

CA 19-9 is a carbohydrate antigenic determine expressed on a high molecular weight mucin (Wu and Nakamura 1997). CA 19-9 is related to the Lewis A blood group substance (Duffy 1998), and only those cancer patients that are Lea⁺b⁻ or Lea⁻b⁺ will be CA 19-9 positive (Wu and Nakamura 1997). CA 19-9 is the gold standard tumor marker for pancreatic cancer (Maestranzi et al. 1998). CA 19-9 must be used with other diagnostic tests, such as computed tomography (CT) scans, to distinguish benign from malignant disease, because CA 19-9 may be elevated in benign pancreatic disease such as pancreatitis. It is reported that approximately 20% of patients with pancreatic cancer may have normal levels of CA 19-9 (Duffy 1998). This marker is not organ specific and is
elevated in other adenocarcinomas including biliary tract cancers (Maestranzi et al. 1998), lung, colorectal, and other gastrointestinal cancers (Wu and Nakamura 1997). Duffy reports that in one study, 24 reports of CA 19-9 in pancreatic cancer were evaluated and reflected a mean sensitivity of 81% and specificity of 90% at a cut-off value of 37 U/mL (1998). If the cut-off increased to 100 U/mL, sensitivity decreased to 68% and specificity increased to 98% (Duffy 1998). In one study of 20,517 patients including 160 with pancreatic diseases, 322 with biliary tract diseases, and 20,035 asymptomatic controls, CA 19-9 for patients with pancreatic cancer had a sensitivity of 76.7% and specificity of 87.1% (Kim et al. 1999). In another study including 144 patients, 58 of which had pancreatic carcinoma, CA 19-9 sensitivity was 81% and the specificity was 95% (Pasquali et al. 1994). One study of 100 patients, 40 of whom had pancreatic cancer, reported CA 19-9 sensitivity at cut-off values of >37 U/ml and >75 U/ml were 90% and 80%, respectively, with specificity values of 70% and 85%, respectively (Nazli 2000).

CA 125 is currently the best serum-based tumor marker for ovarian cancer. It is elevated most consistently in epithelial ovarian carcinomas of serous and endometrioid histological types and less in mucinous ovarian carcinomas (Vergote et al. 1987; Kudoh et al. 1999; Zygmunt et al. 1999; Engelen et al. 2000). CA 125 is a high molecular weight glycoprotein recognized by the OC 125 monoclonal antibody derived from mice immunized with the human serous cystadenocarcinoma cell line OVCA 433 (Bast 1981). CA 125 is elevated in numerous benign conditions and non-ovarian malignancies as shown in Table 2 (Bast et al. 1983; Bast et al. 1998; Thompson 1998; Markman 2000). Sensitivity of CA 125 for second look surgery is 46% and specificity is 99% (Way and Kessler 1996). It is not recommended for screening due to the low prevalence of ovarian cancer and its elevation in many benign conditions (Way and Kessler 1996). In the United States, ovarian cancer occurs at a relatively low incidence among post-menopausal women, approximately 40-50 per 100,000 (Bast et al. 1998). With such a low incidence, a screening test for the general population would require a specificity of 99.6% and a minimum sensitivity of 80% to limit the number of unnecessary surgical
procedures per every case of ovarian cancer diagnosed to 10 (MacDonald and Jacobs 1999). Assuming that a screening procedure had a sensitivity of 80% and a specificity of 98%, there could potentially be 50 surgical procedures for every cancer diagnosis (Teneriello and Park 1995). One study of 90 women, 30 having proven epithelial ovarian cancer, with a set specificity of 95%, had a sensitivity of 76.7% (Fayed et al. 1998). Berek and Bast (1995) have reported CA 125 as having a sensitivity of 85% and a specificity of 99.7%. In a study including 39 patients with serous adenocarcinoma of the ovary, sensitivity for those at FIGO stage I was 50% while sensitivity for those at FIGO stage I-IV was 89.7% (But and Gorisek 1996).
Carcinoembryonic antigen (CEA) is a family of related glycoproteins that express multiple antigenic determinants. CEA, an oncofetal protein, is detectable at low concentrations in normal persons but is elevated in fetal tissue and tumors. Initially, CEA was hoped to be a specific and sensitive marker for colorectal cancer and other malignancies of the gastrointestinal tract. However, CEA is not specific for colon cancer and not all colon tumors produce CEA; only 50-60% of patients with colon carcinoma show elevated CEA levels (Wu and Nakamura 1997). CEA is elevated in smokers and those with renal failure and other numerous benign diseases such as liver and bowel
diseases (Fletcher 1986). Diseases of the liver and kidneys affect the clearance of CEA and, therefore, lead to elevated concentrations of CEA (Fletcher 1986; Wu and Nakamura 1997). At a cut-off level of 2.5 ng/mL, CEA sensitivity ranges from 30% to 85% depending on the stage of the disease in colorectal cancer (Fletcher 1986). CEA is not likely to detect colorectal cancer at an early stage of disease, especially in asymptomatic patients. Depending on the population studied, undetected colorectal cancer has a prevalence of 0.4 to 7/1000 (Fletcher 1986). If colorectal cancer occurred at a prevalence of 1/1000 with a sensitivity set at 40% and a specificity of 90% (for stage A & B cancers), 250 false-positive tests for every one patient with cancer would occur, and 60% of all cancers would be missed (Fletcher 1986). According to Carriquiry and Pineyro (1999), 40% of 209 preoperative patient serums with colorectal cancer had elevated CEA values with highest values correlating to stage IV of disease. In the same study, CEA was an accurate marker for recurrence of disease having a sensitivity of 77% and specificity of 98% (Carriquiry and Pineyro 1999). CEA has limited value for diagnosis, prognosis, and monitoring of patients with colorectal cancer but is not useful for screening due to its low sensitivity and specificity at early stage disease (Fletcher 1986).

Prostate specific antigen (PSA) is a single-chain glycoprotein of 33-34 kD produced by the epithelial cells lining the ducts and acini of the prostate gland and functions as a protease upon ejaculation to liquefy the gelatinous seminal fluid once inside the female reproductive tract Oesterling 1991; Loo and Betancourt 1998). It is used as the gold standard of serological markers for prostate cancer. However, PSA is not prostate cancer-specific but is prostate tissue-specific (Oesterling 1991). Not all prostate cancers have elevated PSA levels. PSA may be elevated in acute urinary retention, acute prostatitis, transrectal needle biopsy and prostate surgery (Coley et al. 1997). Furthermore, Oesterling (1991) reports that of 319 patients with prostate cancer studied, 43% have normal PSA levels while 25% of 597 men with benign prostatic hyperplasia (BPH) have elevated PSA levels. When distinguishing BPH from prostate cancer, if the
cut-off level for PSA is set at > 4 ng/mL and at > 10 ng/mL, the resulting sensitivities are 57% and 23%, respectively, with specificities of 68% and 96%, respectively (Oesterling 1991). PSA’s usefulness as a tumor marker depends upon age and race. The prostate gland increases in size with age, and this demands age-specific reference ranges for men. By using these specific ranges (Table 3), the sensitivity of PSA should increase, aiding in the early detection of cancers in men in their 40s and 50s (Loo and Betancourt 1998). Using the age-specific ranges, sensitivity of PSA is 95% and specificity is ~88% (Way and Kessler 1996). Furthermore, Asians have smaller prostates than Caucasians, and African Americans have more prostate cancer (Loo and Betancourt 1998). Despite these variations, PSA is a valuable screening tool when used in conjunction with the digital rectal examination (DRE) or transrectal ultrasound as well as being a valuable tumor marker for monitoring disease progression and effects of treatment (Wu and Nakamura 1997; Loo and Betancourt 1998).

<table>
<thead>
<tr>
<th>Age</th>
<th>Japanese</th>
<th>African American</th>
<th>Caucasians</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 to 49</td>
<td>0 to 2.0</td>
<td>0 to 2.0</td>
<td>0 to 2.5</td>
</tr>
<tr>
<td>50 to 59</td>
<td>0 to 3.0</td>
<td>0 to 4.0</td>
<td>0 to 3.5</td>
</tr>
<tr>
<td>60 to 69</td>
<td>0 to 4.0</td>
<td>0 to 4.5</td>
<td>0 to 4.5</td>
</tr>
<tr>
<td>70 to 79</td>
<td>0 to 5.0</td>
<td>0 to 5.5</td>
<td>0 to 6.5</td>
</tr>
</tbody>
</table>

*Source: Loo and Betancourt 1998.

Table 3 reveals normal age-specific reference ranges are shown for Asians, African Americans, and Caucasians. Using age-specific ranges makes PSA more sensitive in younger men and more specific in older men. Japanese have smaller prostates compared to Caucasians of the same age while African Americans have more prostate cancer.
A summary of the sensitivities and specificities of the above markers is shown in Table 4. All of these tumor marker assays measure the shedding of a particular protein from the surface of tumor cells. The reasons why there are low sensitivities and specificities with these tumor markers are that the antigen may be on normal tissues (low specificity), and the antigens are shed giving a false positive result. Furthermore, the antigens may be bound so tightly, or are non-existent on some tumors, that no shedding takes place (low sensitivity). In addition, there is a trade-off between sensitivity and specificity, as mentioned above. Increasing sensitivity will decrease specificity and vice versa.
Table 4
Tumor Marker Sensitivity / Specificity
(Measurements From Multiple Studies)
N = 50 to over 3000 patients in studies

<table>
<thead>
<tr>
<th>Tumor Marker</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-fetoprotein (AFP)</td>
<td>65\textsuperscript{a}, 99\textsuperscript{b}, 67\textsuperscript{c}, 98\textsuperscript{d}</td>
<td>93\textsuperscript{a}, 82\textsuperscript{b}, 100\textsuperscript{c}, 65\textsuperscript{d}</td>
</tr>
<tr>
<td>CA 125</td>
<td>55\textsuperscript{e}, 86\textsuperscript{e**}, 78\textsuperscript{f}, 77\textsuperscript{g}, 71\textsuperscript{h}, 80\textsuperscript{i}, 78\textsuperscript{j}</td>
<td>\textsuperscript{-e}, \textsuperscript{-e**}, 77\textsuperscript{f}, 95\textsuperscript{g}, \textsuperscript{-h}, 82\textsuperscript{i}, 77\textsuperscript{j}</td>
</tr>
<tr>
<td>CEA</td>
<td>21\textsuperscript{k}, 36\textsuperscript{l}, 40\textsuperscript{m}, 77\textsuperscript{n}, 83\textsuperscript{o}</td>
<td>95\textsuperscript{k}, 87\textsuperscript{l}, -\textsuperscript{m}, 98\textsuperscript{n}, 72\textsuperscript{o}</td>
</tr>
<tr>
<td>CA 15-3</td>
<td>25\textsuperscript{o}, 33\textsuperscript{p}, 61\textsuperscript{q}, 29\textsuperscript{r}, 13\textsuperscript{s}, 68\textsuperscript{t}, 57\textsuperscript{u}, 26\textsuperscript{v}, 29\textsuperscript{w}</td>
<td>90\textsuperscript{o}, -\textsuperscript{p}, 91\textsuperscript{q}, 90\textsuperscript{r}, 92\textsuperscript{s}, 98\textsuperscript{t}, 97\textsuperscript{u}, -\textsuperscript{v}, -\textsuperscript{w}</td>
</tr>
<tr>
<td>CA 19-9</td>
<td>90\textsuperscript{x}, 81\textsuperscript{y}, 90\textsuperscript{z}, 77\textsuperscript{aa}, 81\textsuperscript{bb}, 70\textsuperscript{cc}</td>
<td>85\textsuperscript{x}, 95\textsuperscript{y}, 70\textsuperscript{z}, 87\textsuperscript{aa}, 90\textsuperscript{bb}, \textsuperscript{cc}</td>
</tr>
<tr>
<td>PSA</td>
<td>84\textsuperscript{dd}, 73\textsuperscript{ee}, 78\textsuperscript{ff}, 65\textsuperscript{ff}</td>
<td>90\textsuperscript{dd}, 77\textsuperscript{ee}, 87\textsuperscript{ff}, 98\textsuperscript{ff}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} only grade 1 ovarian tumors tested.
\textsuperscript{b} only grade 2 ovarian tumors tested.
\textsuperscript{c} Sensitivity – detects those who have cancer.
\textsuperscript{d} Specificity – detects those who do not have cancer.
\textsuperscript{e} Xianyan et al. 1998.
\textsuperscript{f} Khalifa et al. 1999.
\textsuperscript{g} Tsai et al. 1995.
\textsuperscript{h} Way and Kessler 1996.
\textsuperscript{i} But and Gorisek 1996.
\textsuperscript{j} Berek and Bast 1995.
\textsuperscript{k} Fayed et al. 1998.
\textsuperscript{l} Ind et al. 1997.
\textsuperscript{m} Peters-Engl et al. 1995.
\textsuperscript{n} Woolas et al. 1995.
\textsuperscript{o} Fernandez-Fernandez et al. 1996.
\textsuperscript{p} Fletcher 1986.
\textsuperscript{q} Carriquiry and Pineyro 1999.
\textsuperscript{r} Lopez et al. 1999.
\textsuperscript{s} Eskelinen et al. 1997.
\textsuperscript{t} Guadagni et al. 2001.
\textsuperscript{u} Lumachi et al. 1999.
\textsuperscript{v} Gion et al. 1999.
\textsuperscript{w} Barrenetxea et al. 1998.
\textsuperscript{x} Pectasides et al. 1996.
\textsuperscript{y} Rodriguez de Paterna et al. 1995.
\textsuperscript{z} Heinze and Lichtenegger 2000.
\textsuperscript{aa} D’Alessandro et al. 2001.
\textsuperscript{ab} Pamies and Crawford 1996.
\textsuperscript{ac} Pasquali et al. 1994.
\textsuperscript{ad} Nazli et al. 2000.
\textsuperscript{ae} Kim et al. 1999.
\textsuperscript{af} Duffy 1998.
\textsuperscript{ag} Slesak et al. 2000.
\textsuperscript{ah} Ceriani et al. 1997.
\textsuperscript{ai} Ghafoor et al. 1998.
\textsuperscript{aj} Heyns et al. 2001.
Building on the research of Thornthwaite (2000), a new human tumor antigen from MCF-7 cultured carcinoma cells has been isolated. Chapter 2 discusses the development of an enzyme-linked immunosorbent assay (ELISA) for the detection of cancer by quantifying the IgM antibody that is specific for this new cancer antigen and on quantifying an IgM antibody response to the cancer antigen. Interestingly, this IgM antibody, unlike the normal immune response, does not undergo a heavy-chain class switch to IgG. To date, there are no tumor marker assays that quantify the human antibody response to cancer. To have the ability to quantify both antibody targeted to a cancer antigen and the antigen itself could be a valuable asset in clinical cancer diagnosis and prognosis. Chapter 3 thus focuses on developing an assay to quantify the cancer antigen, both the total amount of antigen and the amount of bound antigen in an immune complex (antigen-antibody complex). Total antigen includes both bound and free, unbound circulating cancer antigen. The IgM-binding epitope of the cancer antigen has been termed LT11 and is used for immunizing chickens to produce an IgY polyclonal chicken antibody.

*Chicken IgY antibodies.* Avians produce three principal immunoglobulin classes: IgM, IgG, and IgA. The chicken IgG is referred to as IgY and is functionally homologous to the 150kD mammalian IgG (Haak-Frendscho 1994; Sharma 1997). The yolk of eggs that are laid by immunized hens are excellent source of polyclonal antibodies. Egg-laying hens pass IgY along to their offspring in the yolk reflecting a passive immunity much like that of mammals. The IgY is continuously absorbed by the embryo throughout embryogenesis until the 2nd day after hatching (Li et al. 1998). Furthermore, the phylogenetic differences of avians and mammals allow chickens to produce antibodies against conserved mammalian proteins more quickly and specifically than if those antibodies were produced in another mammalian organism such as rabbits (Hansen et al. 1998; Li et al. 1998). For example, Gassmann et al. (1990) failed to
produce antibodies against a highly conserved mammalian replication protein in rabbits and tried immunization of chickens with this protein. They were able to isolate from one hen a total of 4 g of IgY, from which 130 mg was specific against their target protein (Gassmann et al. 1990).

There are other advantages of using chicken IgY for production of polyclonal antibodies. The concentration of antibodies in yolk is higher than that in the serum. For example, in one week a hen produces IgY antibodies equivalent to 180-200 mL of whole blood (Larsson et al. 1993). An immunized rabbit, repeatedly bled, will only yield approximately 20 mL of whole blood per week (Larsson et al. 1993). Furthermore, IgY does not bind to Fc-receptors, and it does not interfere with rheumatoid factors (Jensenius et al. 1981; Hansen et al. 1998). Larsson (1992) has shown that it does not activate human complement. It also does not cross-react with mammalian IgG (Haak-Frendscho 1994; Kummer and Li-Chan 1998). The antibody can be retrieved from egg yolk as opposed to bleeding the animal. In addition, more specific antibody is obtained from one egg yolk than from the serum of a rabbit and at less cost (Hansen et al. 1998; Li et al. 1998). It has been reported that this high yield of IgY per yolk is greater than 100 mg, approximately 10% of which is antigen specific (Kummer and Li-Chan 1998), and specific antibody production continues at a high titer for long periods of time (Gassmann et al. 1990; Sturmer et al. 1992).

Sturmer et al. (1992) immunized chickens with an enzyme isolated from rat medullary thyroid carcinoma, and subsequently used the IgY antibodies to construct a competitive ELISA to quantify the enzyme. Immunizing a hen with this new cancer antigen, LT11, provides an abundant source of an anti-LT11 polyclonal IgY that can be used for several experiments, such as is proposed in this paper. The anti-LT11 is used for trapping LT11 out of the serum of patients in the quantitative ELISA (Chapter 3) and for immunocytochemical staining of carcinoma tissues (Chapter 4).
Immunocytochemical staining, the use of labeled antibodies as specific reagents for localizing certain tissue components, has become an invaluable tool in cancer diagnosis. MRIs, CTs, and X-rays are able to reveal the areas occupied by large tumors. However, microscopic evaluation of surgically removed tumors provides information regarding their grade and histogenesis (Alison and Sarraf 1997). Furthermore, microscopic examination is important because it yields information regarding the extent of invasion and the involvement of small blood vessels, lymphatics, and lymph nodes (McKinnell et al. 1998). Immunocytochemistry plays a vital role in diagnostic tumor pathology when morphology alone cannot reliably be used to infer tissue of origin. It is also useful in understanding the biology of tumor growth, such as the expression of growth factors and their receptors, cell adhesion molecules, and transcription factors (Alison and Sarraf 1997). Some products of cell differentiation that can be detected by immunocytochemistry and that are useful in tumor diagnosis are shown in Table 5. It is important to realize that the markers in Table 5 are present in normal tissues as well as in benign and malignant tissues.

In the present dissertation, Chapter 4 describes using the polyclonal IgY antibody, anti-LT11, to stain tumors and normal tissues in order to evaluate its effectiveness in identifying cancer from normal tissue. Anti-LT11, hopefully, may prove to be a valuable tumor marker for histopathologists as well as be a significant tool for detecting early cancers.
Table 5
Products Of Cell Differentiation That Can Be Detected By Immunocytochemistry\textsuperscript{a}

<table>
<thead>
<tr>
<th>Product</th>
<th>Cell type recognized</th>
<th>Often markers for</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intermediate filament</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytokeratins</td>
<td>Epithelial cells</td>
<td>Carcinomas, mesotheliomas</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Mesenchymal cells</td>
<td>Mesenchymal tumours, lymphomas</td>
</tr>
<tr>
<td>Desmin</td>
<td>Muscle cells</td>
<td>Smooth and striated muscle tumours</td>
</tr>
<tr>
<td>Neurofilaments</td>
<td>Neural cells</td>
<td>Neural tumours</td>
</tr>
<tr>
<td>Glial fibrillary acidic</td>
<td>Glial cells</td>
<td>Gliomas</td>
</tr>
<tr>
<td>protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tissue specific proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-100</td>
<td>Ubiquitous</td>
<td>Gliomas, schwannomas melanomas</td>
</tr>
<tr>
<td>Prostatic acid phosphatase</td>
<td>Prostatic epithelia</td>
<td>Prostate tumours</td>
</tr>
<tr>
<td>Factor VIII-related antigen</td>
<td>Endothelial cells</td>
<td>Angiosarcoma</td>
</tr>
<tr>
<td>Alpha-foetoprotein</td>
<td>Foetal or regenerating hepatocytes</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>Striated muscle</td>
<td>Striated muscle tumours</td>
</tr>
<tr>
<td>CEA</td>
<td>Various epithelia</td>
<td>? colonic adenocarcinomas</td>
</tr>
<tr>
<td>hCG</td>
<td>Syncytiotrophoblastic cells</td>
<td>Trophoblastic tumours and tumours of germ cells</td>
</tr>
<tr>
<td>Neurone-specific enolase</td>
<td>Various, especially endocrine cells</td>
<td>Endocrine neoplasms</td>
</tr>
<tr>
<td>Chromogranin</td>
<td>Endocrine cells</td>
<td>Endocrine neoplasms</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>Thyroid follicular cells</td>
<td>Thyroid carcinomas</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>C cells of thyroid</td>
<td>Medullary carcinoma of thyroid</td>
</tr>
<tr>
<td>Leukocyte common antigen</td>
<td>All leukocytes</td>
<td>Most leukocyte malignancies</td>
</tr>
<tr>
<td>Pituitary hormones</td>
<td>Appropriate pituitary cell</td>
<td>Corresponding adenoma</td>
</tr>
<tr>
<td>Hormones of DNES</td>
<td>Appropriate cell</td>
<td>Corresponding tumour</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Source: Adapted from Alison and Sarraf 1997.

Table 5 reveals some of the common products of cell differentiation used for immunocytochemistry, the associated cell type recognized by the molecule, and the type of cancer with which the molecules are associated.
CHAPTER 2
THE CARE ANTIBODY TEST

Introduction

The overall mortality rate of cancer has not significantly changed in the last 40 years. Hodgkin’s disease and cancers of the cervix, stomach, and uterus have decreased over this time, but the decrease has been offset by an increase in melanoma, multiple myeloma, non-Hodgkin’s lymphoma, and cancer of the lung, prostate, and breast. Approximately one million new cases of cancer are detected each year (American Cancer Society 2002). This number will increase within a few years to 2.3 million (American Cancer Society 2002). The key to cancer survival and treatment is early detection of the disease. One significant area of clinical research devoted to early detection of cancer is that of tumor markers.

A tumor marker is defined as a substance present in or produced by a tumor, or the host, that can be used for differentiating neoplastic from normal tissue based on measurement of blood or secretions. Tumor markers can be found in cells, tissues, and body fluids such as cerebrospinal fluid, serum, plasma, and milk. They have been categorized as follows: enzymes, hormones, oncofetal antigens, carbohydrate epitopes, and oncogene products. The ideal tumor marker would be useful in diagnosis, prognosis, monitoring, and detecting recurrence and possibly screening of at risk groups. Currently, tumor markers are of limited use in the majority of these applications.

The focus of this chapter will present a unique approach in detection of cancer by using an IgM Cancer Recognition (CARE) antibody (Ab) produced against an IgM binding epitope, LT11, of a new cancer antigen (Ag).
Materials and Methods

*Characterization of the Cancer Antigen (Ag).* The cancer Ag was derived from an MCF-7 mammary carcinoma cell culture line (ATCC HTB-22) by the production of a whole cell, soluble tumor extract. The discovery of the IgM binding cancer Ag was made possible by reacting the IgM from cancer patients with Western blots prepared from the tumor extract and observing the binding of Horseradish Peroxidase (HRP) labeled goat anti-IgM. The positive bands were then extracted. Subsequently, new Western blots were prepared. The IgM binding region of the cancer antigen is called LT11. LT11 is the capture polypeptide in the CARE Ab test and the immunogen for polyclonal antibody development.

*Description of the CARE Ab Test.* As shown in Figure 2, the cancer antigen with LT11 is bound to the well bottoms of 96-well microtiter plates. The exposed plastic surfaces are sequentially blocked with a buffer, PTB, consisting of 1 % BSA in Phosphate Buffered Saline without calcium and magnesium, with 0.05% Tween 20 (Sigma). The PTB solution remains the same for all sample preparations. The plate washing solution (PTWB) is prepared as a 30X concentrate of 255 g NaCl and 15 ml Tween 20 in one liter of deionized water. The blocked LT11 coated wells are washed 3 times with PTWB (Washing Step). Duplicate 100 µl aliquots of patient sera or plasma diluted 1:100 in PTB are added to coated and blocked wells. Serial third dilutions of the positive control standard (pooled patient sera) are prepared in normal human plasma (Sigma), and duplicate aliquots of each 1:100 dilution are further diluted with PTB and added to the wells of the microtiter plate. The negative control is simply normal, human pooled plasma (Sigma). Therefore, each 96-well plate consists of 16 wells for the positive control standard curve, a plate blank of PTB run in duplicate, and 39 patient samples run in duplicate.
Figure 2
CARE Antibody ELISA

Figure 2A is a diagram revealing the first few steps of the CARE Antibody ELISA. The well is coated with the cancer antigen then exposed surfaces blocked with BSA. Patient sera containing CARE IgM is added to the wells and binds to the cancer antigen.

Figure 2B is a diagram revealing the addition of the goat anti-human IgM secondary antibody. Following the addition of the secondary antibody, chromogen is added resulting in a color change of the solution from clear to blue.
After incubation and washing, a fresh 1:10,000 dilution of goat anti-human IgG-HRP conjugated secondary antibody (Biosource) in PTB is prepared. A 100 µl sample is added to each well. Following incubation and washing, 100 µl of freshly diluted tetramethylbenzidine (TMB) chromogen (Bio-Rad) in EIA chromogen diluent (Bio-Rad) is added to each well. After 10 minutes at room temperature, 100 µl of stop solution (1 N H₂SO₄) is added, and the plates are read at 405 nm with a background wavelength of 590 nm.

The difference in optical density (OD) for 405-540 nm [ΔOD(405-540)] for each dilution of the plate blank is subtracted from the reference serum. This yields the NET OD for each dilution of the reference standard. The average NET OD and standard deviation (SD) for each reference dilution is then determined for each duplicate sample. From these data, a calibration curve of NET OD vs log reciprocal dilution for the positive control serum is developed. By definition, the NET OD observed for the undiluted positive control serum is assigned a value of 1, and the NET OD observed for normal human plasma is assigned a value of zero. Curve fitting is accomplished using a standard four-parameter model derived from the KC4 software (Bio-Tek). In a similar manner, the OD for the blank sample subtracted from the OD of the samples is averaged. By reference to the fitted calibration curve, the Mean Relative Concentration (MRC) of each sample is calculated from the NET OD. The equation is solved for the values of the reciprocal of dilution at the NET OD for each test sample. A typical calibration curve obtained with the reference serum as the standard is shown in Figure 3. The standard deviation bars for the duplicate samples are not shown because the SD is too small to show graphically.
Figure 3 is of a typical antibody standard curve for the CARE Ab assay generated by using a standard four-parameter model derived from the KC4 software. A calibration curve of NET OD vs log reciprocal dilution for the positive control serum is developed. By definition, the NET OD observed for the undiluted positive control serum is assigned value of 1 and the NET OD observed for normal human plasma is assigned a value of zero. By reference to the fitted calibration curve, the Mean Relative Concentration (MRC) of each sample is calculated from the NET OD.

Statistical Calculations. Using Minitab™, non-parametric statistical analyses of MRC values of the three HV types, benign/surgical non-cancer, and cancer patients, were performed. The analyses were performed using the Mann-Whitney confidence interval (Wilcoxon rank-sum) and the Kruskal-Wallis rank test. MRC values for cancer patients and HV (total) were compared using the Mann-Whitney test. Likewise, the same test was used to compare MRC values of cancer patients and those of benign/surgical non-cancer patients. Kruskal-Wallis analysis was used to ascertain significance between three subgroups of HV: those with no cancer history, family cancer history, and personal cancer history. OriginLab™ scientific graphing and analysis software was used to prepare scattergrams of data.
Results

Serum stability and comparison with plasma. Positive and negative control values from sera stored up to six months at 4°C. Table 6 shows the ∆OD\(_{(405-590)}\) values from three positive patient sera and one negative serum that were measured for up to 6 months while stored at 4°C. Also, freshly prepared lyophilized human plasma reference standards (Sigma) were used. These data show the precision of the values of OD readings of these data sets without the added benefit of a standard curve (Figure 3).

Table 6
CARE Ab Test Optical Density (OD) Data From Positive Patients, A Healthy Volunteer And Reference Human Plasma Stored At 4 °C

<table>
<thead>
<tr>
<th>Source</th>
<th>OD ± SD</th>
<th>Days Stored at 4 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Patient 1</td>
<td>0.340 ± 0.038</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>n = 14</td>
<td></td>
</tr>
<tr>
<td>Positive Patient 2</td>
<td>0.345 ± 0.038</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>n = 8</td>
<td></td>
</tr>
<tr>
<td>Positive Patient 3</td>
<td>0.176 ± 0.056</td>
<td>179</td>
</tr>
<tr>
<td></td>
<td>n = 15</td>
<td></td>
</tr>
<tr>
<td>Healthy Volunteer</td>
<td>0.053 ± 0.011</td>
<td>206</td>
</tr>
<tr>
<td></td>
<td>n = 21</td>
<td></td>
</tr>
<tr>
<td>Reference Plasma</td>
<td>0.031 ± 0.010</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>n = 13</td>
<td></td>
</tr>
</tbody>
</table>

* Prepared fresh from commercial, lyophilized plasma
Stability of sera at room temperature. Approximately 60 healthy volunteer and patient sera comprised 2 groups, one maintained at –80°C and the other stored at room temperature (21-23°C) for 48 hrs. The data from Figure 4 show the MRC values after storage at the 2 different conditions to be very close with an excellent correlation coefficient (r²) value of 0.97.

Figure 4
Frozen vs. Thawed MRC Values Of The CARE Antibody

Figure 4 displays the correlation of MRC readings from 2 different storage conditions of patient and healthy volunteer sera. Patient sera stored at –80°C is compared to thawed sera kept at room temperature for 2 days.
Plasma versus serum. Plasma and sera were prepared from healthy volunteers (n = 40) and cancer patients (n = 35). The plasma was drawn from the blood at room temperature after 2 hours without centrifugation. The serum was harvested after 1 hour at room temperature, then approximately 2 hours at 4 °C, and subsequent centrifugation (300xg, 4 °C). Figure 5 reveals there is good correlation ($r^2 = 0.92$) between plasma and serum samples.

![Figure 5](image)

Figure 5 is a correlation between serum and plasma MRC values of patients and healthy volunteers.

The IgM isotype of the CARE Antibody. The CARE Ab Test was performed as described above except for the addition of the HRP conjugated goat anti-human isotypes. Samples were probed with goat anti-human IgM, IgG, and IgA. Each of these secondary
antibodies was used at the manufacturer’s (Biosource) suggested dilution. As shown in Figure 6, the CARE Ab was almost exclusively of the IgM type.

Figure 6
Percentage Of Secondary Antibodies Of Patient Serum Samples
With Borderline And Elevated OD Values
(Percentages Based On Mean IgM Set At 100%)

Figure 6 displays percentages of secondary antibodies, IgG and IgA, in patient sera compared to mean IgM set at 100%. The data reveal that the antibody specific for the cancer antigen is predominantly IgM.
*Healthy volunteers.* The total HV (n = 197) were divided into three groups: those with no cancer history (n = 47), those with a family history (n = 126), and those with previous cancer (n = 24). The median CARE Ab MRC values for the subgroups were 26, 34, and 46, respectively. The specificity was 98, 94, and 87%, respectively (Table 7). The median for all HV was shown to be 34. Scattergrams for the healthy volunteer subgroups are shown in Figure 7. A cut-off point between negative and positive MRC values was determined visually at an MRC value of 150.

*Benign and malignant patients.* The benign/surgical non-cancer patients (n = 27) and the cancer patients (n = 61) were also displayed in scattergrams (Figure 7). Cancer types of these patients included melanoma, head and neck, endometrial, breast, colon, lung, sarcoma, leukemia, ovarian, and cervical. The median CARE Ab MRC values were 20 for benign/surgical non-cancer patients and 246 for cancer patients. The specificity was 96% for the benign patients, and the sensitivity was 92% for the cancer patients (Table 8).

*Comparisons of HV, benign/surgical non-cancer, and malignant patient MRC values.* Mann-Whitney evaluation between total HV and cancer patients showed a significant difference between the two groups (p < 0.01). Likewise, when comparing benign/surgical non-cancer patients, the two populations revealed significant differences (p < 0.01). However, Kruskal-Wallis analysis revealed no significant differences between the three subgroups of healthy volunteers (p = 0.53).
Table 7
CARE Antibody Data – Healthy Volunteers

<table>
<thead>
<tr>
<th></th>
<th>No Cancer History</th>
<th>Family History of Cancer</th>
<th>Previous Cancer History</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median MRC Value</td>
<td>26</td>
<td>34</td>
<td>46</td>
</tr>
<tr>
<td>Number of Healthy Volunteers</td>
<td>47</td>
<td>126</td>
<td>24</td>
</tr>
<tr>
<td>Specificity</td>
<td>98%</td>
<td>94%</td>
<td>87%</td>
</tr>
</tbody>
</table>

Table 8
CARE Antibody Data for Cancer and Benign/Surgical Non-Cancer Patients

<table>
<thead>
<tr>
<th></th>
<th>Cancer</th>
<th>Benign Disease (and other surgical conditions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median MRC Value</td>
<td>246</td>
<td>20</td>
</tr>
<tr>
<td>Number of Patients</td>
<td>61</td>
<td>27</td>
</tr>
<tr>
<td>Specificity</td>
<td>NA</td>
<td>96%</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>92%</td>
<td>NA</td>
</tr>
</tbody>
</table>
Figure 7 is a scatterplot of MRC data obtained from healthy volunteers, benign/surgical non-cancer patients and cancer patients. Each “o” represents one patient’s MRC value for the CARE Ab. CA patients vs. all HV types and benign/surgical patients revealed significance of $p < 0.0001$. There was no significance of MRC values between the three HV subgroups ($p > 0.53$).
Monitoring of residual disease. Fifteen patients were monitored for residual disease. Data from 3 of the patients are not shown due to redundancy. While patient numbers are small, the data show in detail the utility of the CARE Ab Test for monitoring residual disease. Figures 8 through 19 show examples of the application of the CARE Ab Test in monitoring residual disease in patients after surgery.

Patient LP (Figure 8) had infiltrating ductal carcinoma of the breast that was classified as stage II, estrogen receptor (ER)/progesterone receptor (PR) negative and aneuploid DNA. During the course of monitoring after surgery, the patient’s MRC values decreased to zero following chemotherapy. Patient BC (Figure 9) had infiltrating ductal carcinoma (stage II) that was ER/PR positive, CA 27.29 negative and aneuploid DNA. MRC values likewise remained low during treatment. Patient LB (Figure 10) had a tumor classified as stage II and ER/PR positive revealing decreasing MRC values after surgery. The MRC values remained in the negative control region during the course of treatment and then increased dramatically to a positive MRC value with subsequent discovery of a new tumor 2 months later.

The monitoring of residual disease in colon cancer after surgery is shown in the next three figures. Patient CH (Figure 11) was classified as stage III colon cancer with periaortic/perirenal cancer with lymph node enlargement. After initial lowering of the MRC value to the control levels, the tumor has increased to positive MRC levels with a record saying, “All treatments for metastatic colon cancer have been exhausted.” Another colon cancer patient, Patient ER (Figure 12), had stage I colon cancer with 0/15 lymph nodes positive. Initial success in lowering the MRC values to zero was short-lived; a dramatic increase was seen about three months later. Patient AS (Figure 13) had a similar pattern with a significant increase in MRC and subsequent death 2 months later. Both Patients CH (Figure 11) and AS (Figure 13) had negative CEA values.

Experience has shown that the CARE Test is also positive for preleukemia (e.g., myelodysplastic disease), leukemia, and lymphomas. Patient JJ (Figure 14) has
myelodysplastic. The MRC values have been consistently elevated during the time of monitoring. Whether this patient develops leukemia remains to be seen. Patient RP (Figure 15) has chronic myelocytic leukemia and is Philadelphia chromosome positive with an elevated MRC value. Patients MD (Figure 16) and GC (Figure 17) were diagnosed with Stage II malignant lymphoma and were PSA negative during the course of measurement. However, while Patient MD was consistently negative for MRC, Patient GC revealed a positive MRC initially. After treatment with 6 cycles of CHOP (Cytoxan, Adriamycin, Oncovin, and Prednisone), the MRC was negative. After 2 months, the MRC became positive again with a radiation treatable lesion.

When a patient with a positive MRC has obvious tumor load, the MRC value may decrease due to the immune system’s being depressed and unable to produce antibodies as a result of the assault on it by the chemotherapeutic agents. Patients MF (Figure 18) and RM (Figure 19) show the effects of chemotherapy on the patients’ ability to produce the CARE antibodies during treatment for metastatic disease.
Figure 8 reveals the mean relative concentration (MRC) of CARE antibody levels of Patient LP following surgery on 4/21/99. Antibody levels remained normal throughout the monitoring course.
Figure 9 reveals MRC CARE antibody levels of Patient BC following surgery on 6/26/99. MRC values were normal throughout monitoring.
Figure 10 shows Patient LB’s MRC levels decreased following surgery on 9/1/99 and remained low throughout the course of treatment. However, there was a sharp increase in patient MRC values prior to the discovery of a 2.4 cm tumor.
Figure 11 reveals extremely high patient MRC values throughout treatment. Surgery occurred on 5/17/99. Notice that CEA levels are negative, and there are periaortic and perirenal tumors with lymph node enlargement diagnosed on 10/22/99. All methods of treatment were exhausted on this patient.
Figure 12 reveals patient MRC values increased slightly after surgery followed by a dramatic decrease in MRC values. Nevertheless, there was a sharp increase in MRC indicating that treatment may have not been successful.
Figure 13
CARE Test In Monitoring Residual Disease Of Patient AS With Colon Cancer Following Surgery

Figure 13 reveals MRC values staying elevated for a period of two months after surgery (5/12/99). A dramatic decrease in antibody concentration was later seen. MRC values stayed low for less than 1 month, after which time a significant increase in MRC values occurred followed by death 2 months later. Interestingly, CEA values were found to be negative during the monitoring course.
Figure 14 shows MRC values for Patient JJ suffering from myelodysplastic disease, a preleukemic condition, diagnosed 12/30/98. Whether this patient develops leukemia remains to be seen.
Figure 15 shows MRC values for Patient RP suffering from chronic myelocytic leukemia (CML). Diagnosis occurred in 2/99. The MRC values remain high during the monitoring course.
"Since this is a low grade lymphoma, there is no indication to start treating unless he has symptoms." 4/6/00

Figure 16 shows consistently negative MRC antibody levels for Patient MD suffering from malignant lymphoma. Surgery occurred on 4/21/99. PSA levels were also negative.
Figure 17 shows positive MRC antibody levels during chemotherapy treatment for Patient GC suffering from malignant lymphoma stage II diagnosed 3/29/99. At the completion of treatment there was a decrease to normal MRC values but then a dramatic increase was detected with the detection of a secondary tumor that was treated with radiation.
Figure 18 reveals negative MRC values for Patient MF suffering from Stage IV breast cancer. The immune system is possibly being compromised due to the effects of chemotherapeutic agents on the immune system. CA 27.29 reveals increasing tumor load.
Figure 19
CARE Test Antibody Values Revealing Possible Compromising Of The Immune System In Patient RM

Figure 19 reveals MRC values for Patient RM suffering from stage IV breast cancer with liver metastases. The negative antibody values may indicate that chemotherapeutic treatments have compromised the immune system. As noted, chemotherapeutic treatment options had been exhausted.

4/3/00 Tried Chemotherapy and Arimidex
"not believe anymore chemotherapy will help"
Discussion

The clinical usefulness of a tumor marker becomes almost totally dependent on its specificity, sensitivity, and prevalence of disease. While prevalence will not be discussed due to the small numbers of any particular anatomical tumor type, the importance of prevalence in determining the best criterion for a good tumor marker is recognized. Sensitivity is the ability of a test to detect patients who actually have cancer. Specificity is the ability of a test to distinguish those patients who do not have cancer from those who do. It is unfortunate that none of the tumor markers discovered so far (Table 4) can be said to approach the 100% specificity and sensitivity of an ideal tumor marker. Sensitivity and specificity are inversely related as seen by altering the cutoff level (or the upper normal level) selected for a particular tumor marker. If a lower cutoff level is selected, more patients without cancer will show positive values and fewer patients with cancer will have negative results, resulting in lower specificity but higher sensitivity. Conversely, if the cutoff is set higher to ensure that no cancer-free patient has a positive test result, then more patients with cancer will be missed resulting in lower sensitivity but higher specificity. An MRC value of 150 was chosen graphically as a reasonable balance between the two. The intermediate range where retesting and monitoring would be warranted has been arbitrarily set between MRC values of 100 and 150. Larger databases will allow the positive predictive value (PPV) and negative predictive value (NPV) to be used to better define the cut-off. In any event, the 150 cut-off is well above the median of the HV population to minimize false positives.

Diagnosis is a procedure that determines definitively whether a person has cancer. The problems of both specificity and sensitivity associated with most tumor markers preclude their measurement for the diagnosis of cancer. The frequency of raised levels of tumor markers in nonmalignant diseases and the overlap observed between normal concentrations and the concentrations of tumor markers in patients with proven cancer discourage their use in diagnosis. Most tumor markers used at present fail to clearly
distinguish malignant from benign diseases. They have, however, been used successfully as an adjunct test for cancer monitoring. Several approaches have been suggested recently to improve the diagnostic specificity of many tumor markers. The use of multiple markers is an approach that has received wide acceptance. The major drawbacks in using multiple tumor markers include the accruing cost and the rigors of proper selection of tumor markers to be included in the panel. Another approach to improving the specificity and sensitivity of a tumor marker, as in the case of the serum PSA test, is to perform frequent measurements, some as short as 1 month intervals, to obtain the doubling rate of the tumor marker level (Pollack et al. 1994). While the exact growth kinetics of each tumor is difficult to generalize, patients with positive MRC values (>150) should be monitored every 1 to 3 months to see if the CARE Ab Test values stay elevated or if the values decrease to a control level indicating a false positive. These suggested time intervals between tests are based in part on the data in Figures 8 through 19, which show measurable changes in MRC values within these time frames. The CARE values increase in healthy volunteers (2% of this group) only to decrease to normal levels after 30-90 days (data not shown). This apparent false positive may result from a tumor presenting itself and the immune system eventually destroying it in an immune surveillance fashion.

One of the most useful applications of tumor markers is in monitoring the course of a disease, especially during treatment. Most other clinical procedures lack the sensitivity and convenience for such frequent examinations. The levels of the CARE Ab give information as to whether the patient is experiencing remission or relapse and help determine the effectiveness of the treatment. During the course of chemotherapy, the level of the tumor marker may indicate if there is a need for a redesign of medication, because some tumor cells may have developed drug resistance. It is important to determine whether any observed changes in the levels of tumor marker during a serial measurement are due to clinical changes such as tumor activity, treatment, or impaired
immune function or are due to possible problems revolving around the precision of the test. The precision of the CARE Ab Test under certain conditions is shown in Figures 3, 4, and 5 and Table 6. Figure 3 shows the data from a typical standard curve of duplicate measurements that overlap so one cannot visualize the standard deviation bars. The OD readings (Table 6) and comparisons under different conditions (Figures 4 and 5) again illustrate the precision and accuracy of the CARE Ab Test.

When interpreting results of any tumor marker test, any rise or fall in the tumor marker concentration has to be at least larger than the 95% confidence limit (approximately 2 standard errors) of the previous level to be considered significant. Attention must also be paid to the slope (the increase of the levels of tumor marker over time) of the serial tumor marker values. A steep slope indicates not only a poor prognosis but also the need for more frequent monitoring. Figures 8 through 19 illustrate the change in serum levels of several tumor types during the course of cancer in a patient. The serum level of the CARE Ab marker correlates well with the success of surgery and the efficacy of chemotherapy as shown in Figures 8 and 9 for low stage breast cancer.

Elevated levels of a tumor marker after surgery may indicate metastatic disease and/or primary recurrence. There are occasions when the serum level of a tumor marker may fall or rise unexpectedly. For example, serum PSA has been known to rise transiently during radiotherapy. Furthermore, the decline of serial CA 125 levels has been known not to be associated with remission of the tumor (Wu and Nakamura 1997).

Monitoring for the detection of recurrence following the surgical removal of the tumor is an important application of the CARE Ab test. Six-month to 12-month intervals are usually sufficient. However, whenever there is any suspicion of recurrence, tests should be conducted more frequently, e.g., at monthly intervals. Because the levels of other tumor markers in benign and malignant diseases overlap, successful treatment may be too late when unambiguous elevations are finally recognized. The CARE Ab test is measuring an IgM antibody response, which is inherently more sensitive than measuring
antigen shedding and is directly proportional to the tumor load. Figures 8 through 19 show examples of the utility of the CARE Ab Test for monitoring residual disease. The MRC IgM level appears to respond rapidly and may be directly proportional to the amount of tumor load. Future studies with measurable tumor, e.g., imaging of tumor size, will be necessary to confirm this relationship.

PSA is the only tumor marker that, because of its tissue specificity, will indicate recurrence in an organ site when it becomes detectable following prostatectomy. As is the case for all other tumor marker tests, the CARE Ab test is not specific for tumor type. For oncologists who develop protocols that are not dependent on the anatomical site, such as antiangiogenic treatments, the CARE Ab test may be of great value.

Traditional methods of treatment such as chemotherapy and radiation depress the immune system. Consequently, this may decrease the IgM in the blood and, therefore, decrease the MRC in the CARE Ab test. Figures 18 and 19 may show the effects of chemotherapy on the patients’ abilities to produce the CARE antibodies during treatment for metastatic disease. An alternative explanation is that the CARE antigenic sites are not expressed in metastatic tumors. Histologic examination of metastatic versus primary tissues may be useful to explain low levels of CARE Ab during metastasis.

Large studies of many different tumor types and defined normal populations must be conducted before the CARE Ab test can be used for screening. In addition, this test may complement other tumor markers in this process. Furthermore, a larger database will most assuredly diminish the significant data presented here. This is in part because of error introduced in multicenter studies; the chief of which is determining the correct pathological diagnosis.
CHAPTER 3
A QUANTITATIVE IMMUNOASSAY FOR THE DETECTION OF A NOVEL CANCER ANTIGEN USING CHICKEN ANTIBODIES

Introduction

Based upon the development of an ELISA assay that quantifies a specific IgM antibody directed against a new cancer antigen, the work described in this chapter involves the development of a new quantitative immunoassay for the detection of this antigen by using chicken antibodies. The cancer antigen was isolated from a human mammary carcinoma cell line and contains a IgM-specific binding epitope, LT11. LT11 is used to immunize egg-laying hens for the production of LT11-specific polyclonal IgY antibodies. The polyclonal antibody is used to trap the antigen out of the serum of patients and healthy volunteers. The antigen may exist in 2 forms in the serum: it may be free or it may be bound to the IgM-specific antibody as an immune complex. Bound and total antigen can be quantified in the assay. Furthermore, the CARE Ab assay (see Chapter 2) was run in parallel with the antigen assay. Healthy volunteers (n = 146) were divided into three subgroups: those with a personal cancer history (n = 13), those with no personal or family history of cancer (n = 36), and those with a family history of cancer (n = 97). Cancer patients’ (n = 26) bound and total antigen levels were significantly higher from those of the total healthy volunteers (p < 0.005). Bound and total antigen levels of cancer patients were also significantly higher than those of the healthy volunteers with no personal or family history of cancer and those with a family history of cancer (p < 0.01). However, no significant differences existed between the bound (p > 0.120) and total antigen levels (p > 0.076) of cancer patients and the patients with a personal cancer history. The IgM concentration using the CARE Ab assay was performed on the same cancer patients and healthy volunteers. The cancer patients had significantly higher
concentrations of IgM than those of the healthy volunteers \((p < 0.0001)\). Also, when the IgM concentrations of the cancer patients were compared to each healthy volunteer subgroup, the cancer patients’ values were significantly higher in all three groups \((p < 0.001)\).

**Materials and Methods**

*Patient sera.* Whole blood was collected by venipuncture into sterile red-top vacutainer tubes. Tubes were labeled and placed in upright racks and left undisturbed at room temperature for 1 hour. Tubes were then placed in a 2-8°C environment for no more than 3 hours. Tubes were then centrifuged at 1500 x g for 15 minutes in a refrigerated centrifuge kept at 4°C. Supernatants (serum) were transferred to clean 3.6 mL Nunc CryoTube™ vials (#366524) and were labeled and stored at -80°C until ready for use. One hundred seventy-seven samples were taken from 146 healthy volunteers. The samples were separated into three categories: healthy volunteers with no personal or family history of cancer, healthy volunteers with a family history of cancer, and healthy volunteers with a personal history of cancer that are either under remission or cured of the disease. Thirty-nine samples were taken from 36 healthy volunteers having no personal or family history of cancer. One hundred six samples were taken from 97 healthy volunteers having a family history of cancer, and 32 samples were taken from 13 healthy volunteers with a personal history of cancer. Forty-five samples from 21 cancer patients were obtained from Richard Hankenson, M.D., in Jackson, TN, from 1999 through 2001, and 5 serum samples of 5 cancer patients were obtained from the Cooperative Human Tissue Network (CHTN) from 2000-2002. These serum samples were obtained from patients positive for breast, lung, liver/gastric, lymphoma, leukemia, colon, rectal, prostate, head and neck, melanoma, cervical, or ovarian cancer.
The reference standard, a positive pooled serum, is kept in stock at -80°C until ready for use. This serum will be serially diluted into pooled normal plasma to artificially create a set of samples containing a full range of defined antigen concentrations in a constant background of serum/plasma proteins.

**Antibody specificity.** One volume 0.2% (w/v) Triton X-100 was added for 3 cell pellets of MCF-7 cells (ATCC, HTB-22) to be homogenized. The cells were sonicated on ice using a Branson 450 sonifier fitted with a 1/8 inch microtip set at 40% power /50% duty cycle for 1 minute. The solution was allowed to cool for 2 minutes. The sonication was repeated for 1 minute. The solution was incubated at 4°C for 15-30 minutes. The preparation was centrifuged at 10,000 x g for 30 minutes at 4°C. The supernatant was then removed and saved. The cell pellet was vortexed then resuspended in 1 volume 0.2% (w/v) Triton X-100. The suspension was incubated at 4°C for 15 minutes and subsequently centrifuged at 10,000 x g for 30 minutes at 4°C. The supernatants were combined and the pellet discarded. The preparation was frozen in a polypropylene tube by placing it in a dry ice/acetone bath. The tube was then thawed, and the precipitated material removed by centrifugation at 10,000 x g for 30 minutes at 4°C. The typical protein concentration of the undiluted preparation is 15-20 mg/ml. The homogenate was combined 1:1 with BioRad sample buffer containing 5% β-mercaptoethanol, and the mixture was heated at 95°C for 15 min. After brief centrifugation to remove insoluble material, the supernatant was applied to lanes of 15% SDS-PAGE Criterion gel (BioRad). Prestained Precision molecular weight markers (BioRad) were added to the outer lane, and the gel was run according to the manufacturer’s instructions. Following electrophoresis, the proteins were transferred to prepared Immobilon P membranes (Millipore Corp.) using a semi-dry transfer apparatus for 1 hour at 0.8 mA/cm² and then passively overnight. On day 2, the membranes were briefly immersed in 100% MeOH, allowed to air dry completely, and then cut into strips containing 1 lane each. Individual strips were immersed in diluted preparations of preimmune (20 µg/ml) and immune (20
µg/ml) chicken IgYs in a Phosphate-Tween-BSA (PTB) buffer. After 1 hour of gentle rocking, the antibody solutions were removed, and the strips were washed 3 times for 20 seconds with PBS. Following a 1-hour incubation with HRP-labeled secondary goat anti-chicken antibody, the membrane strips were washed 4 times with PBS and then immersed in insoluble tetramethylbenzidine (TMB) substrate for membranes (Sigma) until bands were visualized. The reaction was quenched by the addition of a large volume of deionized water.

Development of LT11 ELISA (Ag Assay). Horseradish peroxidase (HRP) conjugated to goat anti-human IgM was purchased from Biosource (Camarillo, CA). Anti-LT11 IgY was obtained from the yolks of immunized chickens (Commonwealth Biotechnologies Incorporated, Richmond, VA). Anti-LT11 IgM was purified by fast performance liquid chromatography (FPLC) from sera of human donors shown to be positive for the antibody in the CARE Antibody ELISA. Pooled normal plasma was obtained from Sigma (St. Louis, MO). Ninety-six well microtiter plates were purchased from Dynex. Bovine serum albumin (BSA), Fraction V, was purchased from Sigma. Phosphate buffered saline (PBS) was obtained from Gibco (Carlsbad, CA). A concentrated stock solution of sulfuric acid was obtained from Fisher (Suwanee, GA). Sodium chloride and Tween-20 were purchased from Sigma. TMB chromogen and chromogen diluent were purchased from BioRad (Hercules, CA).

A Phosphate (Sigma)-Tween-20 (BioRad)-BSA (Sigma) (PTB) buffer was prepared by dissolving 10g of BSA in 1L PBS and then 0.5ml Tween 20 was added. This mixture was stored at 4°C and was used as a blocking solution and to dilute antibodies for the assay. A wash buffer was prepared at 30X concentrate by dissolving 255g sodium chloride and 15ml Tween-20 in 750ml distilled H₂O, q.s. to 1L. This solution was stored at 4°C. Prior to running the assay, a 1X wash buffer solution was prepared and left at room temperature. The coating solution is a dilution of the chicken anti-LT11 antibody to 20 µg/ml in PBS. This solution is prepared immediately before use. The primary
antibody is also prepared immediately before use by diluting human anti-LT11 IgM 1:1000 in PTB. Secondary antibody, HRP conjugated goat anti-human IgM, is prepared immediately before use at a 1:10,000 dilution (5 µl to 50 ml PTB). TMB substrate solution is composed of thawed TMB chromogen diluted 1:100 in TMB diluent. The stop reagent is a 1N H₂SO₄ solution.

Equipment required for the LT11 indirect sandwich ELISA includes the following: 1) a Phenix-ST Sunrise Touchscreen dual wavelength microplate reader equipped with 405 and 540 nm filters, Magellan Version 2.22 and BioTek Instruments, Inc., KC4 Version 2.7 Revision 8 plate reader software with data analysis package and hardware lock; 2) a Wallac 96 PW OEM Version plate washer; 3) a 12 channel multichannel pipettor; and 4) repeater pipettes and repeater multichannel pipette.

The following procedure is used to measure the LT11 antigen: 100 microliters of a coat solution (20 µg/ml chicken anti-LT11 in PBS) is added to each well of a microtiter plate. One plate can sufficiently assay 16 antigen samples (total and bound, in duplicate) with 8 standards. The plate is sealed with a silicone mat and placed in a humidified chamber to incubate overnight at 4°C.

The next day, the coated test plate is washed with wash buffer using a wash program composed of 3 cycles of rinsing, soaking (45 secs.), and aspirating. Unreacted binding sites are blocked by adding 300 µl of PTB to each well. The plate is left to incubate in the humid chamber for 1 hour at room temperature. During this incubation period, the 8 standard mixtures are prepared along with the patient samples. Standard 1 is composed of 100 µl of positive control pooled serum. Standards 2-7 consist of positive control serum serially diluted by thirds into pooled normal plasma: 1/3, 1/9, 1/27, 1/81, 1/243, and 1/729. Standard 8 is composed of 100 µl of pooled normal plasma. Five microliters of each standard mixture or test sample is added to individual tubes containing 500 µl of PTB.
After each 1-hour, room temperature incubation, starting with the blocking step, the plate is washed with plate washing buffer according to the wash program (3 cycles). One hundred microliters of standards and test samples are added to duplicate wells in columns 1-6 on the plate. One hundred microliters of the same standards and test samples are added to duplicate wells in columns 7-12 on the plate, as shown in Figure 20. The plate is left to incubate in a humid chamber at room temperature for 1 hour and subsequently washed as described above.

One hundred microliters of human anti-LT11 purified IgM (diluted 1:1000 in PTB) is added to the wells in columns 1-6. One hundred microliters of PTB is added to the wells in columns 7-12. The plate is left to incubate at room temperature in a humid chamber for 1 hour and subsequently washed.

One hundred microliters of secondary antibody, goat anti-human IgM-HRP conjugate (diluted 1:10,000 in PTB) is added to each of the wells. The plate is left in a humid chamber to incubate at room temperature for 1 hour. The TMB substrate is prepared during the last 15 minutes of the aforementioned incubation period. One hundred microliters of substrate solution is added to each well and allowed to incubate at room temperature for 10 minutes. The enzymatic reaction is stopped by adding 100 µl of stop solution (1 N H₂SO₄) to all wells. The plate is then read by the Phenix-ST Sunrise Touchscreen dual wavelength microplate reader at 405 and at 540 nm yielding results in optical density (OD) readings.

The 4-parameter standard curve is produced by using the KC4 V. 2.7 software (Figure 21). The ∆OD(405-540) for the plate blank sample is subtracted from each ∆OD(405-540) for each dilution of reference serum to yield the NET OD value. The average of the NET OD readings for each reference sample is calculated. The calibration curve is created of NET OD vs. log reciprocal dilution for the reference serum. By definition, the NET OD observed for the undiluted reference serum is assigned a value of 1.0, and the NET OD observed for normal human plasma is assigned a value of 0.0. The curve is fit...
using a 4-parameter model where the governing equation is \( y = \frac{a-d}{1+(x/c)^b} + d \). In this equation: \( a \) = theoretical response at concentration equal to 0; \( b \) = measure of the slope of the curve at the inflection point; \( c \) = value of the response at the inflection point; \( d \) = theoretical response at infinite concentration; \( x \) = concentration and \( y \) = response NET OD value. In a similar manner, the NET OD for each test sample is determined. By reference to the fitted calibration curve, the Mean Relative Concentration (MRC) of each sample is calculated from the OD readings. The above equation is solved for the value of \( x \) (reciprocal of dilution) at the NET OD for each test sample. A typical standard curve for the LT11 is shown in Figure 21. It is important to note that the standard deviation bars for duplicate measurements are shown for every dilution. If no standard deviation bar is seen, then the duplicates are too close to being equivalent to graphically resolve. The concentrations of LT11, bound and total, are calculated from the optical density readings obtained by reference to the fitted curve.
Figure 20

Template Of Microtiter Plate With Standards And Samples

<table>
<thead>
<tr>
<th></th>
<th>TOTAL</th>
<th></th>
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</tr>
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<tr>
<td>5</td>
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<td>5</td>
<td>SPL 9</td>
</tr>
<tr>
<td>6</td>
<td>SPL 1</td>
<td>6</td>
<td>SPL 9</td>
</tr>
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<td>7</td>
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<tr>
<td>12</td>
<td>SPL 9</td>
<td>12</td>
<td>SPL 9</td>
</tr>
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</table>

Figure 20 is a template of the 96-well microtiter plate used in the antigen assay. The standard curve for the total antigen using a positive control serum in third (1/3) dilutions are placed in decreasing order in columns 1-2 / rows A-H. (The standards and samples are run in duplicate.) Standards for the bound antigen are placed in rows A-H / columns 7-8. Sixteen samples can be run on 1 plate. Samples for total antigen quantification are placed in duplicate in columns 3-6. Likewise, samples for bound antigen are placed in duplicate in columns 9-12.
Figure 21
Typical Standard Curve For Total LT11 Antigen

Figure 21 is of a typical standard curve of the total antigen using a positive control serum revealing concentration and optical density (OD) values. The curve is a 4-parameter curve calculated by the above equation. Standards were run in duplicate and the standard deviation of the 2 measurements obtained. Standard deviation bars are shown. If no standard deviation bar is seen, duplicate measurements were too close to reveal standard deviation bars.
**CARE Antibody (Ab) assay.** The serum samples of the cancer patients and healthy volunteers were subjected to the CARE Antibody assay to evaluate the concentrations of IgM, specific for LT11, present. Immulon-2 96-well microtiter plates (Dynex) were coated with MCF-7 homogenate antigen preparation. The unreacted sites of the plate are blocked with 300 µl of a Phosphate (Sigma)-Tween-20 (BioRad)-BSA (Sigma) (PTB) buffer. The plate was then incubated for 1 hour at room temperature. During the incubation, standard mixtures of a positive control serum were prepared for generation of a standard curve. The 7 reference standards were diluted in successive 1:3 dilutions into pooled normal plasma. The first standard was composed of 100 µl of positive control serum. Standards 2-7 were subsequently diluted 1:3. Standard 8 was simply 100 µl of pooled normal plasma. Standards and samples for the assay were prepared by adding 5 µl of each standard mixture or test sample to individual test tubes containing 500 µl of PTB. After each 1-hour, room temperature incubation, starting with the blocking step, the plate was washed with plate washing buffer 3 times (one complete cycle). One hundred microliters of standards and test samples were added to duplicate wells (Figure 22). The plate was allowed to incubate for 1 hour at room temperature in a humid chamber and subsequently washed. The secondary antibody, goat anti-human Igs-HRP (Biosource), was diluted 1:10,000 in PTB. One hundred microliters of goat anti-human Igs-HRP conjugate working solution was added to each well. The plate was allowed to incubate for 1 hour at room temperature and then was washed. One hundred microliters of freshly diluted TMB chromogen was added to all wells and allowed to incubate for 10 minutes. After 10 minutes, 100 µl of 1 N H₂SO₄ stop solution was added to each well. The plate was then read at 405 and 540 nm by the Phenix-ST Sunrise Touchscreen dual wavelength microplate reader yielding results as optical density (OD) readings. The MRC values were obtained as explained above. A typical standard curve for the CARE Antibody assay is shown in Figure 23.
Figure 22
Template Of Plate For Antibody ELISA

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<td>SPL 9</td>
<td>SPL 10</td>
<td>SPL 11</td>
<td>SPL 12</td>
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<tr>
<td>B</td>
<td>SPL 1</td>
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<td>SPL 3</td>
<td>SPL 4</td>
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<td>SPL 15</td>
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<td>SPL 18</td>
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<td>E</td>
<td>SPL 25</td>
<td>SPL 26</td>
<td>SPL 27</td>
<td>SPL 28</td>
<td>SPL 29</td>
<td>SPL 30</td>
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<td>SPL 28</td>
<td>SPL 29</td>
<td>SPL 30</td>
<td>SPL 31</td>
<td>SPL 32</td>
<td>SPL 33</td>
<td>SPL 34</td>
<td>SPL 35</td>
<td>SPL 36</td>
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<tr>
<td>G</td>
<td>STD 1 1,000</td>
<td>STD 2 1/3 dilution of G1</td>
<td>STD 3 1/3 dilution of G2</td>
<td>STD 4 1/3 dilution of G3</td>
<td>STD 5 1/3 dilution of G4</td>
<td>STD 6 1/3 dilution of G5</td>
<td>STD 7 1/3 dilution of G6</td>
<td>STD 8 1/3 dilution of G7</td>
<td>Normal Plasma</td>
<td>Plate Blank</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>STD 1 1,000</td>
<td>STD 2 1/3 dilution of G1</td>
<td>STD 3 1/3 dilution of G2</td>
<td>STD 4 1/3 dilution of G3</td>
<td>STD 5 1/3 dilution of G4</td>
<td>STD 6 1/3 dilution of G5</td>
<td>STD 7 1/3 dilution of G6</td>
<td>STD 8 1/3 dilution of G7</td>
<td>Normal Plasma</td>
<td>Plate Blank</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 22 is a template for the 96-well microtiter plate used in the CARE Antibody assay. Standards of a positive control serum at 1/3 dilutions are placed in duplicate by decreasing concentration in rows G-H / columns 1-7. The negative control is in rows G-H / column 8. The plate blank is also run in duplicate in rows G-H / column 9. The samples are run in duplicate in vertical wells of the same column from rows A-F / columns 1-12.
Figure 23 is of a typical standard curve for the CARE Antibody assay. The curve is based on standards run in duplicate. The readings are reflected in optical density readings (OD) and by the Mean Relative Concentration (MRC). The curve is calculated from the 4-parameter equation shown above. Standard deviation bars are shown. If no standard deviation bars are seen, duplicates are too close to being equivalent to graphically resolve.

Statistical analysis. Origin™ V. 7.0 scientific graphing and analysis software was used for normality testing and to obtain histograms. The Mann-Whitney U and Kruskal-Wallis tests in Minitab™ statistical software V. 13.32 were used to ascertain significance of the data.
Results

Antibody specificity. The reactivity of chicken antibody with LT11 polypeptide is shown in Figure 24. Prestained Precision molecular weight markers (BioRad) are in the outer lane and marked accordingly at 50, 37, 25, 15, and 10kD. MCF-7 cell homogenate was placed in 3 lanes. IgY from a preimmunized hen was placed in the 2nd lane, and IgY from hens immunized with cancer antigen was added to the 2 remaining lanes. In Figure 15, a 10kD band and a band between 37kD and 50kD was strongly detected with chicken anti-LT11. The corresponding regions in the preimmune IgY preparations did not contain these strongly staining bands. There were additional bands present in most, if not all of the lanes, including those containing preimmune IgY. This suggests that at least some may be due to non-specific binding of antibodies to proteins in the MCF-7 homogenate.
Figure 24 is of a Western blot of MCF-7 cell homogenate. Molecular weight markers are in the outer left lane. MCF-7 cell homogenate is placed in the next 3 lanes. IgY from a pre-immunized hen is placed in the 2nd lane. IgY from immunized hens with the cancer antigen is placed in duplicate lanes (3 and 4). A strong band between 37 and 50kD and one at 10kD are detected with immune IgY.

*LT11 ELISA.* Forty-five serum samples from 21 cancer patients were obtained from Richard Hankenson, M.D., in Jackson, TN, from 1999-2001, and 5 serum samples
of 5 cancer patients were obtained from the Cooperative Human Tissue Network (CHTN) from 2000-2002. Each sample was subjected to the LT11 Antigen assay to quantify the amount of bound and total antigen in the serum. The histograms of the bound and total antigen data from cancer patients are shown in Figure 25. One hundred seventy-seven serum samples from 146 healthy volunteers were obtained and subjected to the LT11 ELISA. Of the 177 samples, 32 samples were from 13 healthy volunteers with a personal cancer history, 39 samples were from 36 volunteers with no personal or family history of cancer, and 106 samples were from 97 volunteers with a family history of cancer. Histograms for the subgroups of healthy volunteers’ bound antigen levels are shown in Figure 26. Likewise, histograms for the subgroups of healthy volunteers’ total antigen levels are shown in Figure 27.

Normality testing was performed in Origin™ V. 7.0 scientific graphing and analysis software using the Shapiro-Wilk test (OriginLab Corporation 2002). The distribution of cancer patients’ bound antigen levels was determined to be not normal at a 0.05 level of significance (p < 0.007). Likewise, the distribution of all healthy volunteers’ bound antigen levels were shown to be not normal (p < 0.00001). Furthermore, cancer patients’ total antigen levels were shown to be not normal (p < 0.02) as were the total antigen levels of the healthy volunteers (p < 0.00001).
Figure 25
Antigen Levels Of Cancer Patients

A is a histogram of the bound antigen levels of 26 cancer patients. Seven patients have levels less than 200 while the remaining 19 patient have values greater than 200. Nine of which are greater than or equal to 1000. This histogram reveals a distribution that is not normal (p < 0.007).

B is a histogram of total antigen levels of 26 cancer patients. Twenty patients have total antigen levels greater than 300; 11 of which have total antigen values greater than or equal to 1000. This distribution of data is not normal (p < 0.02).
Figure 26
Bound Antigen Levels Of Healthy Volunteer Subgroups

A is a histogram of the bound antigen levels of the 13 healthy volunteers that have a personal history of cancer. The histogram displays a nonnormal distribution (p < 0.007).

B is a histogram of bound antigen levels of 36 healthy volunteers with no personal or family history of cancer. Distribution is not normal (p < 0.00001).

C is a histogram of bound antigen levels of 97 healthy volunteers with a family history of cancer. Distribution is not normal (p < 0.00001).
A is a histogram of total antigen levels of 13 healthy volunteers with a personal history of cancer. This is the only subgroup displaying a normal distribution (p < 0.07).

B is a histogram of total antigen levels of 36 healthy volunteers with no personal or family history of cancer (p < 0.00003).

C is a histogram of total antigen levels of 97 healthy volunteers with a family history of cancer (p < 0.00001).
Considering that the data are not normally distributed, nonparametric methods were used to determine if there were significant differences between any of the groups. The Mann-Whitney U is suggested for 2 independent means of nonparametric data (Bon et al. 1996; Kopczynski and Thielemann 1998; Silvestrini et al. 1998; Xu et al. 1998; Tuxen et al. 1999; Johnson and Kuby 2000). Using the Mann-Whitney U in Minitab for all healthy volunteers with bound antigen (n = 146) versus the cancer patients with bound antigen (n = 26), there was a significant difference between the levels of bound antigen in the 2 groups (p < 0.005) (Table 9). Also, when comparing the healthy volunteers’ total antigen level (n = 146) to that of the cancer patients’ total antigen (n = 26) there was a very significant difference between the total antigen levels of the 2 groups (p < 0.002) (Table 9). Cancer patients’ bound and total antigen levels were also compared to the 3 subgroups of healthy volunteers (Table 10). When compared to the bound antigen level of healthy volunteers with a personal history of cancer (n = 13), there was a not a significant difference in bound antigen levels of the 2 groups (p > 0.120). Furthermore, there was no significant difference between the total antigen of cancer patients and the total antigen of healthy volunteers with a personal history of cancer (p > 0.076).

Concerning bound antigen levels of healthy volunteers with no personal or family history of cancer (n = 36), a significant difference (p < 0.005) was found to exist between the bound antigen levels of cancer patients and those of healthy volunteers with no personal or family history of cancer. When the total antigen levels of cancer patients and healthy volunteers with no personal or family history of cancer (n = 36) were evaluated, there again was a significant difference between the 2 groups (p < 0.004). Furthermore, when bound and total antigen levels of cancer patients were compared to those of the healthy volunteers with a family history of cancer (n = 97), there was a very significant difference between the 2 groups’ bound antigen levels (p < 0.008) and total antigen levels (p < 0.004).
Table 9
Levels Of Total And Bound Antigen From Cancer Patients And Healthy Volunteers

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Population</th>
<th>N</th>
<th>Median</th>
<th>P value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOUND*</td>
<td>Cancer</td>
<td>26</td>
<td>413</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td></td>
<td>Healthy</td>
<td>146</td>
<td>146</td>
<td></td>
</tr>
<tr>
<td>TOTAL**</td>
<td>Cancer</td>
<td>26</td>
<td>744</td>
<td>&lt; 0.002</td>
</tr>
<tr>
<td></td>
<td>Healthy</td>
<td>146</td>
<td>243</td>
<td></td>
</tr>
</tbody>
</table>

* Bound = IgM-antigen complex  
**Total = Bound + Free antigen  
† P value < 0.05

With these results, comparisons between the 3 subgroups of healthy volunteers were done using Kruskal-Wallis to determine if there existed any significant differences between the 3 groups (Zar 1984; Medl et al. 1995; Kopczynski and Thielemann 1998; Silvestrini et al. 1998; Xu et al. 1998; McClave et al. 2001). Kruskal-Wallis nonparametric analysis of the three subgroups showed that there existed no significant differences in bound and total antigen levels (p > 0.840) between any groups at a family error rate of p < 0.05.
Table 10
Antigen Serum Levels In Cancer Patients Versus Subgroups Of Healthy Volunteers

<table>
<thead>
<tr>
<th>Antigen</th>
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<th>N</th>
<th>Median</th>
<th>P value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bound</td>
<td>Cancer</td>
<td>26</td>
<td>413</td>
<td>&gt; 0.120</td>
</tr>
<tr>
<td></td>
<td>Personal Cancer History</td>
<td>13</td>
<td>127</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>Cancer</td>
<td>26</td>
<td>744</td>
<td>&gt; 0.076</td>
</tr>
<tr>
<td></td>
<td>Personal Cancer History</td>
<td>13</td>
<td>279</td>
<td></td>
</tr>
<tr>
<td>Bound</td>
<td>Cancer</td>
<td>26</td>
<td>413</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td></td>
<td>No Personal or Family History of Cancer</td>
<td>36</td>
<td>126</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>Cancer</td>
<td>26</td>
<td>744</td>
<td>&lt; 0.004</td>
</tr>
<tr>
<td></td>
<td>No Personal or Family History of Cancer</td>
<td>36</td>
<td>266</td>
<td></td>
</tr>
<tr>
<td>Bound</td>
<td>Cancer</td>
<td>26</td>
<td>413</td>
<td>&lt; 0.008</td>
</tr>
<tr>
<td></td>
<td>Family History of Cancer</td>
<td>97</td>
<td>155</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>Cancer</td>
<td>26</td>
<td>744</td>
<td>&lt; 0.004</td>
</tr>
<tr>
<td></td>
<td>Family History of Cancer</td>
<td>97</td>
<td>221</td>
<td></td>
</tr>
</tbody>
</table>

† P value = level of significance

*CARE Antibody assay.* The CARE Antibody assay was performed on all serum samples in addition to the newly developed LT11 Antigen assay. The concentrations of LT11-specific antibody, IgM, in the serum of cancer patients and healthy volunteers were calculated from the KC4 V. 2.7 software generated 4 parameter curve. Histograms are shown for the antibody concentrations (MRC) obtained by the CARE Antibody assay for
the cancer patients (Figure 28), total healthy volunteers (Figures 28) and subgroups of healthy volunteers (Figure 29). Normality testing was performed in Origin™ V. 7.0 scientific graphing and analysis software using the Shapiro-Wilk test. Distributions for cancer patients (p < 0.01) and all healthy volunteer subgroups (p < 0.00001) were shown to be not normal. Therefore, nonparametric methods were used to determine significance. Mann-Whitney U analysis revealed there to be a significant difference in the mean relative concentrations (MRC) of antibody in the serum of cancer patients versus those of the healthy volunteers (p < 0.0001) (Table 11). Furthermore, when cancer patients’ MRC antibody values were compared with those of each of the subgroups of healthy volunteers, there were significant differences with all groups (p < 0.0001) (Table 12). The Kruskal-Wallis nonparametric test for comparisons between the 3 healthy volunteer subgroups revealed no significant difference between any pair of the 3 subgroups (p > 0.658) at a family error rate of p > 0.05.
Figure 28
Antibody Levels Of Cancer Patients And Total Healthy Volunteers

A is a histogram of CARE antibody levels of 26 cancer patients. ----- represents a 150 cutoff level. Normality testing reveals non-normal distribution (p < 0.01).

B is a histogram of CARE antibody levels of 146 healthy volunteers. ----- represents a 150 cutoff level. Normality testing reveals non-normal distribution (p < 0.00001).
Figure 29
CARE Antibody Levels Of Three Subgroups Of Healthy Volunteers

A is a histogram of CARE Antibody levels of 13 healthy volunteers with a personal history of cancer. ----- represents a 150 cutoff level. Normality testing reveals non-normal distribution (p < 0.02).

B is a histogram of CARE Antibody levels of 36 healthy volunteers with no personal or family history of cancer. ----- represents a 150 cutoff level. Normality testing reveals non-normal distribution (p < 0.00001).

C is a histogram of CARE Antibody levels of 97 healthy volunteers. ----- represents a 150 cutoff level. Normality testing reveals non-normal distribution (p < 0.00001).
Table 11
CARE Antibody Concentrations Of Cancer Patients And Healthy Volunteers

<table>
<thead>
<tr>
<th>Population</th>
<th>N</th>
<th>Median MRC Value</th>
<th>P value †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>26</td>
<td>249</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Healthy Volunteers</td>
<td>146</td>
<td>32</td>
<td></td>
</tr>
</tbody>
</table>

*MRC = Mean Relative Concentration of antibody
† P value = level of significance

Table 12
CARE Antibody Concentrations Of Cancer Patients And Healthy Volunteer Subgroups

<table>
<thead>
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<th>Median MRC Value</th>
<th>P value †</th>
</tr>
</thead>
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<tr>
<td>Cancer</td>
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<td>249</td>
<td></td>
</tr>
<tr>
<td>Personal Cancer History</td>
<td>13</td>
<td>50</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>No Personal or Family Cancer History</td>
<td>36</td>
<td>26</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Family History of Cancer</td>
<td>97</td>
<td>30</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

*MRC = Mean Relative Concentration of antibody
† P value = level of significance
Discussion

This chapter focuses on a new cancer-associated polypeptide, LT11, and the development of a sensitive enzyme-linked immunoassay (ELISA) for the detection and quantification of LT11 in serum of patients suspected of cancer. This antigen has been found to elicit almost exclusively an IgM response in humans as described in Chapter 2. The immunologic response of IgM-producing lymphocytes can be quantified using a similar ELISA assay that evaluates concentrations of IgM in cancer patients and healthy controls, revealing a significant difference between the amount of IgM present in cancer patients and those of healthy volunteers (Chapter 2). The CARE Antibody assay was run parallel to the newly developed LT11 Antigen assay to evaluate differences between not only LT11 bound and free concentrations in cancer patients and healthy volunteers, but also in the IgM (LT11-specific antibody) differences between the groups. This chapter, building upon the antigen-elicited IgM response, focuses on determining if the antigen is present in the serum and secondly, if present, quantifying the amount of antigen in the serum of patients, both the antigen bound to IgM in the serum and the total antigen present in the serum.

There was a significant difference (p < 0.005) between the amounts of bound and total antigen present in cancer patients’ serum versus that of healthy volunteers’ serum. When comparing antigen levels in the serum of cancer patients against those of the healthy volunteer subgroup of those with a personal cancer history, there was no significant difference between the bound antigen (p > 0.120) of the 2 populations nor was there a significant difference between the total antigen levels of the 2 populations (p > 0.076). In contrast, significant differences were found between bound antigen levels of cancer patients and healthy volunteers with a family history of cancer (p < 0.008) and those without a personal or family history of cancer (p < 0.005). Furthermore, total antigen levels of cancer patients were significantly higher than those of healthy
volunteers with a family history of cancer (p < 0.004) and those without a personal or family history of cancer (p < 0.004).

For further analysis, the 3 subgroups of healthy volunteers were evaluated using the Kruskal-Wallis test for nonparametric analysis for more than 2 independent means. Results reflected that there were no significant differences (family error rate p > 0.05) between the healthy volunteer subgroups. The LT11 assay data from this chapter indicate that total antigen concentration in serum is significantly elevated in cancer patients compared to that of healthy volunteers, with the exception of those who have a personal cancer history.

When the CARE Antibody assay was run in parallel to the LT11 assay, cancer patients revealed a significantly (p < 0.0001) higher IgM anti-LT11 antibody concentration than that of healthy volunteers. Kruskal-Wallis nonparametric analysis for more than 2 independent means revealed no significant differences between any pair of the 3 subgroups of healthy volunteers at a family error rate of p < 0.05. The results of these data, LT11 antigen and CARE Ab assays, may prove to be very promising for prognostic and monitoring purposes of those with cancer. Nevertheless, larger studies need to be done to ascertain the appropriate cut-off values and normal ranges.

The data presented in this chapter suggest that LT11 is an antigen that is present in both normal and cancerous tissues; however, it is significantly elevated in cancer. The reasons for this increased expression are not known at this time but are the focus of continued research. It seems that this antigen may be chemically altered and continue to be present in patients who have a past history of cancer. Another possibility could be that the body continually produces cancer cells expressing this antigen, but the immune system is capable of destroying such cells.
CHAPTER 4
IMMUNOSTAINING OF A NEW CANCER ANTIGEN PRESENT IN CELL MEMBRANES OF PROSTATIC AND OVARIAN CULTURED CELLS AND OVARIAN, BREAST, AND COLON CARCINOMA TISSUES

Introduction

A new cancer antigen has been isolated from a cultured human mammary carcinoma cell line. This new antigen binds an IgM antibody produced by cancer patients. The IgM binding epitope of this new antigen has been isolated and is called LT11. The purpose of the work described in this chapter is to determine if the antigen polypeptide is present in/on normal tissues and/or carcinoma tissues. LT11 was used for chicken polyclonal IgY antibody production. The hen produces an IgY antibody that is passively absorbed into the yolk of the egg. The anti-LT11 IgY is isolated from the egg yolk and purified. Immunohistochemical staining using an anti-chicken HRP-conjugated secondary antibody was performed on 16 normal ovarian tissues, 53 ovarian adenocarcinomas, and 3 borderline ovarian tumors. Lumenal, cytoplasmic, nuclear, and stromal staining were evaluated on all tissues in addition to the Surrounding Uninvolved Epithelium (SUE) of the 53 adenocarcinoma specimens. Using nonparametric statistical analysis, lumenal staining of the tumors was significantly more (p < 0.0001) than the cytoplasmic and nuclear staining of the cells within the tumor. Furthermore, lumenal staining of the adenocarcinomas compared to that of the SUE and normal tissues was very significant (p < 0.001). There was no significant difference in lumenal staining of the borderline ovarian tumors and the adenocarcinomas (p > 0.05). Cytoplasmic staining of the tumors was significant from the SUE (p < 0.01) and the normal ovarian tissues (p < 0.05). In contrast, nuclear staining of the tumor was not significantly different from the SUE or the normal ovarian tissues (p > 0.05). There was no stromal staining in any of the tissues, both normal and cancerous.
Furthermore, normal ovarian, colon, and breast tissues underwent immuno-fluorescent staining in addition to carcinomas of the respective tissues. Also, ovarian carcinoma and prostatic carcinoma cultured cells were subjected to immuno-fluorescence for confocal microscopy. In summary, fluorescence imaging reveals that LT11 is localized to the cell membranes of the carcinoma cells but is absent from the normal tissues. Furthermore, the cultured carcinoma cells reveal that LT11 is not only associated with the cell membrane but may be related to a cortical intermediate filament.
Materials and Methods

Ovarian tissues for immunohistochemistry. Paraffin embedded tissues were obtained from the Cooperative Human Tissue Network (CHTN) at the University of Alabama at Birmingham (UAB) in the Pathology Department. Patients included 16 normal ovarian (3 with endometriosis), 3 borderline ovarian carcinoma, 29 serous adenocarcinomas, and 24 endometrioid adenocarcinomas of the ovary. Paraffin sections (5µm) were obtained using a Leica RN2135 microtome. Of the 16 normal patients, 15 were evaluated for staining of the surface epithelium. Lumenal, cytoplasmic, and nuclear staining was scored. Delete slides for background subtraction were also evaluated. Delete slides were coated with 3% goat serum versus the primary antibody, anti-LT11. Only 1 normal tissue of the 16 normals could not be scored for the surface epithelium due to the absence of a delete slide with surface epithelium. Of the 16 normal tissue slides, 5 specimens contained inclusion cysts of which the lumenal, cytoplasmic, and nuclear staining were all scored. Stromal staining was scored for all 16 tissues. Lumenal, cytoplasmic, and nuclear staining scores were also given to all tumor specimens. Nineteen of the 53 specimens contained surrounding areas of uninvolved epithelium (SUE) while the remainder of specimens contained tumor only. The lumenal, cytoplasmic and nuclear areas of the surrounding uninvolved epithelium (SUE) were also scored. Stromal staining was scored for all 53 specimens.

Tissues for immunofluorescence. Paraffinized tissues were obtained from the CHTN at UAB’s Pathology Department. Tissues included 1 normal ovary, 2 papillary serous adenocarcinomas of the ovary, 1 endometrioid adenocarcinoma of the ovary (moderately to poorly differentiated), 1 moderately differentiated adenocarcinoma of the colon, 1 normal colon, 1 infiltrating ductal carcinoma of the breast and 1 normal breast. Tissue sections of 5µm were obtained using a Leica RN2135 microtome. Sections were placed on microscope slides and incubated at 65°C for 60 minutes to melt the paraffin.
Cell culture. Ovarian cancer cells (OVCAR3) (ATCC HTB-161) and prostate cancer cells (LNCaP) (ATCC CRL-1740) were maintained in cell culture flasks with DMEM for the OVCAR3 cells and RPMI 1640 for the LNCaP cells, supplemented with 10% fetal calf serum, 2mM glutamine, and an antibiotic antifungal solution (10,000 IU penicillin, 10,000 mcg/ml streptomycin and 25 mcg/ml amphotericin B). Adherent cell cultures were passaged using 0.1% trypsin in HBSS (Sigma). Cells were trypsinized from culture flasks, seeded onto sterile glass coverslips, and placed in 6-well microtiter plates. Cells were grown to ~ 50-75% confluency before permeabilization in methanol or acetone for 20 minutes at –20°C. Cells were subsequently washed in PBS and then fixed with 3% formaldehyde for 30 minutes at room temperature.

Immunohistochemistry (HRP staining) of ovarian tissues. Paraffin sections (5µm) were treated with 3.0% hydrogen peroxide to prevent endogenous peroxidase activity following deparaffinization with xylene, alcohol, and TRIS buffer and antigen retrieval with citric acid and microwave exposure. Sections were blocked with 3% goat serum and subsequently incubated with polyclonal anti-cancer antigen (LT11) IgY at a concentration of 15 µg/ml for 1 hour at room temperature. Following the incubation and rinse with TRIS buffer, sections were incubated with goat anti-chicken HRP-conjugated secondary monoclonal antibody (Southern Biotech). Delete slides were incubated with 3% goat serum in PBE buffer (PBS, EDTA, sodium azide, and BSA). For visualization of the indirect antibody-antigen reactivity, diaminobenzidine tetrahydrochloride (Biogenes, San Ramon, CA) was used as a substrate. Sections were stained lightly with hematoxylin and then dehydrated from 70% alcohol to 95% to absolute alcohol and finally xylene. Coverslips were added using Permount™ (Fisher Scientific). The degree and location of immunohistochemical was determined. The intensity of the immunostaining was graded based on a scale of 0 (no staining) to +4 (intense staining). The percentage of cells at each intensity was estimated, and the decimal equivalent of the percentage was multiplied by the appropriate intensity score to obtain a weighted average.
of the intensity score (Poczatek et al. 1999). This average is the immunostaining score. Delete slides were also scored for background and subtracted from the slide stained with anti-LT11. Statistical analysis was performed in Minitab™ version 13.32 and in OriginLab™ scientific graphing and analysis software version 7.0. Normality testing using the Shapiro-Wilk normality test was performed in OriginLab™ for staining scores of all tissues. Mann-Whitney U nonparametric analysis was performed on peroxidase staining scores for comparisons of degree and location of immunostaining in all specimens.

Staining procedure of paraffinized tissues for basic fluorescence imaging. Slides were deparaffinated and rehydrated in consecutive steps starting with xylene. Slides were immersed in a glass box containing xylene for 2 minutes. Slides were subsequently immersed twice in xylene for 2 minutes each, followed by immersion in 100% ethanol for 2 minutes, 95% ethanol for 2 minutes, 70% ethanol for 2 minutes, and phosphate buffer solution (PBS) for 2 minutes. To further expose the antigenic site of the protein within the carcinoma tissues, antigen retrieval was employed. Water was heated in a plastic container for 8 minutes in a microwave. Coplin jars filled with 10mM citric acid were then added to the heated water bath and subsequently placed in the microwave for a period of 6 minutes. Slides were then added to the Coplin jars that were then heated for another 6 minutes. The water bath was then cooled with running cool water. Slides were washed in PBS for a few seconds then treated with 3.0% hydrogen peroxide for 5 minutes to prevent endogenous peroxidase activity. Tissues were then washed with distilled water and subsequently blocked with 3.0% goat serum (Sigma) in a humidity chamber for 1 hour at room temperature. Slides were then drained, and the diluted primary antibody, anti-LT11, was added to the tissues and allowed to incubate for 1 hour in a humidity chamber. The anti-LT11, a stock solution of 24.6 mg/ml, was diluted to 7.5 mg/ml in PBE. Controls were incubated with the preimmune LT11 chicken antibody (13.4 mg/ml stock) diluted to 7.5 mg/ml. Slides were placed in PBS for 5 minutes and then subjected
to 1 more washing of PBS. Slides were blocked with 3.0% goat serum for 30 minutes in a humidity chamber and subsequently washed in fresh PBS. The secondary antibody, AlexaFluor 594 goat anti-chicken antibody (Molecular Probes), was diluted in PBE 1:100 and added to the tissue sections for an incubation period of 1 hour under plastic coverslips in the humidity chamber. Tissue sections were then washed with PBS and counterstained with a nuclear dye, Hoescht 33258 (Sigma), that was diluted 1:1000 in PBS from a 20μg/ml stock solution. After incubation for 4 min., the slides were rinsed twice in a PBS bath. The slides were mounted with a 1:9 v/v PBS/glycerol solution containing 1% p-phenylene diamine (PPD) (Sigma). The coverslips were sealed with nail polish and left to dry. Slides are stored at -20°C in the dark.

**Staining of cultured cells for confocal microscopy.** OVCAR3 and LNCaP cells were grown to ~ 50-75% confluency on coverslips in 6-well sterile culture plates. The cells were rinsed twice with PBS and then permeabilized with methanol or acetone for 20 minutes at -20°C. The cells were rinsed twice with PBS and subsequently fixed in 3% formaldehyde for 30 minutes at room temperature. Cells were blocked with 3% BSA and PBS for 30 minutes at room temperature. The primary antibody, IgY anti-LT11 diluted in PBE to a concentration of 7.5 μg/ml, was then added to the coverslips and incubated for 1 hour at room temperature. Coverslips were washed 5 minutes in PBS for 3 cycles and subsequently blocked with 3% BSA and PBS for 15 minutes at room temperature. The secondary antibody, anti-chicken AlexaFluor 594, diluted 1:100 in PBE was added to the coverslips and incubated for 1 hour at room temperature. Coverslips were subsequently washed for 5 minutes in PBS for 3 cycles. Coverslips were then blocked with 3% BSA in PBS for 15 minutes at room temperature. The cells were subsequently counterstained with the nuclear dye, Hoescht 33258 (Sigma), diluted from stock 1:1000 in PBS for 4 minutes at room temperature. Coverslips were rinsed for 5 minutes in PBS for three cycles and were then mounted on microscope slides with a 1:9 v/v PBS/glycerol
solution containing 1% p-phenylene diamine (PPD) (Sigma). The coverslips were sealed with nail polish and left to dry. Slides are stored at -20°C in the dark.

**Basic fluorescence imaging.** Basic imaging was performed using an upright Leitz Orthoplan epifluorescence microscope with brightfield, phase contrast and Hoffman modulation contrast optics, Roper Photometrics CH250 liquid-cooled camera and a Vario-Orthomat II camera system. The scope was attached to a Macintosh workstation for image acquisition and image analysis. The software employed for the image acquisition and quantification included the IPLab Spectrum by Scanalytics. Additional processing of the images was done in Adobe PhotoShop and Microsoft PowerPoint.

**Confocal microscopy.** Confocal microscopy was performed using the the Leica Confocal Imaging Spectrophotometer TCS SP UV unit with a Coherent Laser Group Enterprise UV laser and Krypton ion lasers, an acoustooptical tunable filter (AOTF), and 3 detector channels. The instrument is mounted on a Leica DMIRBE inverted research microscope equipped for epifluorescence. Images were captured using the 100x Plan Apochromatic objective and processed using Leica Confocal software.
Results

**Immunohistochemistry.** Sixteen normal ovarian patient tissues, 3 borderline ovarian tumors and 53 adenocarcinomas from patients were subjected to immunohistochemical staining. The 16 normal ovarian patient tissues were evaluated, and staining scores were given for inclusion cysts, if present, stroma, and surface epithelium. Five of the 16 normal ovarian tissues contained inclusion cysts. One of the 16 normal tissues lacked surface epithelium but did possess an inclusion cyst for evaluation. The inclusion cysts and surface epithelium were evaluated for luminal, cytoplasmic, and nuclear staining. Three borderline ovarian tumors, 29 serous adenocarcinomas, and 24 endometrioid adenocarcinomas were also evaluated. One tumor specimen was evaluated as both serous and endometrioid carcinoma due to its pathological consistency containing both tumor types. Staining scores were given for the stroma and luminal, cytoplasmic, and nuclear areas of the tumor. Also, 19 of the 53 tumor specimens contained uninvolved surrounding epithelium. The luminal, cytoplasmic, and nuclear areas of the SUE were also scored. Data analysis was performed in Minitab™ and OriginLab™. Immunostaining scores from all data groups with 1 exception were shown to be not normal according to the Shapiro-Wilk normality test performed in OriginLab™. The data of the luminal staining of the tumors was shown to be normal. With these results from the normality testing, nonparametric testing, i.e. Mann-Whitney U, was performed in Minitab™ to ascertain significance among the groups.

Tumor luminal staining data were compared to tumor nuclear and cytoplasmic staining. Luminal staining of the tumors was shown to be very significant compared to that of the cytoplasmic and nuclear staining within the same tumor specimens (p < 0.0001). Furthermore, the tumor luminal surface stains significantly more than the lumens of the inclusion cysts, the surface epithelium of the normal ovary (Figure 30) and the surrounding uninvolved epithelium (p < 0.0001) (Figure 31). However, there was no significant difference between the luminal staining of the borderline ovarian and
carcinoma tissues ($p > 0.05$). Two of the 3 borderline tumors had lumenal staining scores of 2.3. By visual estimation, an arbitrary cut-off line was set at 0.8. Cytoplasmic staining of the tumors was significant compared to the SUE ($p < 0.01$) (Figure 32) and the normal ovarian tissues ($p < 0.05$). In contrast, nuclear staining of the tumor was not significantly different from the SUE or the normal ovarian tissues ($p > 0.05$) (Figure 33). There was no stromal staining in any of the tissues. Figure 34a demonstrates absence of staining of ovarian epithelium and stroma. Figure 34b is taken from a borderline ovarian tumor and illustrates significant staining around lumenal areas of epithelial cells.

In summary, with one exception, the normal tissues did not have lumenal staining, where 52 of 53 cancer tissues revealed significant lumenal staining. The nucleus and cytoplasm of cancerous and noncancerous tissues did not stain to any significant degree; neither did the periphery of the tumors. This is very important because anti-LT11 may prove to be an important tool for examining margins of tumors.
Figure 30
Lumenal Staining Of Tumor And Normal Tissues

Figure 30 is a scattergram revealing staining intensities of the lumenal surface of normal and cancerous tissues. “□” represents surface epithelium. “○” represents inclusion cysts. Five of the 16 normal ovarian tissues contained inclusion cysts. One normal tissue did not possess surface epithelium for evaluation, only an inclusion cyst. Three borderline tissues, 29 serous carcinomas and 24 endometrioid carcinomas were also evaluated. Each “□” or “○” represents an immunostaining score for each individual specimen slide. The ---line represents a subjective cutoff for discerning cancerous from normal ovarian tissues.
Figure 31 is a scattergram revealing lumenal staining of the tumors and the lumenal staining of the surrounding uninvolved epithelium (SUE) around the tumors. Nineteen of the 53 tumor specimens evaluated contained surrounding uninvolved epithelium. Each "●" represents an immuno-staining score for each individual specimen slide. The ----- line represents a subjective cutoff for staining intensity distinguishing carcinoma tissues from the surrounding uninvolved epithelium.
Figure 32

Cytoplasmic Staining Scores Of Tumors, Surrounding Uninvolved Epithelium, Borderline Tissue, Inclusion Cysts, And Normal Ovarian Tissue Surface Epithelium

Figure 32 is a scattergram revealing scores of cytoplasmic staining intensity of tumors, borderline carcinomas, surrounding uninvolved epithelium (SUE) around the tumor, inclusion cysts and surface epithelium of normal ovarian tissue. Fifty-three of the tumor specimens, 19 of that had surrounding uninvolved epithelium, were evaluated for cytoplasmic staining. Likewise, 3 borderline carcinomas and the 16 normal ovarian tissues were scored. Of the 16 normal ovarian tissues, 5 had inclusion cysts and 15 possessed surface epithelium. Each “□” represents an immunostaining score for each individual specimen slide. The ----- line represents a subjective cutoff for the staining intensity based upon lumenal staining.
Figure 33 is a scattergram revealing nuclear immunostaining scores of ovarian tumors, borderline carcinomas, surrounding uninvolved epithelium (SUE) around the tumors, inclusion cysts and surface epithelium of normal ovarian tissue. Fifty-three tumor specimens, 19 of that having surrounding uninvolved epithelium, were evaluated for nuclear staining. Likewise, 3 borderline carcinomas, 15 normal ovarian tissues with surface epithelium and 5 normal ovarian tissues with inclusion cysts were scored. Each “□” represents an immunostaining score for each individual specimen slide. The ----- line represents a subjective cutoff for the staining intensity based upon lumenal staining.
Figure 34
Immunohistochemical Staining Of Normal Ovarian And Borderline Ovarian Carcinoma Tissues With Anti-LT11 IgY

A) Normal ovarian tissue stained with chicken anti-LT11 primary antibody and goat anti-chicken HRP conjugated secondary antibody.

B) Borderline ovarian carcinoma stained with chicken anti-LT11 primary antibody and goat anti-chicken HRP conjugated secondary.
**Immunofluorescence.** Basic immunofluorescence imaging was performed on 5µm paraffin-embedded tissue sections. Images of 1 normal ovary, 2 papillary serous adenocarcinomas of the ovary, and 1 endometrioid adenocarcinoma of the ovary were acquired and imaged using a Leitz upright epifluorescence microscope. Negative controls consisted of carcinoma tissue stained with the preimmune chicken polyclonal antibody. Both the normal ovarian tissue and the ovarian carcinoma stained with preimmune IgY (Figure 35) revealed very faint and diffuse, non-specific staining. In contrast, papillary serous (Figure 36) and endometrioid adenocarcinomas (Figure 37) revealed intense specific staining localized to the cell membrane of the epithelial cells. There was no stromal staining.

To further demonstrate the presence of LT11 in cancer tissues, breast and colon carcinomas as well as normal breast and colon tissues were subjected to immunofluorescence imaging. Normal breast tissue was stained with preimmune IgY revealing an absence of any staining save the nuclear staining with Hoescht 33258 (Figure 38). Two infiltrating ductal carcinomas were stained revealing intense and distinct localization of LT11 to the cell surface (Figure 39).

Furthermore, normal colon tissue was stained with anti-LT11 revealing faint, diffuse staining (Figure 40). Adenocarcinoma of the colon revealed the same intense distinct staining of the cellular surface as those of the ovarian and breast carcinoma tissues suggesting again LT11 is localized to the cell surfaces of carcinoma tissues (Figure 41).
Figure 35
LT11 Staining Of Normal Ovarian Tissues And Ovarian Carcinoma Stained With Preimmune IgY


B) Normal ovarian tissue stained with chicken anti-LT11 and Hoescht 33258 nuclear counterstain. Bar equals 25 μm.

C) Ovarian carcinoma stained with preimmune IgY. Bar equals 25 μm.

D) Ovarian carcinoma stained with preimmune IgY and Hoescht 33258 nuclear counterstain. Bar equals 25 μm.
Figure 36
Papillary Serous Ovarian Adenocarcinoma Stained With Chicken Anti-LT11

A) Papillary serous ovarian adenocarcinoma stained with chicken anti-LT11 and Hoescht 33258 nuclear counterstain. Bar equals 25 µm.

C) Papillary serous ovarian adenocarcinoma stained with chicken anti-LT11 and Hoescht 33258 counterstain. Bar equals 25 µm.

A) Endometrioid ovarian adenocarcinoma (moderately to poorly differentiated) stained with chicken anti-LT11 and Hoescht 33258 nuclear counterstain. Bar equals 25 µm.

B) Endometrioid ovarian adenocarcinoma (moderately to poorly differentiated) stained with chicken anti-LT11. Bar equals 25 µm.
Figure 38

Normal Breast Tissue Stained With Preimmune IgY

A) Normal breast tissue stained with preimmune IgY and Hoescht 33258 nuclear counterstain. Bar equals 50 µm.

B) Normal breast tissue stained with preimmune IgY. Bar equals 50 µm.
Figure 39
Infiltrating Ductal Carcinoma Of The Breast Stained With Chicken Anti-LT11

A) Infiltrating ductal carcinoma of the breast stained with chicken anti-LT11 and Hoescht 33258 nuclear counterstain. Bar equals 50 µm.

B) Infiltrating ductal carcinoma of the breast stained with chicken anti-LT11. Bar equals 50 µm.
C) Infiltrating ductal carcinoma of the breast stained with chicken anti-LT11 and Hoescht 33258 nuclear counterstain. Bar equals 50 µm.

D) Infiltrating ductal carcinoma of the breast stained with chicken anti-LT11. Bar equals 50 µm.
Figure 40
Normal Colon Tissue Stained With Chicken Anti-LT11

A) Normal colon tissue stained with chicken anti-LT11 and Hoescht 33258 nuclear counterstain. Bar equals 50 µm.

B) Normal colon tissue stained with chicken anti-LT11. Bar equals 50 µm.
Figure 41
Adenocarcinoma Of The Colon Stained With Chicken Anti-LT11

A) Adenocarcinoma of the colon stained with chicken anti-LT11 and Hoescht 33258 nuclear counterstain. Bar equals 50 µm.

B) Adenocarcinoma of the colon stained with chicken anti-LT11. Bar equals 50 µm.
Confocal microscopy. Prostate carcinoma cells (LNCaP) were cultured and prepared for confocal microscopy. Figure 42 is a composite displaying still images of a 3-dimensional rotation of 3 prostatic cells stained with anti-LT11 and a Hoescht 33258 nuclear counterstain. The krypton laser was used to excite the Alexa Fluor 594 secondary antibody. The images reveal very specific staining of the cell membrane as well as some filamentous staining, again emphasizing that LT11 may be a portion of an intermediate filament. Figure 43 is of the same prostatic cancer cells stained with chicken anti-LT11 and Hoescht 33258 but displaying a step-by-step movement through the cells using a 0.6 µm step-size.

Ovarian carcinoma cells (OVCAR3) were also cultured and prepared for confocal microscopy. Figure 44 displays still images of a 3-dimensional rotation of the ovarian cells stained with anti-LT11, again emphasizing surface staining and some filamentous cortical staining. Figure 45 is the same as Figure 44 with the additional Hoescht 33258 nuclear counterstain. Figure 46 is a step-by-step movement through the same ovarian carcinoma cells using a 0.5 µm step-size to reveal outer membranous staining of LT11. Figure 47 is the same as Figure 46 with the additional Hoescht 33258 nuclear counterstain. Video clips for stepping through cells and the 3D rotations can be seen at: http://cancerfoundation.com/care.html - videos.
Figure 42
Prostate Carcinoma Cultured Cells Stained With Anti-LT11 And Hoescht
(3D Rotation Sequential Individual Images)
Figure 43
Prostate Carcinoma Cells Stained With Anti-LT11 And Hoescht
(Sequential Individual Stepping Images)
Figure 44
Ovarian Carcinoma Culture Cells Stained With Anti-LT11
(3D Rotation Sequential Images)
Figure 45
Ovarian Carcinoma Culture Cells Stained With Anti-LT11 and Hoechst
(3D Rotation Sequential Individual Images)
Figure 46
Ovarian Carcinoma Culture Cells Stained With Anti-LT11
(Sequential Individual Stepping Images)
Figure 47
Ovarian Carcinoma Cultured Cell Stained With Anti-LT11 And Hoescht Nuclear Dye
(Sequential Stepping Images)
Discussion

Neoplastic cells of tumors produce numerous different molecules that may be elevated during malignancy. Such molecules, i.e., mucins, enzymes, hormones, cytokeratins, and cell surface components, may be used as tumor markers that are potentially useful in cancer screening, diagnosis, prognosis, and monitoring treatment of those with a diagnosed disease. A new potential tumor marker, cancer antigen LT11, has been isolated from a human carcinoma cell line. This tumor marker has been shown to mount an immune response in cancer patients, specifically an IgM antibody response, and can be quantified using an enzyme-linked immunosorbent assay. LT11 is a new cancer antigen obtained from MCF-7 cells that binds to the variable region of the IgM found in sera of cancer patients. The work described in this chapter further investigates the usefulness of LT11 as a tumor marker in the pathology setting, namely immunohistochemistry.

For production of the anti-LT11 polyclonal antibody, egg-laying hens were immunized with LT11, and IgY antibodies were purified on protein A columns from the egg yolks. Using the IgY anti-LT11, different carcinoma paraffin-embedded tissues were immunostained. Immunohistochemical staining with peroxidase revealed significantly more intense staining of ovarian carcinoma tissues than that of the normal ovarian tissues. The staining in the carcinoma tissues was mainly localized to the epithelial lumenal surfaces with very little staining of the cytoplasm. No stromal staining was observed. In contrast to the carcinoma tissues, the normal tissues revealed insignificant staining. These results reflect that LT11 is abundantly present in the epithelial cells of ovarian carcinoma compared to that of normal ovarian tissue.

Furthermore, LT11 was used to stain ovarian, breast, and colon carcinomas in immunofluorescence. The fluorescence imaging revealed very intense and specific staining of the cell surface in the carcinoma tissues (Figures 36, 37, 39, 41). In contrast, preimmune staining of ovarian carcinoma (Figure 35c,d) and normal ovarian (Figure
35a,b), normal breast (Figure 38), and normal colon (Figure 40) revealed diffuse, non-specific staining. These images further emphasize LT11 as a cell surface component found on carcinoma tissues of the ovary, breast, and colon but not on normal ovarian, breast, and colon tissue.

Confocal imaging of an ovarian carcinoma cell line (OVCAR3) and a prostate cell line (LNCaP) showed more surface staining with LT11 as was expected; however, there also seemed to be cortical staining of a filamentous network. These animations further emphasize that LT11 is a cell surface component; however, it may also be related to an internal network of intermediate filaments. However, because this staining was not seen on the paraffin-embedded tissue carcinoma samples, it may also be possible that the cortical filamentous staining may be the result of the manipulated and artificial characteristics of cells grown in culture.

This chapter has shown LT11 to be a cell surface protein present on the cell membranes of ovarian, breast, and colon carcinoma tissues along with ovarian and prostate cultured cells. LT11 is not present on the cell membranes of normal tissues. LT11 is a cancer antigen that can be detected by immunohistochemistry and fluorescence that may become a valuable tool for histopathologists in ascertaining tissues to be either cancerous or normal. More work needs to be done to qualify this antibody as such a valuable tool. For instance, the presence or absence of LT11 in other cancers, e.g., pancreas, lung, liver, and kidney, needs to be determined, and its presence or absence in benign tumors must be defined.

Not only is this new cancer antigen proving to be valuable in pathohistology, it may also be very valuable for cancer screening, diagnosis, prognosis and monitoring. For instance, the IgM anti-LT11 can be quantified in patients with cancer at elevated levels compared to healthy volunteers. Currently, no other tumor marker assay quantifies the amount of antibody specific for a cancer antigen. This may prove LT11 to be a very
valuable tumor marker that may be used in the clinical setting and may be useful in the
immersing field of antibody imaging and therapy.
CHAPTER 5
FUTURE DIRECTIONS FOR LT11 ANTIGEN RESEARCH

To determine the presence of the LT11 Antigen (Ag) in serum, an ELISA assay was developed using polyclonal chicken antibodies, IgY, directed against LT11. The IgY traps the antigen out of the serum. The antigen may exist in 2 forms: free or bound to IgM as an antigen-antibody complex. The LT11 Ag assay quantifies total (bound + free) and bound antigen. The results of the Ag assay to date revealed a significant difference (p < 0.005) between the bound and total LT11 concentrations in cancer patients versus healthy volunteers. Healthy volunteers consisted of 3 subgroups: those with a past cancer history, those with a family history of cancer, and those with no family history of cancer. There were no significant differences between any of the 3 healthy volunteer subgroups’ bound and total antigen levels (p > 0.840).

The results of this study suggest that the LT11 Ag assay may be valuable in distinguishing cancer patients from those without cancer. It is important to note that there are no other tumor marker assays that quantify antibodies produced against cancer by the patient. The assays available today such as CEA, CA 125, etc., are antigen assays that use monoclonal antibodies directed against the antigen.

Improvements on the LT11 Ag assay. The development of a monoclonal antibody for detection against LT11 would most likely increase the specificity of the LT11 assay. In this study, chickens were immunized with the LT11 antigen to produce polyclonal antibodies. When animals are injected with an antigen, they respond by producing polyclonal antibodies, i.e. the production of a mixture of antibodies from different B-cell clones. This “cocktail” of immunoglobulins is directed against different epitopes of the antigen of interest. The antibodies vary in their variable regions but all react only with the relevant antigen (Chapel et al. 1999).
Monoclonal antibodies tend to be the preferable detecting antibody in the research industry because of their specificity. Monoclonal antibodies are the product of a fused B cell and an immortal myeloma cell. This fusion results in a hybridoma. Each hybridoma is derived from a single B lymphocyte that produces an immunoglobulin of only 1 antigen specificity; therefore, the secreted antibody is the product of a single B lymphocyte clone, i.e., monoclonal (Johnstone and Thorpe 1996). Provided that there are no genetic mutations in the hybridoma cell, the amount and specificity of the monoclonal antibody production would remain unlimited (Peters and Baumgarten 1992). The advantage of using monoclonal antibodies is their absolute specificity. However, polyclonal antibodies with reactivity for multiple epitopes on the antigen molecule will result in stronger and more effective labeling than achieved with a comparable monoclonal antibody (Brooks and Schumacher 2001). In the antigen assay, the polyclonal anti-LT11 IgY could continue to be used to trap the antigen and the immune complexes from the serum while the detecting antibody would be monoclonal in nature. This would theoretically increase specificity.

In addition, having both the antibody and antigen assays could greatly increase sensitivity and specificity for cancer detection; however, the study at the current time is too small to determine sufficient cut-off values and normal ranges. Furthermore, after a larger study is performed, the LT11 Ag test may be shown to be complementary to the other tumor marker assays with regards to reliability, precision, and accuracy. The hope, of course, is that this assay will be better than some of the more common tumor marker tests.

Another aspect that could be investigated concerning the antigen assay would be to monitor cancer patients from diagnosis through their treatment. Working cooperatively through an IRB, an oncologist could enter 5-10 patients in a monitoring study. Their blood would be obtained from the time of diagnosis through treatment, e.g., surgery, chemotherapy and radiation. The patients would preferably consist of those with
The study would focus on the changes in antigen levels, bound and total, from the time of diagnosis through the course of treatment. The antigen assay would yield information regarding effects of different types of treatment on different cancers and the immune system. Hopefully, these data would provide background information for funding of a much larger study.

The nature of LT11. The sequence of the LT11 antigen has just recently been completed by Commonwealth Biotechnologies Incorporated (see Appendix). The LT11 IgM-binding epitope has been narrowed to a 16 mer polypeptide; however, the sequence has not been published due to patents that are pending. Protein databases, such as those of the National Center for Biotechnology (NCBI), are being used to evaluate if LT11 is homologous or very similar to other known proteins. This will hopefully lead to the location of the protein on the genome using other BLAST searches. Basic imaging revealed that the antigen is associated with the plasma membrane in carcinoma tissues but absent in normal tissues. Furthermore, confocal imaging of the cultured carcinoma cells revealed cell membrane association. Prostatic carcinoma cultured cells appeared to have cortical staining resembling a filamentous network, possibly cytoskeletal in nature. However, this staining pattern was not observed with the paraffinized tissue specimens. The filamentous staining pattern of the cultured cells may be due to their artificial nature. One experiment that may prove very significant is to double-stain carcinoma tissues and cultured carcinoma cells using anti-LT11 IgY and antibodies directed against cytoskeletal elements.

Evaluating the data from the CARE Ab and the LT11 Ag assays, it seems that the antigen is present in both healthy volunteers and cancer patients, although quite elevated in the latter group. The presence of LT11 in normal tissues is not strikingly surprising. The determination is that markers in current use can be synthesized by normal tissue and levels in serum can be elevated in certain benign conditions (Duffy 2001). Again, a larger study of cancer patients and healthy volunteers would help to set ranges for the normal
amounts of LT11 present in the serum of healthy volunteers. In addition to a larger study, the question arises concerning this antigen’s function both in normal tissues, and how is this function, if at all, different in cancer tissues.

One theory regarding the presence of LT11 in the serum of healthy individuals is that this antigen may be an intracellular protein that is shed when normal cells die by apoptosis or by some cytotoxic mechanism. The idea in this theory is that the conformation of the protein is such that the antigenic epitope is not exposed. As was shown by the immunofluorescence of the carcinoma and normal tissues, there was no significant staining of the normal tissue. Therefore, the antigen may be an intracellular protein that, upon cell death, is released and the antigenic determinate exposed. One possible study that could be done to test this hypothesis would be to grow a normal culture cell line, perhaps human umbilical vascular endothelial cells (HUVEC). The supernatant could be used to run the LT11 Ag assay for a baseline level of antigen concentration. Next, the cells would be subjected to an apoptotic and/or cytotoxic molecule for a small period of time. The supernatant would be obtained and the LT11 assay performed to determine any increase in LT11 concentration. Furthermore, histologic staining of apoptotic cells could also be performed to determine the presence of the antigen using fluorescent immunostaining.

Another theory about the elevated expression of LT11 in cancer patients versus healthy volunteers is that perhaps LT11 is present in normal cells but is a modified intracellular protein that somehow is being abnormally expressed on the surface of cells. The LT11 polypeptide is a portion of a larger molecule that possibly penetrates the plasma membrane of cancer cells. If LT11 is exposed, what is its role? It may possibly be a receptor for sequential enzymatic or immunologic action. Although one cannot say this would be the case, LT11 may act as a receptor that initiates at least one step in the metastatic process. One proposal for this hypothesis would be to perform a competition assay. Labeled anti-LT11 IgY would be incubated with LT11 to get a baseline reading
for total IgY binding. Subsequently, increasing concentrations of labeled metastatic molecules, such as plasminogen, could be incubated with anti-LT11 IgY. It is projected that there may be a decrease in IgY binding should LT11 prove to be a receptor for a metastatic molecule.

*Investigating the CARE Ab to understand the LT11 Ag.* With regard to the immune system, cell-mediated immunity, i.e., T cells, has been the most extensively studied while humoral immunity, i.e., B cells and antibodies, have not been as thoroughly investigated. The IgM antibody that is specific for LT11 does not undergo a heavy-chain class-switch to IgG, which is the common immunologic response when challenged with a new antigen. The B cells begin producing IgG molecules that are specific for the antigen; they are the memory B cells that respond upon the second challenge with the antigen (Chapel et al. 1999; Roitt and Delves 2001). In the case of IgM and LT11, there is no switching to IgG and, therefore, no memory cells produced that are specific for LT11. This may be a possible explanation as to why the cancer growth exceeds the ability of the immune system to control its growth. IgM may possess some intrinsic cytotoxic or apoptotic activity that destroys cancer cells or may rely on complement activation; however, when growth of the tumor cells is beyond the capacity of IgM to control and without IgG, the tumor growth will overwhelm the immune system. For IgM to convert to IgG, there must be T-cell interaction (Chapel et al. 1999). This implies that the cancer or some other factor is preventing this interaction and, therefore, prevents T cell help in addition to the inhibited IgG production. Furthermore, antigen processing is necessary. If the antigen is so incorporated into the cell membrane, it may not be available to cause transition from IgM to IgG. These are possible explanations for cancer cells being able to avoid the immune system’s control.

Experiments that could be done to determine the function of IgM include the commercialized apoptosis or cytotoxicity assays such as ApoAlert™ (Clontech) and CytoTox 96™ (Promega). IgM could be incubated with cancer cells, such as K-562
leukemia cells. Viability would be determined initially. Increasing levels of IgM would be added to the cultured cells at different time intervals. Viability for each concentration and time interval would be determined. Furthermore, should IgM prove not to possess intrinsic apoptotic or cytotoxic ability, another study using IgM with human complement would be employed. IgM has the ability to bind complement factors and, therefore, may prove to be of some defense against cancer cells.

_A therapeutic humanized antibody against LT11._ As mentioned earlier, a monoclonal antibody is desired despite the specific polyclonal IgY molecule because monoclonals have an absolute specificity. Another reason a monoclonal would be of benefit would be in humanizing the antibody. Monoclonals are raised in mice; therefore, a murine antibody is needed to undergo the grafting process necessary to form a humanized antibody. Monoclonal antibodies from mice immunized with a specific cancer antigen have been applied in experimental therapeutic methods. Injecting rodent antibodies into humans, however, has proven to be extremely difficult due to ethical and technical reasons. Recognition of the foreign murine protein stimulates a human anti-mouse antibody response, HAMA. This response causes toxic reactions and rapid elimination/neutralization of the antibody, thus interfering with the therapeutic purpose of the monoclonal antibody (Birch and Lennox 1995). Furthermore, HAMA limits the ability to re-treat patients and also has major effects on the pharmacokinetics of the monoclonal (Birch and Lennox 1995). Furthermore, monoclonals from rodents lack the human effector functions, such as complement fixation and antibody-dependent cell-mediated cytotoxicity (ADCC), needed to carry out an effective immune response to destroy the tumor (Mendelsohn et al. 2001). Due to these drawbacks of murine monoclonal antibodies, humanized antibodies have recently been explored in clinical trials. Humanized antibodies offer the advantages of 1) reducing the HAMA response, 2) enhancing effector functions, 3) altering pharmacokinetics of plasma and whole-body clearance, 4) increasing penetration into tumor masses, 5) increasing affinity, and 6)
allowing for more effective delivery of therapeutic molecules (Mendelsohn et al. 2001). To understand the production of a humanized antibody, it is necessary to understand the structure of immunoglobulins.

An IgG molecule is composed of 2 heavy and 2 light chains linked together by disulfide bonds. It contains 2 variable regions (antigen-binding sites) and a constant, Fc, region (Figure 48). The constant region is responsible for binding to cell surface receptors and for fixing complement, a group of serum proteins involved in an enzymatic cascade resulting in cell lysis or labeling of the antigen for phagocytosis. The light chain has 2 domains: a variable (VL) and a constant (CL) domain. The heavy chain of an IgG molecule has 5 domains: a variable domain (VH), CH1, hinge, CH2, and CH3 (Birch and Lennox 1995; Mendelsohn et al. 2001). The VL and VH domains each possess 3 hypervariable loops that form the antigen-binding site of an antibody and are known as the complementarity-determining regions (CDRs) (Birch and Lennox 1995; Mendelsohn et al. 2001). The loops are connected to β-sheet framework regions that provide a supportive element (Mendelsohn et al. 2001). Together the framework and the CDRs determine the specificity and affinity of an antibody for binding to an antigen. By swapping the constant domains of the rodent antibody with the human constant domains, chimeric monoclonal antibodies are formed (Figure 49). This allows the constant domains of the human antibody to interact with the human effector immune functions while carrying the specificity of the rodent variable regions. In addition, the chimeras may have increased in vivo stability (Birch and Lennox 1995). Nevertheless, it is plausible that the variable regions of the rodent antibody may be seen as foreign.
Figure 48
Generalized Antibody Structure

Figure 48: Schematic representation of an antibody molecule depicting 4-polypeptide chains (2 heavy and 2 light chains). The heavy chains are glycosylated. The hinge region is part of the heavy chain that contains the disulfide bonds. Each chain is divided into domains as illustrated (VL and CL for the light chain; VH, CH1, CH2, and CH3 for the heavy chain). These domains are the globular regions of the protein that consist of 3 to 4 loops stabilized by β-pleated sheet secondary structure. Three variable loops in the light chain and 3 in the heavy chain constitute the complementarity-determining regions (CDRs), which together form the antigen-binding site (Mendelsohn 2001).
Humanized Antibody Production

A murine monoclonal antibody (MAb) can be altered to produce a chimeric MAb. Chimeric MAbs are produced by swapping the Fc region of the murine MAb with that of a human antibody. Humanized MAbs are made by grafting the CDRs (along with some framework residues) from the murine MAb into a human antibody framework (Mendelsohn 2001).
By manipulating the chimeric antibodies, humanized antibodies can be formed. This is done by CDR-grafting. The CDRs of the mouse are grafted into the human antibody framework (Figure 49). Campath-1, a humanized antibody, has been shown to be very effective in treatment of non-Hodgkin’s lymphoma (Birch and Lennox 1995).

**Improvements on detection of the LT11 Ag in tissues and cells.** In addition to the future projects mentioned above, anti-LT11 IgY will be used as a primary antibody in immunofluorescence as a histopathological tool in several other tissues. For instance, despite improvements in imaging techniques, early diagnosis to distinguish between chronic pancreatitis and pancreatic adenocarcinoma is still very difficult (Slesak et al. 2000). Having an immunofluorescent tool that would identify pancreatic cancer but not pancreatitis would be of tremendous benefit in histopathology. The anti-LT11 IgY should detect other tumors as well. Pancreatic carcinoma, hepatocarcinoma, renal carcinoma, lung carcinoma and other cancerous tissues will be subjected to immunofluorescence in addition to their benign counterparts and normal tissues.

Furthermore, frozen and fresh tissues need to be evaluated as to the consistency of the staining of LT11. The cultured cells presented in Chapter 4 were fresh cells that underwent immunostaining. It will be interesting to evaluate biopsied fresh and frozen tissues to ascertain consistency of staining.

From looking at the tissues presented in the study, the polyclonal antibody significantly stains the cell membrane as well as some cortical staining. This information is a baseline for further studies using immunostaining. Another improvement in the staining of the tissues would be the development of the monoclonal antibody against LT11. This should greatly decrease background noise while increasing the specificity of binding to LT11, allowing for improved images of LT11 staining of the cell membrane.

As mentioned earlier, immunofluorescent staining of sarcomas would be warranted at this time. Furthermore, leukemia and lymphoma cells could be isolated.
from cancer patients. These cells could be isolated from the blood and then fixed for staining.

The discovery of this new cancer antigen and its specific antibody, IgM, has led to a multitude of new theories and ideas that need to be explored. First and foremost is to try to improve both the assay and staining procedures using a monoclonal antibody. Secondly, the properties and functions of the antigen will be addressed, its role in carcinoma tissues, its effect on the immune system, and the IgM antibody’s function against this new cancer antigen. Thirdly, the possibility of developing a therapeutic humanized antibody against the antigen will be explored once the monoclonal is developed.
BIBLIOGRAPHY


APPENDIX

LETTER TO THE COMMITTEE FROM JERRY THORNTHWAITE, Ph.D.

Richard Skalko, PhD
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November 6, 2002

Dear Dick:

I recognize this dissertation does not include data on the biochemical nature, including the sequence, of the L.T II antigen. While these data have been completed, they are not included here for proprietary reasons while under review for patent considerations.

There are very important characteristics of the antigen revealed in this dissertation that lay the groundwork for understanding the biological importance in diagnosis, prognosis, monitoring for residual disease, screening and therapy.

I respectfully thank you and the rest of the committee for allowing Emily to conduct her research in my laboratory.

Respectfully yours,

Jerry T. Thornthwaite, Ph.D.
Associate Professor of Chemistry and Engineering Sciences
VITA

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